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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME *c* OXIDASE

XVII. AN EPR STUDY OF THE PHOTODISSOCIATION OF CYTOCHROME $a_3^{2+} \cdot CO$*

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SUMMARY

1. The photodissociation reaction of the cytochrome *c* oxidase–CO compound was studied by EPR at 15 °K. Illumination with white light at both room and liquid N_2 temperatures of the partially reduced cytochrome *c* oxidase (2 electrons per 4 metals) in the presence of CO, causes the appearance of a rhombic ($g_x = 6.60$, $g_y = 5.37$) high-spin heme signal.

This signal disappears completely upon darkening of the sample and reappears upon illumination at room temperature; accordingly the photolytic process is reversible. Under these conditions, no great changes in the intensities are observed, neither of the copper signal at $g = 2$, nor of the low-spin heme signal at $g = 3$, 2.2 and 1.5.

2. In the presence of ferricyanide (2 mM) and CO, both the low-spin heme signal ($g = 3.0$, 2.2 and 1.5) and the copper signal of the partially reduced enzyme have intensities about equal to those of the completely oxidized enzyme in the absence of CO. Upon illumination of the carboxy-cytochrome *c* oxidase in the presence of ferricyanide, it was found that the rhombic high-spin heme signal appears without affecting appreciably the copper of low-spin heme signals. Thus, in the presence of ferricyanide the EPR-detectable paramagnetism of the illuminated carboxy-cytochrome *c* oxidase is higher than in the untreated oxidized enzyme.

3. The membrane-bound cytochrome *c* oxidase reduced with NADH in the presence of CO and subsequently oxidized with ferricyanide shows a similar rhombic high-spin heme signal ($g_x = 6.62$, $g_y = 5.29$) upon illumination at room temperature. This signal disappears completely upon darkening and reappears upon illumination at room temperature.

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* For practical reasons we will in this paper maintain the cytochrome *a* and cytochrome a_3 terminology. Cytochrome a_3 then is that part of the protein, the heme group of which is less readily reduced (cyanide and azide) or oxidized (CO and NO) in the presence of ligands.

INTRODUCTION

As discovered originally by Warburg and Negelein [1], the CO compound of cytochrome *c* oxidase, as in most oxygen-binding hemo-proteins, is photodissociable. Although the photodissociation reaction of the fully reduced enzyme has been extensively studied by Chance and co-workers [2, 3] and Yonetani and co-workers [4, 5] using optical spectroscopy methods, relatively little is known about the photodissociation reaction of the CO compound in the partially reduced enzyme and its effect on the EPR spectrum. Leigh and Wilson [6] demonstrated that upon illumination of the CO compound at 10 °K and at a potential of 300 mV, the EPR spectrum of the particulate enzyme showed a transition from low- to high-spin heme. This effect is the opposite of the changes occurring upon addition of CO to the partially reduced sample [7].

It has been reported [8–11] that when the CO compound of fully reduced cytochrome *c* oxidase is treated with ferricyanide, the CO compound remains in its ferrous state, while the other heme component, the 830-nm chromophore and the EPR-detectable copper are reoxidized. This is of particular interest because the paramagnetic interaction suggested to be present [12, 13] between either heme *a*–copper or heme *a*–heme *a* and copper–copper is likely to be broken under these conditions.

In this paper we report on changes in the EPR spectrum occurring upon illumination of the CO compound of the partially reduced enzyme in the presence and absence of ferricyanide. It is found that dissociation of the CO compound, both in the absence and presence of ferricyanide, brings about an increase in the EPR-detectable paramagnetism.

MATERIALS AND METHODS

Beef-heart cytochrome *c* oxidase was prepared as described before [14, 15]. The absorbance coefficient of cytochrome *c* oxidase (red-ox) was 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [16]. The preparations contained 8–10 μmoles heme *a* per gram protein. The beef-heart submitochondrial particles were a generous gift of Dr I. Y. Lee.

