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AN EPR STUDY OF THE PHOTODISSOCIATION REACTIONS OF OXIDISED CYTOCHROME *c* OXIDASE-NITRIC OXIDE COMPLEXES

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Complexes of oxidised cytochrome *c* oxidase with NO in the absence and presence of ligands such as formate, fluoride and cyanide are photodissociable. After photodissociation at 10 K the EPR spectrum of the high-spin cytochrome a_3^{3+} in the absence of ligands or in the presence of fluoride or formate disappears – as does the EPR spectrum of the low-spin cytochrome a_3^{3+} in the presence of cyanide. The action spectra of the photodissociation reaction of these complexes show slight differences but all have maxima at 640–660 nm and below 400 nm, and are assigned to a diamagnetic Cu_B^+-NO^+ complex. The differences in the action spectra in the presence of various ligands are due to binding of these anions to the cytochrome (a_3-Cu_B) couple. The disappearance of the cytochrome a_3 signal upon photodissociation of the Cu_B^+-NO^+ complex is explained by a magnetic interaction between cytochrome a_3^{3+} and Cu_B^{2+} in the photodissociated complex. The temperature at which NO recombines with Cu_B^{2+} is about 30 K and slightly affected by the presence of added ligands. It is suggested that in the oxidised ligand-cytochrome *c* oxidase complexes the coupling ligand between cytochrome a_3^{3+} and Cu_B^{2+} is cyanide, fluoride and formate. The observation that two ligands may bind simultaneously to the cytochrome a_3-Cu_B couple leads to further support for the notion that during turnover of cytochrome *c* oxidase both metal ions are involved in binding and reduction of oxygen.

Introduction

Cytochrome *c* oxidase, the enzyme that catalyses the electron transfer from cytochrome *c* to oxygen, contains four redox centres: two haem *a* groups associated with cytochrome *a* and cytochrome a_3, and two copper atoms [1]. The haem in cytochrome *a* and one of the copper atoms, Cu_A, are involved in the uptake of electrons from cytochrome *c* and the electron transfer to the oxygen binding site [2,3]. The haem of cytochrome a_3 is able to bind and reduce oxygen [4]. The other copper atom, Cu_B, is located very close to the haem iron of cytochrome a_3 [5]. From EPR spectra of cytochrome *c* oxidase in the presence of azide and NO it was possible even to estimate a distance of 3.4 Å between cytochrome a_3 and Cu_B [6,7]. It is generally accepted that the Cu_B atom is also involved in the oxygen binding and reduction mechanism [6–8].

Oxidised cytochrome *c* oxidase showed EPR signals of one low-spin haem, assigned to cytochrome *a*, and of a copper atom, Cu_A [9]. The EPR signals of cytochrome a_3 and Cu_B are difficult to detect because of an antiferromagnetic coupling between the high-spin haem of cyto-

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Abbreviation: EXAFS, extended X-ray absorption fine structure.

chrome a_3 and Cu_B [9]. A number of reports have appeared, however, in which it was shown that oxidized cytochrome *c* oxidase exhibited '$g = 12$' signals [10,11] which can be assigned to an $S = 2$ system [12,13]. Both metal atoms can be made visible by EPR. When fully reduced cytochrome *c* oxidase is allowed to react with oxygen at cryogenic temperatures, EPR signals of Cu_B are observed [14–16]. Upon reduction of Cu_B in the oxidised enzyme, uncoupling of the antiferromagnetic complex occurs and the EPR signals of the high-spin haem of cytochrome a_3 become visible. This EPR signal changes in shape when cytochrome a_3 binds ligands such as fluoride and formate [9,11] or can be converted into low-spin states when it binds ligands such as cyanide, sulphide, azide and hydroxide [9,17–19]. Also, upon addition of nitric oxide to oxidised cytochrome *c* oxidase, EPR signals of a high-spin haem become visible [6], due to the binding of nitric oxide to Cu_B resulting in a diamagnetic Cu_B^+-NO^+ complex [20] and leaving cytochrome a_3 in an uncoupled high-spin state. In our previous paper [20], we showed that the EPR signal disappears upon illumination below 30 K and that the light action spectrum of the reaction was that of a copper chromophore. When NO is allowed to react with cytochrome *c* oxidase in the presence of azide, a complex is formed that exhibits EPR signals characteristic of a triplet species. The triplet signal originates from nitrosylferrocytochrome a_3 interacting with Cu_B [6,7] and also this signal is photodissociable [20].

