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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME *c* OXIDASE

XIX AN EPR STUDY OF THE PHOTODISSOCIATION OF CARBOXY-CYTOCHROME *c* OXIDASE IN THE PRESENCE OF AZIDE

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SUMMARY

1 The photodissociation reaction of the cytochrome *c* oxidase–CO compound in the presence of azide was studied by EPR at 15 °K Addition of CO in the dark to cytochrome *c* oxidase, partially reduced (2 electrons per 4 metal ions) in the presence of azide brings about a decrease in intensity of the azide-induced low-spin heme signal at $g = 2\,9$, 2 2 and 1 67 and an increase in intensity of both the low-spin heme signal at $g = 3$ and the copper signal at $g = 2$ Subsequent illumination with white light at room temperature of this sample causes an enhancement of the azide-induced signal at $g = 2\,9$, and a decrease in intensity of both signals at $g = 3$ and $g = 2$ It is shown that these changes in the EPR spectrum are reversible

2 These results demonstrate that upon photodissociation, CO is replaced by azide wheras upon incubation in the dark CO expels azide from its binding site in cytochrome *c* oxidase

3 Concomitantly with the binding of CO and dissociation of the azide molecule, and vice versa, electron redistributions occur as inferred from the changes in the intensity of the copper signal at $g = 2$

4 The results are explained in a model of cytochrome *c* oxidase with either a common binding site (cytochrome a_3)* for CO and azide or in a model with anti-cooperative interaction between two different sites of binding

5 Similar types of experiments with cyanide instead of azide show that cyanide is more firmly bound to partially reduced cytochrome *c* oxidase than CO and azide The affinity of ligands for partially reduced enzyme decreases in the sequence cyanide, CO (dark), azide and CO (illuminated)

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

INTRODUCTION

Recently Leigh et al [1, 2] and Wever et al [3] demonstrated that the photodissociation reaction of the cytochrome a_3^{2+} CO compound in partially reduced cytochrome *c* oxidase is accompanied by formation of a rhombic high-spin heme signal at $g = 6$ Leigh et al [1, 2] also observed changes in the intensity of the low-spin heme signal at $g = 3$ and conclude that the effect is due to a spin-state transition in cytochrome *a* from low- to high-spin iron upon dissociation of the CO molecule However, we could not demonstrate a clear-cut relation between the formation of the rhombic high-spin heme signal and the changes in the low-spin heme signal at $g = 3$ Therefore, we assign the rhombic high-spin heme signal to carboxy-cytochrome *c* oxidase with the heme iron of cytochrome a_3 in the ferric state, since the rhombicity of the signal appearing upon photodissociation of the cytochrome a_3^{2+} CO compound differs considerably from that in the partially reduced enzyme in the absence of CO [3]

In this paper we report on some changes in the EPR spectrum occurring upon illumination and darkening of partially reduced carboxy-cytochrome *c* oxidase in the presence of azide The aim of this investigation is to determine whether azide and CO have a common or different [4] binding sites It will be shown that upon photodissociation of CO, azide is bound and that upon recombination of CO with cytochrome a_3 in the dark azide is expelled from its binding site

MATERIALS AND METHODS

Beef-heart cytochrome *c* oxidase was prepared as described before [5, 6] The absorbance coefficient of cytochrome *c* oxidase (red–ox) was 24 0 mM^{-1} cm^{-1} at 605 nm [7] The preparations contained 8–10 μmoles heme *a* per g protein The beef-heart ATP-Mg submitochondrial particles prepared according to the method of Low and Vallin [8] were a generous gift of Dr I Y Lee

Chemicals were of analytical grade, mainly obtained from British Drug Houses NADH, grade 2 was purchased from Boehringer The concentration was calculated with an absorbance coefficient of 6 22 mM^{-1} cm^{-1} at 340 nm [9] Phenazine methosulphate was from Sigma

The anaerobic titrations and illumination of the samples were performed as previously described [3]