Chemicals were analar grade, mainly obtained from British Drug Houses. NADH, grade 2, came from Boehringer, the concentration was calculated with $\varepsilon = 6.22\ mM^{-1} \cdot cm^{-1}$ at 340 nm [17]. Phenazine methosulphate was from Sigma.

The experiments were carried out anaerobically in modified EPR tubes equipped with a special gas holder for the anaerobic addition of CO (Matheson gas products). The titrations with NADH and phenazine methosulphate were performed as previously described [16, 18]. Ferricyanide was added to frozen (77 °K) partially reduced carboxy-cytochrome *c* oxidase, after which the specially equipped EPR tube was made anaerobic and filled with CO. After thawing of the sample the enzyme and ferricyanide were mixed.

Anaerobiosis was achieved by repeated evacuation and flushing with N_2 gas washed through an alkaline pyrogallol column containing catalytic amounts of iron and copper ions. The CO was also freed of traces of oxygen by passing it through an alkaline pyrogallol column.

For illumination of the sample an Aldis slide projector with a lamp of 500 W

was used. The light beam was passed through a cuvette filled with water in order to reduce heat development in the sample. The efficiency of illumination was increased by reflecting the beam with a concave mirror into a silvered liquid nitrogen Dewar, where the sample was frozen.

EPR spectra were recorded using a Varian E-3 EPR spectrometer. The microwave frequency was determined with a Hewlett–Packard frequency counter (5246 L) with frequency converter (5255 A). The magnetic field was calibrated using an AEG Magnetfield meter (GA 11-22.2). Samples were cooled to 15 °K by a helium transfer system (Air Products Inc. model LTD-3-100) with automatic temperature controller. The temperature was measured with the aid of a calibrated carbon resistor and thermocouple (Au-chromel) located just below the sample.

RESULTS

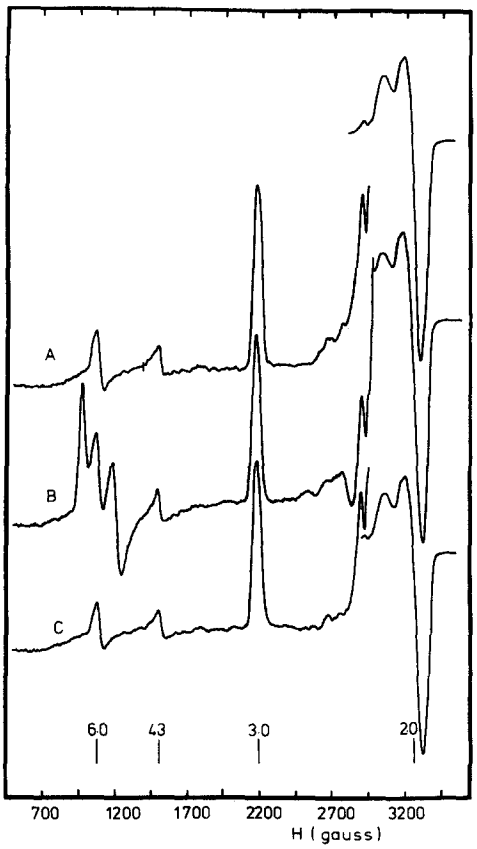
In Fig. 1, Spectrum A shows the EPR spectrum of partially reduced enzyme (2 electrons per 4 metals) after anaerobic addition of CO. Since the $a_3^{2+} \cdot CO$ compound is photodissociable, CO addition, incubation and subsequent freezing of the sample were carried out in the dark. Under these conditions, the EPR spectrum of the enzyme at 15 °K shows a small signal at $g = 6$ and an intense signal at $g = 3$ originating from high- and low-spin ferric heme *a*, respectively [7]. After thawing this sample, and exposing it at room temperature to intense white light and subsequent freezing under illumination in liquid N_2, there appears a large high-spin heme signal at $g = 6$ with a high degree of rhombicity ($g_x = 6.60$, $g_y = 5.37$; Fig. 1, Spectrum B). This signal is stable in liquid N_2 in the dark for days, but disappears when the EPR tube is subsequently thawed, darkened for a few minutes and frozen in liquid N_2 (Spectrum C). This light-dependent process is reversible since it can be repeated several times. It is evident that photodissociation of the 'EPR-silent' $a_3^{2+} \cdot CO$ compound in partially reduced enzyme is linked to the appearance of the rhombic high-spin heme signal. The central line of the signal at $g = 6$, observed in the presence of CO both after illumination and in the dark, may arise from a heme species of cytochrome *c* oxidase more axial in symmetry and unaffected by CO.