In the presence of cyanide and nitric oxide, cytochrome a_3 shows an EPR signal characteristic of a low-spin haem-cyanide complex [6,12]. In this paper the photodissociation reactions of such oxidised cytochrome a_3^{3+} ligand complexes in the presence of nitric oxide are reported. It will be shown that these complexes all have the light action spectrum of a copper chromophore.

Methods

Cytochrome *c* oxidase was isolated from bovine heart as described in Refs. 21 and 22. The absorbance coefficient of cytochrome *c* oxidase (reduced – oxidised) was 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [23]. Chemicals were mainly from BDH (Analar Grade). Nitric oxide (Matheson Gas Products) was purified by leading the gas through a cold trap.

Cyanocytochrome *c* oxidase was prepared by addition of cyanide to a solution of cytochrome *c* oxidase under turnover conditions in the presence of ascorbate and cytochrome *c* and through which oxygen was bubbled for 30 min. Thereafter the reduced cyano complex was re-oxidised with an excess of potassium ferricyanide. Unreacted ferricyanide was removed on a Sephadex G-50 column against 1% (w/v) cholate and 50 mM Tris-H_2SO_4 (pH 8.0). Cyanocytochrome *c* oxidase was concentrated by $(NH_4)_2SO_4$ precipitation and centrifugation at 50000 rpm for 15 min.

The cytochrome *c* oxidase precipitate was dissolved in 50 mM Tris-H_2SO_4 (pH 8.0), 1% (w/v) cholate. The absorbance coefficient of cyanocytochrome *c* oxidase was 22 $mM^{-1} \cdot cm^{-1}$ at 593 nm [22]. The sample was stored in liquid nitrogen.

The high-spin cytochrome *c* oxidase samples were dissolved in 50 mM potassium phosphate (pH 7.4), 0.5% (v/v) Tween 80 and 100 mM potassium fluoride or sodium formate.

All EPR experiments were carried out anaerobically in Thunberg-type cuvettes. Anaerobiosis was achieved by equilibration against helium as described previously [20]. After addition of nitric oxide the sample was thoroughly mixed and immediately frozen in liquid nitrogen.

Optical spectra were obtained on a Cary-219 recording spectrophotometer; EPR spectra were obtained as described before [20]. Illumination was performed by irradiation with a 150 W xenon lamp, through a light guide on the grid of the cavity. Intensities and wavelengths of the irradiated light were varied by interference filters and the temperature of the EPR sample was measured as described in Ref. 20.

Results

When nitric oxide was added to oxidised cytochrome *c* oxidase, which shows the EPR signals from cytochrome a^{3+} and Cu_A^{2+}, a slightly rhombic, high-spin haem EPR signal was formed. This signal has been attributed to cytochrome a_3^{3+} and arises from the binding of NO to Cu_B^{2+}, resulting in a diamagnetic Cu_B^+-NO^+ complex and paramagnetic cytochrome a_3^{3+} ($S = 5/2$) [6,20]. This