EPR spectra were recorded using a Varian E-9 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 at 15 °K of partially reduced enzyme (2 electrons per 4 metal ions) in the presence of 100 mM azide Under these

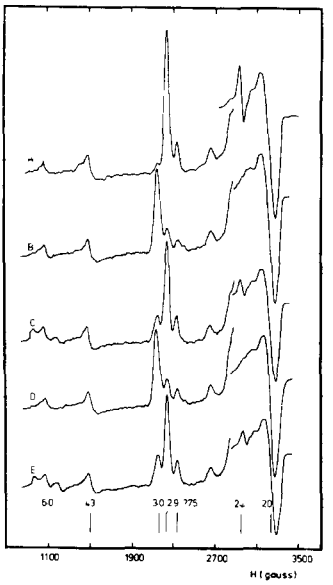

Fig 1 Effect of CO on the EPR spectrum of azido-cytochrome *c* oxidase in the dark and upon illumination A EPR spectrum of partially reduced (2 electrons from NADH per 4 metal ions) cytochrome *c* oxidase (0 2 mM) in the presence of 100 mM azide The enzyme was dissolved in 100 mM potassium phosphate, pH 7 2, and 1 0 % (v/v) Tween-80 B After anaerobic addition of CO to A and incubation in the dark at room temperature for 50 min C After illumination of B at room temperature for 45 s and subsequent freezing under illumination in liquid N_2 D After incubation of C in the dark at room temperature for 10 min E After illumination of D at room temperature for 45 s and subsequent freezing under illumination in liquid N_2 Conditions of EPR spectroscopy were frequency 9 080 GHz, microwave power, 2 mW, modulation amplitude 10 G, scanning rate 500 G min^{-1}, time constant, 1 0 s, temperature 15 °K The signal around $g = 2$ was recorded at a 10-fold lower receiver gain except for A which was recorded at a 5-fold lower gain

conditions, an intense signal at $g = 2\,9$ is observed and small signals at $g = 2\,75$, $g = 3\,0$ and $g = 6$ Upon anaerobic addition of CO and a subsequent incubation of 50 min at room temperature in the dark, Spectrum B is obtained The low-spin heme signals at $g = 2\,9$ and at $g = 2\,75$ disappear to a great extent, whereas the low-spin heme signal at $g = 3$ and the copper signal at $g = 2$ increase in intensity After thawing this sample and exposing it at room temperature to intense white light and subsequent freezing under illumination in liquid N_2 (Spectrum C), the signals at $g = 2\,9$ and 2 75 intensify, while the copper signal at $g = 2$ and the low-spin heme signal at $g = 3$ decrease in height In addition a weak high-spin heme iron signal with a high degree of rhombicity is observed at $g = 6$ This spectrum is stable in liquid N_2 for hours, but after thawing of the EPR tube and darkening for 10 min Spectrum D is obtained, which is very similar to Spectrum B The light-dependent reactions reversible, since the process can be repeated (Spectrum E)

From these observations it is clear that photodissociation of the cytochrome $a_3{}^{2+}$ CO compound is linked to the appearance of both a small rhombic high-spin heme signal at $g = 6$ and the azide-induced low-spin heme signal at $g = 2\,9$ Concomitantly reversible changes are observed in the intensities of both the low-spin

heme signal at $g = 3$ and the copper signal at $g = 2$ These results demonstrate that in the dark, when CO binds firmly to cytochrome *c* oxidase, azide is expelled from its binding site by CO, whereas upon illumination the event is reversed

Though essentially the same results (not shown) are obtained with lower concentration of azide (5 mM), one observation deserves further attention The intensity of the signal at $g = 2\,9$ appearing upon illumination of the partially reduced enzyme in the presence of CO is smaller with 5 mM azide than with 100 mM azide, whereas the intensity of the rhombic high-spin heme signal at $g = 6$ is greater with 5 mM azide Thus, the presence of the rhombic signal at $g = 6$ suggests an incomplete binding of azide to partially reduced cytochrome *c* oxidase