In Fig. 1 it can also be seen that light only slightly affects the intensities of the copper signal and the low-spin heme signal. Since these changes are small and the signals show a gradual decrease in intensity with time, the effects are probably not due to an internal redistribution of electrons in which the EPR-detectable components of the oxidized enzyme participate, but rather to a slow continuous reduction of the sample. Such a reduction has also been observed spectrophotometrically with the oxidized enzyme incubated anaerobically with CO [19].

At 77 °K, the photolytic process is also observed (Fig. 2, Spectrum A). Illumination at this temperature of a frozen sample, previously stored in the dark, produces approximately one-third of the intensity of the rhombic high-spin heme signal as shown in Fig. 1, Spectrum B.

Fig. 2, Spectrum B, shows the appearance of the rhombic $g = 6$ signal in a similar sample of carboxy-cytochrome *c* oxidase, but incubated at room light. This observation demonstrates that laboratory light already brings about a photodissociation of the $a_3^{2+} \cdot CO$ compound in the partially reduced enzyme (cf. ref. 7).

After reduction of cytochrome *c* oxidase in the presence of CO, it was found



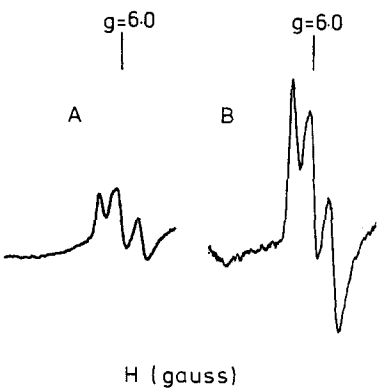


Fig. 1. Effect of illumination on the EPR spectrum of carboxy-cytochrome *c* oxidase. (A) 0.4 mM cytochrome *c* oxidase in 100 mM potassium phosphate, pH 8.2. The enzyme was partially reduced with two electrons (from NADH) per four metals, after which CO was added anaerobically. Incubation in the dark at room temperature for 5 min. (B) After illumination of A at room temperature for 45 s and subsequent freezing under illumination in liquid N_2. (C) After incubation of B in the dark at room temperature for 5 min. Conditions of EPR spectroscopy were: frequency, 9.115 GHz; microwave power, 2 mW; modulation amplitude, 10 G; scanning rate, 312 G · min^{-1}; time constant, 1.0 s; temperature, 15 °K. The copper signal was recorded at a 10-fold lower receiver gain.

Fig. 2. EPR spectra of the $g = 6$ region of the high-spin heme signal. (A) Same sample as in Fig. 1, prepared in the dark at room temperature. Illuminated in the frozen state at 77 °K for 105 s. (B) Similar sample, but incubated at room temperature with laboratory light. Conditions of EPR spectroscopy as in Fig. 1. Gain in B 2.7-fold higher than in A.

[8–11] that the EPR-detectable copper, the 830-nm chromophore and cytochrome *a* are reoxidized by ferricyanide without affecting the redox state of the $a_3^{2+} \cdot CO$ compound. Under these conditions, it seems likely that the magnetic coupling between the $a_3^{2+} \cdot CO$ compound and some other component of the enzyme is broken. This led us to study the effects of illumination on ferricyanide-reoxidized carboxy-cytochrome *c* oxidase.