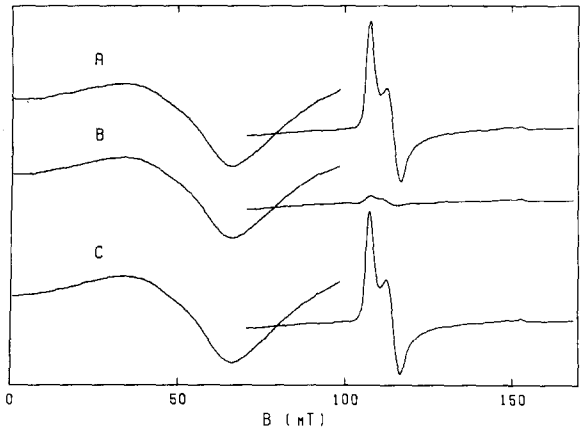

Fig. 1. The effect of light on the EPR spectrum of formate-cytochrome *c* oxidase in the presence of NO. (A) 0.25 mM formate-cytochrome *c* oxidase in 50 mM potassium phosphate (pH 7.4), 100 mM sodium formate, 0.5% Tween 80, $p_{NO} = 60$ kPa. (B) After illumination of A at 10 K. (C) After warming B to 40 K. Conditions of EPR spectroscopy: frequency 9240 MHz; microwave power 6 dB; modulation amplitude, 1 mT; scanning rate 25 mT/min; time constant 0.1 s; temperature 10 K. The $g = 12$ signal was recorded on a 10-fold higher gain.

complex was found to be sensitive to light and could be photodissociated at low temperature, in agreement with our previous results [20]. After warming the sample, the original high-spin haem EPR signal reappeared (not shown).

The experiment was repeated in the presence of formate and fluoride, ligands of cytochrome a_3 which leave the haem iron in the high-spin state [9,11,24]. Upon addition of nitric oxide to oxidised cytochrome *c* oxidase in the presence of sodium formate, also a high-spin haem EPR signal became visible (Fig. 1) whereas the other signals at $g = 3$ from cytochrome a^{3+}, at $g = 2$ from Cu_A^{2+}, and the broad signal at $g = 12$ remained unaffected. The high-spin haem signal in the presence of formate was characterised by *g* values slightly different from those in the absence of formate (Table I). Also, this complex could be photodissociated and re-formed by heating.

In the presence of fluoride the same effects were observed (Fig. 2). Addition of nitric oxide leads to formation of a slightly rhombic, high-spin haem signal, which disappears upon illumination, and the effect of light can be reversed by warming the sample to 40 K. The broad signal at '$g = 3$' upon which the $g = 3.03$ line of cytochrome *a* is superimposed, observed in the presence of fluoride, was not sensitive to NO or light. The high-spin haem signal in the presence of fluoride also shows *g* values different from those in the oxidised enzyme (Table I), indicating that fluoride is bonded to cytochrome a_3^{3+}.

TABLE I

EPR PARAMETERS OF CYTOCHROME a_3^{3+} IN OXIDISED CYTOCHROME *c* OXIDASE-NITRIC OXIDE COMPLEXES

The *g* values were obtained from the difference spectrum (dark minus light).

Added ligand	g_x	g_y	g_z
None	6.17	5.75	–
Formate	6.22	5.71	–
Fluoride	6.13	5.80	–
Cyanide	–	–	3.42 (major)
	–	–	3.31 (minor)

Another well known ligand of cytochrome a_3 is cyanide [17,22]. When nitric oxide was added to oxidised cyanocytochrome *c* oxidase, an EPR signal at $g = 3.42$ (Fig. 3) was found, attributed to cytochrome a_3^{3+}-CN^- in agreement with the re-