The photolytic process is also observed at 77 °K (Fig 2) Illumination at this temperature of a sample previously stored in the dark (Spectrum A) produces the rhombic high spin heme signal (Spectrum B), without significantly affecting the intensity of the copper and the low-spin heme signals at $g = 3$, 2 9 and 2 75 This demonstrates that at 77 °K photodissociation does occur without, however, binding of azide

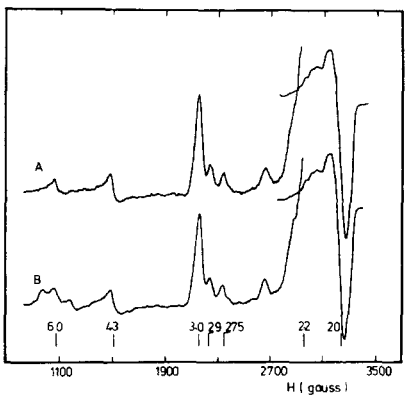

Fig 2 EPR spectra of carboxy-cytochrome *c* oxidase in the presence of 100 mM azide A Same sample as in Fig 1, incubated in the dark at room temperature B After illumination of A in the frozen state at 77 °K for 60 s Conditions of EPR spectroscopy as in Fig 1

The changes occurring in the spectra after darkening of an illuminated sample are relatively slow When an illuminated sample (Fig 3, Spectrum A) is put in the dark at room temperature for about 35 s and 125 s, intermediate types of spectra (Fig 3, Spectra B and C, respectively) are obtained Finally, the spectrum obtained after 10 min of incubation in the dark shows an intense signal at $g = 3$ but hardly any signals at $g = 2\,9$ and 2 75

Since binding of CO is rapid [10] the results demonstrate in accordance with earlier findings [11] that the dissociation of azide is a rather slow process This has also been demonstrated spectrophotometrically (not shown) by following the CO-induced band at 432 nm upon illumination of a similarly treated sample of cytochrome *c* oxidase ($k_{off} = 0\,03\ s^{-1}$ at 20 °C and pH 7 0)

The photolytic reaction can also be demonstrated with the membrane-bound enzyme When submitochondrial particles (beef heart) are incubated with 100 mM azide, followed by the addition of NADH under anaerobic conditions, the EPR

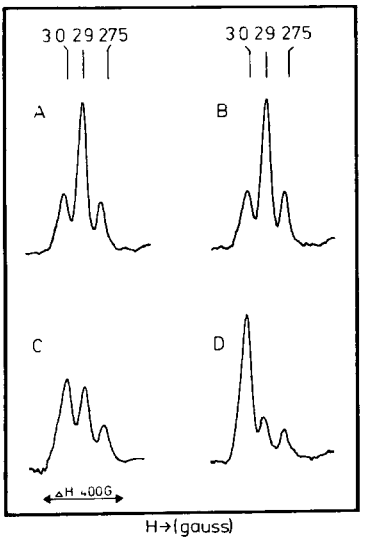

Fig 3 Time course of the changes in the EPR spectrum of the $g = 3$ region occurring after thawing in the dark an illuminated sample of cytochrome *c* oxidase previously incubated with azide and CO Same sample as in Fig 1 A After illumination at room temperature for 45 s and subsequent freezing under illumination in liquid N_2 B After thawing of A in the dark for 35 s and freezing in the dark in liquid N_2 C After thawing of B in the dark for 90 s and freezing in the dark in liquid N_2 D After thawing of C in the dark for 10 min and freezing in the dark in liquid N_2 Conditions of EPR spectroscopy as in Fig 1

spectrum shows the low-spin heme signal at $g = 2\,9$ Upon anaerobic addition of CO and subsequent incubation in the dark at room temperature the azide-induced signal disappears completely, but reappears upon illumination of the sample at room temperature, and vanishes again upon subsequent darkening of the sample