The EPR spectra of oxidized cytochrome *c* oxidase in the presence of 2 mM ferricyanide, and of the enzyme previously reduced in the presence of CO and sub-

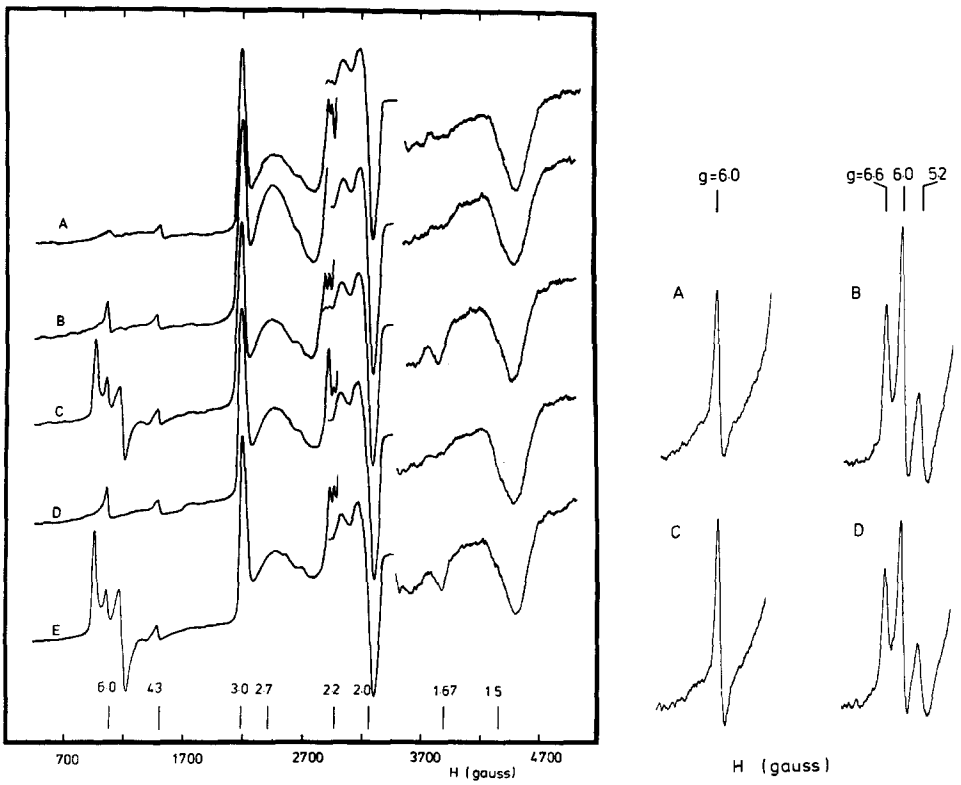


Fig. 3. Effect of illumination on the EPR spectrum of carboxy-cytochrome *c* oxidase in the presence of ferricyanide. (A) 0.4 mM cytochrome *c* oxidase in 100 mM potassium phosphate, pH 8.1, after anaerobic addition of 2 mM ferricyanide. (B) The enzyme of A was partially reduced with two electrons per four metals, after which CO was added anaerobically and the enzyme subsequently oxidized by 2 mM ferricyanide. Incubation in the dark at room temperature for 10 min. (C) After illumination of B at room temperature for 45 s and freezing under illumination in liquid N_2. (D) Incubation of C in the dark at room temperature for 5 min. (E) After illumination of D at room temperature for 45 s and freezing under illumination in liquid N_2. Conditions of EPR spectroscopy as in Fig. 1. Gain in the $g = 1.5$ region, 4-fold higher than in $g = 3$ region.

Fig. 4. EPR spectrum of beef-heart submitochondrial particles in the $g = 6$ region. (A) After anaerobic addition of 0.5 mM NADH in the presence of CO. (B) After a subsequent oxidation of A with 3 mM ferricyanide and illumination for 45 s at room temperature. The sample was frozen in liquid N_2 under illumination. (C) After incubation of B in the dark at room temperature for 5 min. (D) After illumination of C at room temperature for 45 s and freezing in liquid N_2 under illumination. Conditions of EPR spectroscopy were as in Fig. 1.

sequently treated anaerobically with ferricyanide in the dark are shown in Fig. 3, Spectrum A and B, respectively. Judging from the copper line at $g = 2$ and the line at $g = 3$, better visible, however, at $g = 1.5$, since the shape of the line at $g = 3$ is not concealed behind the ferricyanide resonance at $g = 2.7$, ferricyanide causes a complete reoxidation of both the EPR-detectable heme *a* and copper.