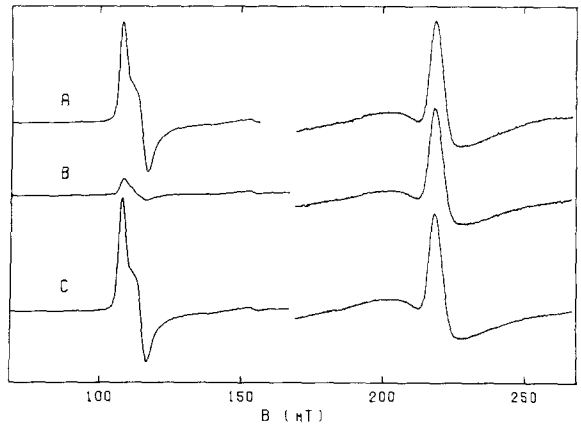

Fig. 2. The effect of light on the EPR spectrum of fluorocytochrome *c* oxidase in the presence of NO. (A) 0.25 mM fluorocytochrome *c* oxidase in 50 mM potassium phosphate (pH 7.4), 100 mM potassium fluoride, 0.5% Tween 80, $p_{NO} = 60$ kPa. (B) After illumination of A at 10 K. (C) After warming of B to 40 K. Conditions of EPR spectroscopy as in Fig. 1: frequency 9242 MHz. The $g = 3$ region is recorded on a 5-fold higher gain.

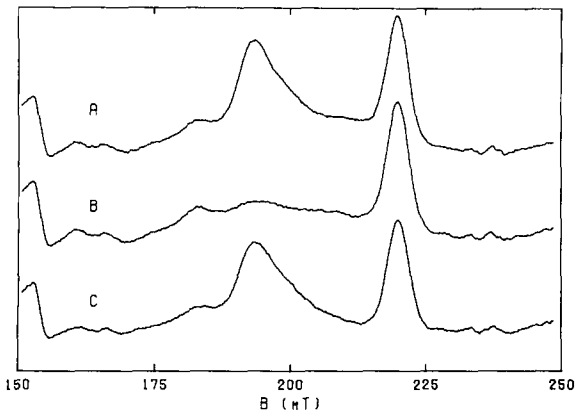

Fig. 3. The effect of light on the EPR spectrum of cyanocytchrome *c* oxidase in the presence of NO. (A) Cyanocytochrome *c* oxidase prepared as outlined in Methods, $p_{NO} = 60$ kPa. (B) After illumination of A at 10 K. (C) After warming of B to 40 K. Conditions of EPR spectroscopy: frequency 9243 MHz; microwave power 14 dB; modulation amplitude 1 mT: scanning rate 12.5 mT/min; time constant 3 s; temperature 10 K.

sults in Refs. 6 and 12. Upon illumination the low-spin haem signal disappears, and warming to 40 K results in re-formation of the $g = 3.4$ signal (Fig. 3).

Photodissociation of the various complexes also led to formation of a broad signal at 350 mT. Fig. 4 shows this signal obtained from a difference spectrum (illuminated minus dark) of oxidised cytochrome *c* oxidase in the presence of the various ligands. Upon warming the sample this broad signal disappeared. We have assigned this signal to NO interacting with the cytochrome a_3-Cu_B couple. In addition, illumination of the sample led to the disappearance of small amounts of cytochrome a_3^{2+}-NO at $g = 2$ and formation of a radical in the cyanide complex. All complexes show formation of the broad signal at 350 mT upon illumination and disappearance of this signal upon heating. This would indicate that a simple photodissociation equilibrium is operative in which light removes NO from the complex and elevation of the temperature causes recombination with Cu_B.

The photodissociation rates, k, of photolabile ligand complexes are proportional to the light intensity I [25]: $k = \varepsilon\phi I$, where ε is the absorbance coefficient of the excited complex and ϕ the quantum efficiency of the photodissociation process. Fig. 5 shows that the dissociation rates of the