The same type of experiments have been carried out with 10 mM cyanide instead of azide Since in the presence of CO the typical low-spin heme signal at $g = 3\,6$ of cytochrome a_3^{3+}–cyanide [12] in the partially reduced enzyme persists under all conditions tested (not shown), it is concluded that cyanide binds more firmly to partially reduced cytochrome *c* oxidase than CO

DISCUSSION

The effect of azide on the EPR spectrum of the partially reduced enzyme is the same as that observed by Van Gelder and Beinert [13] and Wilson and Leigh [4] In spectra of isolated cytochrome *c* oxidase two azide compounds with rhombic symmetry can be detected with signals at *g* values of 2 9, 2 2 and 1 67 and of 2 75, 2 2 and 1 76 The latter signal has also been observed by Van Gelder and Beinert [13] but no further attention will be paid to it, since under our conditions the intensity of this signal is rather small

The assignment of the intense low-spin heme signal at $g = 2\,9$ is ambigious Van Gelder and Beinert [13], using the definition of Keilin and Hartree of cytochrome a_3 [14], consider cytochrome a_3 to be the reactive species, so that the signal at $g = 2\,9$

has been ascribed to a low-spin cytochrome a_3–azide compound On basis of measurements of redox potentials of cytochrome *c* oxidase in the presence of ligands and assuming that the high-potential heme species is cytochrome a_3, Wilson et al [4, 15] concluded that azide binds to oxidized cytochrome *a*, whereas CO combines with reduced cytochrome a_3 Unfortunately, our experiments are not conclusive as to the binding site of azide, since the effect of CO upon addition to azido-cytochrome *c* oxidase and the subsequent dissociation and recombination reactions can be explained in models with either a common or different binding sites for the ligands on the enzyme

It is obvious from our experiments that there is a competition between azide and CO Consequently, a model with different independent binding sites for both ligands can be excluded However, our observations are readily reconciled with a model with strong anti-cooperative interaction between the binding site of azide (cytochrome *a*) and that of CO (cytochrome a_3) Binding of CO to cytochrome a_3 then expels azide from the other ligand-binding site of cytochrome *c* oxidase Recently, it has been pointed out [16–18] that such an allosteric model is consistent with other studies on ligand binding Moreover, a number of groups [1, 2, 4, 10, 15, 17–20] have demonstrated the presence of site–site interactions in cytochrome *c* oxidase

The competition between CO and azide for cytochrome *c* oxidase can also be explained in a model in which CO replaces azide on a common binding site (cytochrome a_3) [14] Since CO stabilizes reduced cytochrome a_3 and azide the oxidized form, this implies that an electron redistribution occurs upon binding of CO to partially reduced azido-cytochrome *c* oxidase Reasoning along these lines then leads to the conclusion that the changes occurring in the copper signal and the low-spin heme signals represent electron redistributions

Since both possibilities, i e the common site of binding for azide and CO or different anti-cooperative binding sites, seem equal likely, we have no preference for either of the models

The absence of any effect of CO on the low-spin heme signal of the cytochrome $a^{2+}a_3{}^{3+}$–cyanide compound at $g = 3\,6$ demonstrates that either cyanide binds more firmly to partially reduced enzyme than CO or the dissociation rate of cyanide is very slow However, the second possibility is unlikely, since the half time of the dissociation reaction of partially reduced cyano-cytochrome *c* oxidase is 6 min [21, 22, cf refs 23 and 24]

In addition in this paper it is shown that the affinity of CO to cytochrome *c* oxidase in the dark but not upon illumination is greater than that of azide Consequently, the affinity of these ligands to the partially reduced enzyme decreases in the sequence cyanide, CO (dark), azide and CO (illuminated) This order is consistent with the results of Van Buuren et al [25] who found that cyanide expels azide from cytochrome *c* oxidase

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