Upon illumination of the CO-containing sample at room temperature (Spectrum C), little or no change occurs in the copper signal or low-spin heme signal at

$g = 3$ and at $g = 1.5$, but a high-spin heme iron signal appears with a high degree of rhombicity ($g_x = 6.60$, $g_y = 5.37$) and a resonance at $g = 1.67$. Upon darkening of the sample at room temperature (Spectrum D) the rhombic high-spin heme signal and the line at $g = 1.67$ disappear completely, again without appreciably affecting the height of either the copper ($g = 2$) or the low-spin heme signal at $g_z = 3$ and $g_x = 1.5$.

This photolytic process is reversible, since upon illumination the signal at $g = 6$ reappears (Spectrum E). Furthermore, as inferred from the gradual decrease of the line at $g = 2.7$ of ferricyanide (Spectra B–E), a slow continuous electron transfer in the system occurs, the origin of which is unknown (cf. ref. 19). Additionally, in some of the spectra a line is observed present as a shoulder on the high-field side of the low-spin heme line at $g = 2.2$. This small line and the line at $g = 1.67$, which are both affected upon illumination of the sample, may be the g_y and g_x parts, respectively, of a signal of a low-spin heme species.

The membrane-bound enzyme also exhibits the photolytic process. When submitochondrial particles (beef heart) are reduced with NADH in the presence of CO, only a signal at $g = 6$, axial in symmetry, is present (Fig. 4, Spectrum A). This signal, relatively intense when compared to the purified enzyme, is likely to be due to the presence of metmyoglobin [20] in the particles. Upon addition of ferricyanide to these particles and subsequent freezing to 77 °K under illumination, a rhombic high-spin heme signal is observed with g_x at 6.62 and g_y at 5.29 (Spectrum B). Both g-values are similar to those observed in the isolated enzyme. Spectra C and D show that this signal disappears and reappears upon darkening and illumination, respectively, of the sample at room temperature. Consequently, the photolytic process is also reversible in the particulate enzyme.

DISCUSSION

The EPR spectra presented here have shown that the photodissociation reaction of the $a_3^{2+} \cdot CO$ compound in the partially reduced enzyme at both room temperature and 77 °K is linked to the appearance of a high-spin heme signal, with a much higher degree of rhombicity than in the partially reduced enzyme in the absence of CO. Although the results can be explained by a slow conformation transition, the high degree of rhombicity of the signal at $g = 6$ suggests that upon illumination the CO molecule perturbs the ligand field of the heme iron. The possibility that CO is loosely bound to the enzyme after photodissociation is supported by the observation that the photolytic process also occurs at 77 °K. At this low temperature, diffusion of the ligand into the frozen solution seems unlikely. If, indeed, upon illumination the CO does not escape from the heme crevice, a nearly instantaneous recombination with the heme iron is expected, which will be independent of the CO concentration. This has been observed by Yonetani [5] for cytochrome *c* oxidase. The observation that the rhombic high-spin heme signal, present after illumination of the carboxy-cytochrome *c* oxidase, is stable in liquid N_2 is consistent with the optical absorption measurements of Chance et al. [3] and Yonetani [5]. They showed, at 77 °K, photolysis of the $a_3^{2+} \cdot CO$ compound of the fully reduced enzyme and found that the process is irreversible at that temperature.

Leigh and Wilson [6] demonstrated that the photodissociation process of the

$a_3^{2+} \cdot CO$ compound in pigeon-heart mitochondria occurs even at 10 °K. Their EPR difference spectra at 10 °K of illuminated minus dark showed formation of two high-spin heme species, one with a rather rhombic signal with lines at 6.58 and 5.40 and the other species showing a rather symmetrical signal at $g = 6$. These g-values are similar to those of the signals observed upon illumination at room temperature in the presence of CO of both the isolated and membrane-bound cytochrome *c* oxidase.