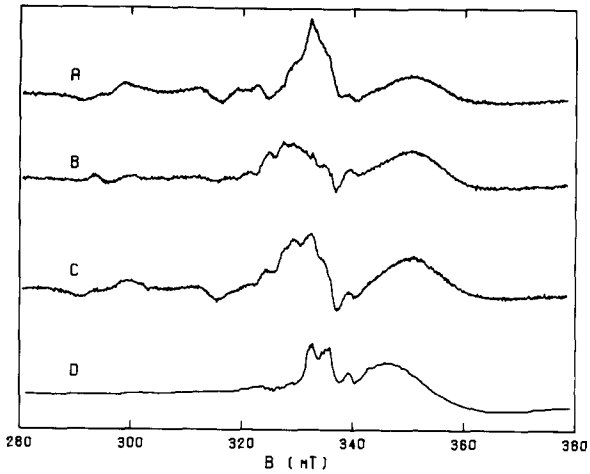

Fig. 4. The effect of light on the EPR spectra of oxidised cytochrome *c* oxidase nitric oxide complexes in the $g = 2$ region. The difference spectra were obtained by subtracting the EPR spectrum obtained after illumination from that before illumination. (A) Without added ligands. (B) In the presence of formate. (C) In the presence of fluoride. (D) In the presence of cyanide. Conditions of EPR spectroscopy: frequency 9249 MHz; microwave power 20 dB; modulation amplitude 1 mT; scanning rate 0.25 mT/min; time constant 0.3 s; temperature 10 K. Other conditions as in Figs. 1–3.

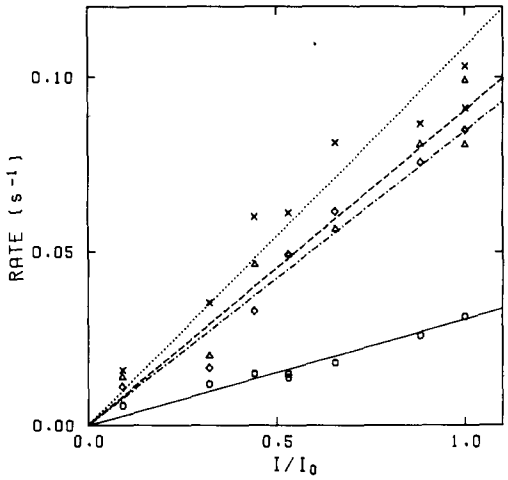

Fig. 5. Dependence of the dissociation rates on the light intensities during illumination of the nitric oxide-cytochrome *c* oxidase complexes. The dissociation rates were calculated from the initial rates of the dissociation reaction. ×, oxidised cytochrome *c* oxidase; Δ, formate-cytochrome *c* oxidase; ◇, fluoro-cytochrome *c* oxidase; ○, cyanocytochrome *c* oxidase. The samples and conditions were as in Figs. 1–3. The irradiation wavelength was 590 nm. The light-induced dissociation of the high-spin haem complexes was followed in the peak of the $g = 6$ signals, whereas the low-spin haem cyanide complex was followed at $g = 3.42$.

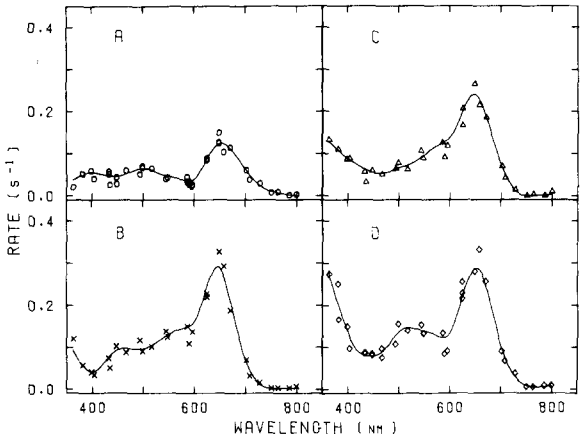

Fig. 6. Action spectra of the photodissociation reactions in cytochrome *c* oxidase-NO complexes. (A) Cyanocytochrome *c* oxidase. (B) Oxidised cytochrome *c* oxidase without additions. (C) Formate-cytochrome *c* oxidase. (D) Fluorocytochrome *c* oxidase. Conditions: as in Fig. 4.

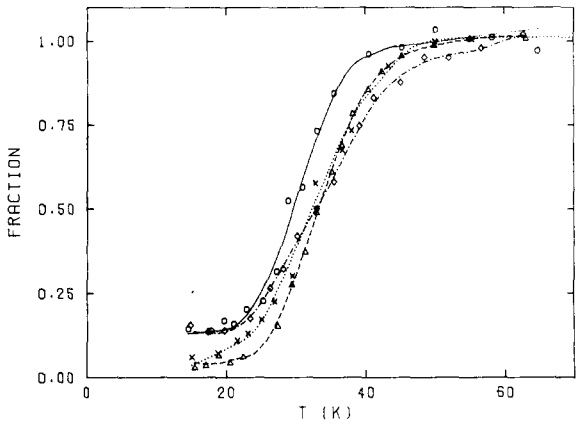

Fig. 7. Fraction of recombined NO complexes of cytochrome *c* oxidase after warming the photodissociated complex to various temperatures. The fraction was calculated by dividing the intensity of the EPR signal after warming by that before illumination. All spectra were recorded at the same low temperature (approx. 15 K); during warming the steady state of the complex was checked by recording the EPR intensity for about 5 min. The complexes were photodissociated by illumination with white light from a xenon lamp filtered with infrared filters for about 10 min. (×·····×) Oxidised cytochrome *c* oxidase; (△-----△) formate-cytochrome *c* oxidase; (◇·–·–·◇) fluorocytochrome *c* oxidase; (○———○) cyanocytochrome *c* oxidase. Conditions: as in Figs. 1–3.

nitric oxide-cytochrome *c* oxidase complexes are proportional to the light intensity. The proportionality constant $\varepsilon\phi$ has similar values for the various high-spin haem complexes, but is much less for the cyano complex.

Since this difference could be caused by a different absorbance spectrum of the cyano complex or a variation in quantum yield compared to the high-spin haem complexes, the light action spectra of the photodissociation of the nitric oxide complexes were measured. These spectra were obtained by measuring the initial rates of the photodissociation reaction in order to minimise inner filtering effects. In Fig. 6 the action spectra for the four nitric oxide complexes studied have been summarised: the spectra for oxidised cytochrome *c* oxidase in the absence and presence of fluoride and formate are very similar in intensity and shape, but the spectrum for cyanocytochrome *c* oxidase has a lower intensity than those of the other three spectra and a slightly different shape. The maxima in the action spectra of all four complexes are around 640 nm and very similar to the action spectrum of oxidised cytochrome *c* oxidase obtained before [20]. The action spectra do not resemble the absorbance spectra of haem compounds and are completely different from the action spectra of cytochrome a_3^{2+}-NO [20]. We have attributed the light-absorbing chromophore to Cu_B in all four nitric oxide complexes.

Though the photodissociation reactions in the four complexes are very similar, it is possible that the presence of ligands for cytochrome a_3^{3+} affects the recombination of nitric oxide at Cu_B^{2+}. Therefore, we studied the temperature dependence of the re-formation of the EPR signals after photodissociation at various temperatures. Fig. 7 shows that the high-spin haem complexes show very similar transition temperatures (32–33 K), whereas that of the cyano complex is only slightly lower (29.6 K), indicating again that the copper complexes are very similar to each other. Furthermore, it was found that the asymmetric $g = 3.42$ signal consisted of two different EPR signals at $g = 3.42$ and $g = 3.31$, that recombined with a transition temperature 1 K different from each other (not shown).

Discussion

The experiments reported here strongly suggest that cytochrome *c* oxidase can bind two ligands in the cytochrome a_3-Cu_B couple at the same time. One of the ligands (NO) is bonded to Cu_B^{2+} and forms a diamagnetic Cu_B^{+}-NO^{+} complex. This would explain the uncoupling of the EPR-invisible cytochrome a_3^{3+}-Cu_B^{2+} couple and the appearance of the EPR signals of cytochrome a_3^{3+} upon addition of nitric oxide to oxidised cytochrome *c* oxidase [6]. The same phenomenon was observed in the presence of ligands such as formate, fluoride and cyanide, which is in agreement with the results of Stevens et al. [6] and Brudvig et al. [12]. The Cu^{+}-NO^{+} complex can be photodissociated, which is in agreement with previous results [20], and the complex shows the action spectrum of a copper chromophore, very similar to the absorbance spectrum of copper proteins such as ceruloplasmin [26] and ascorbate oxidase [27]. After photodissociation, a broad signal was formed at 350 mT attributable to dissociated NO. Upon warming the sample the Cu_B^{+}-NO^{+} complex was formed again. The effects studied are summarised by the equilibrium:

$$Cu_B^{+}\text{-}NO^{+} \underset{\text{temperature}}{\overset{\text{light}}{\rightleftharpoons}} Cu_B^{2+} + NO$$

This equilibrium was slightly affected by the presence of different ligands on cytochrome a_3^{3+}.