Furthermore, they found some decline of the signal at $g = 3$ upon illumination and explained this by transition of low- to high-spin heme. However, under our conditions no relationship could be demonstrated between formation of the high-spin heme and the changes of the $g = 3$ signal. In this context it is noteworthy that under certain conditions we have also observed that the photolytic process at room temperature is accompanied by large changes in the copper ($g = 2$) and low-spin heme signal at $g = 3$ (unpublished). These changes are probably due to a complete dissociation of the CO molecule at room temperature upon illumination.

Because CO has a higher affinity for the ferrous than for the ferric state of the heme iron, the apparent midpoint potential of the heme iron in the ligand complex has increased. As discussed before, upon illumination of the sample the CO molecule becomes more loosely bound, and as a consequence the apparent midpoint potential of the heme iron–CO complex will decrease. This then may give rise to redistribution of electrons. It is suggested, therefore, that an electron transfer occurs from the diamagnetic $a_3^{2+} \cdot CO$ compound to some other electron-accepting group upon illumination, thus causing the formation of the rhombic high-spin iron heme signal at $g = 6$. Since both the low-spin heme *a* signal and the EPR-detectable copper signal are not or only slightly affected upon illumination, a redox shuttle is proposed to be operative between the $a_3^{2+} \cdot CO$ compound and an 'EPR-silent' electron-accepting group. It is possible that this group is the EPR-undetectable copper. If so, this requires that this copper species is at least partially oxidized. Although two electrons per four metals are added, it should be noted that in the absence of ferricyanide the $g = 3$ signal present after CO addition represents only 75% (not shown) of that observed in the oxidized enzyme. Consequently, it can be calculated that part (25%) of the EPR-undetectable copper is in the oxidized state.

Although less likely on a theoretical basis, a charge transfer may occur from the heme–iron to the CO ligand. This then would give rise to a negatively charged CO molecule and a ferric iron. But, this possibility can be ruled out since illumination of the completely reduced enzyme in the presence of CO does not induce a signal at $g = 6$.

It is obvious from our data that after illumination of the ferricyanide-treated carboxy-cytochrome *c* oxidase the intensities of the copper and low-spin heme signals (cf. Fig. 3, Spectra A and C) are almost equal to those in the untreated oxidized enzyme. In addition, a high-spin heme iron EPR signal is observed, demonstrating that paramagnetism as detected by EPR is higher in the illuminated carboxy-enzyme than in the oxidized enzyme, so that part of the EPR-undetectable heme *a* has become EPR detectable.

The intensity of the rhombic high-spin heme *a* signal in terms of total heme can actually not be determined, since data on the zero-field splitting parameter of high-spin heme iron in cytochrome *c* oxidase are not available. But, in order to obtain some estimate of the amount of heme *a* involved in the photolytic process, the high-spin

heme signal of ferricyanide- and light-treated carboxy-cytochrome *c* oxidase was compared with that of acidic metmyoglobin. From the single integrated areas, taken from the low-field end to the point of intersection of the derivative curve with the base line of these signals, it was found that the rhombic high-spin heme signal represents about 50 μM heme *a*. This value amounts to about 6% of the total heme *a* present in the enzyme.

Why, in the presence of ferricyanide, so little heme *a* becomes detectable by EPR upon illumination of the carboxy-cytochrome *c* oxidase seems rather puzzling. An explanation for this finding is that in the presence of CO the electron-accepting component of the redox shuttle is largely reduced. It has been demonstrated that upon oxidation with O_2 [21] and ferricyanide [22] high- and low-potential copper species are present. This observation points to the possibility that in the presence of CO and ferricyanide not only part of the reduced form of the heme has been stabilized but also part of the reduced form of the copper. Possibly, this copper is the EPR-undetectable copper species, since Tsudzuki and Wilson [23] have found that in the presence of CO the EPR-detectable copper has a potential of 255 mV. This then offers an explanation why upon illumination of carboxy-cytochrome *c* oxidase relatively little heme *a* in the enzyme has become EPR detectable

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