The temperature at which 50% of the complex was re-formed after photodissociation ($T_{1/2}$) was 29.6 K for cyanocytochrome *c* oxidase, 32.4 K in the absence of added ligands and 33 K in the presence of fluoride and formate. The intensity and shape of the light action spectra of Cu_B^{+}-NO^{+} in the presence of the various ligands were also slightly different. For the cyanide complex the intensity was less than 30% compared with the other complexes. Furthermore, the cyanide complex showed a very low intensity in the near-ultraviolet region. In the presence of fluoride, the band at 640 nm in the oxidised nitric oxide-cytochrome *c* oxidase complex shifted to 655 nm. The differences in the optical properties of these Cu_B^{+}-NO^{+} complexes may be explained by binding of the various ligands to Cu_B, since it is known that anion binding to copper proteins affects their spectra [28]. The difference in recombination temperature ($T_{1/2}$) of NO to Cu_B in the presence of cyanide can also be caused by binding of cyanide to Cu_B. Since addition of NO to cyano-oxidase results in the formation of a low-spin haem signal with a g_z value close to that observed in partially reduced cyanocytochrome *c* oxidase [17], it is clear that cyanide is bonded to cytochrome a_3. Thus, cyanide is probably a bridging ligand between the iron atom of cytochrome a_3 and Cu_B in cyanocytochrome *c* oxidase.

Ligand binding to Cu_B^{+} in cytochrome *c* oxidase, as observed in the Cu_B^{+}-NO^{+} complexes, is also found upon photodissociation of reduced cytochrome *c* oxidase-CO complexes at low temperature [29]. With Fourier transform infrared spectroscopy it was observed that after photodissociation of cytochrome a_3^{2+}-CO, the freed CO was bound to Cu_B^{+} and that upon warming the sample CO jumped back to the Fe^{2+} in the haem of cytochrome a_3. As outlined below, although not very likely, a similar effect could be present in our system: after photodissociation of Cu_B^{+}-NO^{+} the freed NO then may jump over to cytochrome a_3^{3+}. Photodissociation of the cytochrome a_3^{3+}-C≡N-Cu_B^{+}-NO^{+} complex would in that case lead to cytochrome a_3^{3+}-NO and Cu_B^{2+}-C≡N. This cyano-copper complex would then be responsible for the broad EPR signal at 350 mT. However, the presence of other ligands is then expected to affect strongly the ligand rearrangements after photodissociation and recombination upon warming the sample. This was not observed. Therefore, we prefer a model in which photodissociation of Cu_B^{+}-NO^{+} does not lead to ligand rearrangements and where the bridging ligand between cytochrome a_3^{3+} and Cu_B^{2+} remains bound to both metal ions. The broad EPR signal at 350 mT would then be due to NO interacting with the cytochrome a_3^{3+}-Cu_B^{2+} couple.

In the oxidised cytochrome *c* oxidase-nitric oxide complex there is no evidence that cytochrome a_3^{3+} is involved in direct complexation with NO. Upon addition of NO to oxidised cytochrome *c* oxidase the optical absorbance spectrum of both haems in cytochrome *c* oxidase was unchanged [6]. Furthermore, the photodissociation process showed an action spectrum very different from haem spectra [20]. The observation that NO

is unable to interact directly with cytochrome a_3^{3+} indicates that the sixth ligand-binding site is already occupied. As discussed before [20], in the oxidised cytochrome *c* oxidase-nitric oxide complex the ligand involved is the coupling ligand which is responsible for the antiferromagnetic interaction between cytochrome a_3^{3+} and Cu_B^{2+}. In the other nitric oxide complexes, fluoride, formate or cyanide is bonded to cytochrome a_3^{3+} as indicated by the difference in *g* values in the presence of various ligands.

The best demonstration that a second ligand can be bonded to the cytochrome a_3^{3+}-Cu_B^{2+} couple simultaneously with NO is shown by the oxidised cyanocytochrome *c* oxidase-nitric oxide complex. This complex shows similar properties upon photodissociation and recombination to those of the oxidised cytochrome *c* oxidase-nitric oxide complex originally studied except for the EPR spectrum which clearly showed that the haem iron of cytochrome a_3^{3+} is in a low-spin ($S = 1/2$) state with a g_z value (3.42) close to the g_z value (3.58) of the cytochrome a_3^{3+}-CN^- complex in partially reduced cytochrome *c* oxidase [17,30]. Upon photodissociation of the cyanocytochrome *c* oxidase-nitric oxide complex the EPR spectrum of cytochrome a_3^{3+}-CN^- disappears due to photodissociation of the diamagnetic Cu_B^+-NO^+ compound and coupling of Cu_B^{2+} ($S = 1/2$) and cytochrome a_3^{3+}-CN^- ($S = 1/2$) to a diamagnetic singlet state ($S = 0$) as was proposed for cyanocytochrome *c* oxidase from susceptibility measurements [31] or due to a triplet state ($S = 1$) as was found in recent MCD measurements [32] and Mössbauer studies [33]. Since the latter techniques are more reliable in separating the different redox centres in cytochrome *c* oxidase, we favour the last assignment.

Triplet systems are normally detectable by EPR. In order to explain the absence of EPR signals, large zero-field splittings must be present in this triplet as was already deduced from the MCD measurements [32]. Such large zero-field splittings (greater than 1 cm^{-1}) could not be caused by dipolar interactions between cytochrome a_3^{3+} and Cu_B^{2+} but are due to an anisotropic exchange interaction between cytochrome a_3^{3+} and Cu_B^{2+}. The ligand which is responsible for the exchange interaction could be the CN ligand which is already bonded to cytochrome a_3^{3+}. The cyanide ligand is then a bridging ligand between cytochrome a_3^{3+} and Cu_B^{2+} in oxidised cytochrome *c* oxidase complexes with or without NO and a non-bridging ligand in partially reduced cytochrome *c* oxidase. This would be in agreement with results from near-infrared MCD measurements [30,34]. That cyanide in oxidised cytochrome *c* oxidase is a bridging ligand is consistent with the observed large stability of oxidised cyanocytochrome *c* oxidase compared to partially reduced cyanide derivatives of cytochrome *c* oxidase [35].

In the cytochrome *c* oxidase-nitric oxide complexes with fluoride and formate, these ligands might also bridge between cytochrome a_3^{3+} and Cu_B^+-NO^+. Binding of these anions to cytochrome a_3^{3+} is then responsible for the change in rhombicity of the EPR parameters compared to the oxidised cytochrome *c* oxidase complex. Furthermore, in fluorocytochrome *c* oxidase and formate-cytochrome *c* oxidase $g = 12$ and $g = 3$ signals were present in different proportions. These signals did not change upon addition of nitric oxide, indicating that NO does not bind to the paramagnetic centres of these species of cytochrome *c* oxidase, as was observed already in the oxidised enzyme [10].

The observation that cytochrome *c* oxidase binds two different ligands simultaneously in the cytochrome a_3-Cu_B couple and the finding from EXAFS [5] and EPR measurements [6,7] that the Fe in cytochrome a_3 and the Cu_B are located very close to each other, make it likely that during turnover oxygen is bonded in a bridge between cytochrome a_3 and Cu_B. Furthermore, the accessibility of Cu_B to ligands supports suggestions [7,8,12,16] that Cu_B is involved in binding of the products of the oxygen reduction reaction.

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