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THE CYTOCHROME *c* OXIDASE-AZIDE-NITRIC OXIDE COMPLEX AS A MODEL FOR THE OXYGEN-BINDING SITE

ROLF BOELENS *, HENK RADEMAKER, RON WEVER ** and BOB F. VAN GELDER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam (The Netherlands)

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The complex of cytochrome *c* oxidase with NO and azide has been studied by EPR at 9.2 and 35 GHz. This complex which shows $\Delta m_s = 2$ EPR triplet and strong anisotropic signals, due to the interaction of cytochrome $a_3^{2+} \cdot NO$ ($S = \frac{1}{2}$) and Cu_B^{2+} ($S = \frac{1}{2}$), is photodissociable. Its action spectrum is similar to that of cytochrome $a_3^{2+} \cdot NO$ with bands at 430, 560 and 595 nm, but shows an additional band in the near ultraviolet region. The quantum yield of the photodissociation process of cytochrome $a_3^{2+} \cdot NO$ in the metal pair appears to depend on the redox state of Cu_B. When the photolysed sample was warmed to 77 K, a complex was observed with the EPR parameters of cytochrome $a_3^{3+} - N_3^- - Cu_B^{1+}$ ($S = \frac{1}{2}$). This process of electron and ligand transfer can be reversed by heating the sample to 220 K. It is suggested that in the triplet species azide is bound to Cu_B^{2+} whereas NO is bridged between Cu_B^{2+} and the haem iron of the cytochrome a_3^{2+}. The complex has a triplet ground state and a singlet excited state with an exchange interaction $J = -7.1$ cm^{-1} between both spins. The anisotropy in the EPR spectra is mainly due to a magnetic dipole-dipole interaction between cytochrome $a_3^{2+} \cdot NO$ and Cu_B^{2+}. From simulations of the triplet EPR spectra obtained at 9 and 35 GHz, a value for the distance between the nitroxide radical and Cu_B^{2+} of 0.33 nm was found. A model of the NO binding in the cytochrome a_3-Cu pair shows a distance between the haem iron of cytochrome a_3 and Cu_B of 0.45 nm. It is concluded that the cytochrome a_3-Cu_B pair forms a cage in which the dioxygen molecule is bidentate coordinated to the two metals during the catalytic reaction.

Introduction

Cytochrome *c* oxidase (ferro cytochrome *c*: oxygen oxidoreductase, E.C. 1.9.3.1) contains four redox metal centres, two iron ions in haem *a* groups and two copper ions [1]. These redox centres are involved in the electron transfer from cytochrome *c* to oxygen catalysed by this enzyme. The haem *a* group associated with cytochrome *a* accepts the electrons from cytochrome *c* and transfers the electrons via Cu_A to the haem *a* group associated with cytochrome a_3 [2]. Cytochrome a_3 and the associated Cu_B bind and reduce O_2 [3–5].

The absence of EPR signals attributable to cytochrome a_3^{3+} and Cu_B^{2+} in the oxidized enzyme suggested a strong magnetic interaction between these redox centres [6]. It was proposed from EXAFS measurements that in oxidized cytochrome *c* oxidase Cu_B^{2+} and Fe^{3+} in cytochrome

* Present address: Laboratory of Physical Chemistry, University of Groningen, The Netherlands

** To whom reprint requests should be addressed.

Data supplementary to this article are deposited with, and can be obtained from, Elsevier Science Publishers B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to BBA/DD-279/41489/765 (1984) 196. This supplementary information includes a description of the spin hamiltonians for two single ions and those obtained for an interaction pair. The method by which computer simulations of the pair spectra are carried out is given.

a_3 share a bridging sulphur ligand and are only 0.375 nm apart [7]. Alben et al. [8] concluded from low-temperature infrared studies on the photodissociation of cytochrome $a_3^{2+} \cdot CO$ that Cu_B could bind CO and EPR studies of oxidized cytochrome *c* oxidase-NO complexes have demonstrated that NO could bind to Cu_B [9–12]. These results strongly support models in which both Cu_B and cytochrome a_3 are involved in the oxygen binding and reduction, as proposed by several authors [12–16]. In the reaction of reduced cytochrome *c* oxidase with oxygen an intermediate was detected with an EPR spectrum which could be attributed to Cu_B^{2+} [17]. From this spectrum it could be concluded that the copper ion interacted with another paramagnetic centre, presumably cytochrome a_3 in a low-spin ferryl state [5,17]. Further oxidation led to an intermediate with EPR resonances at $g = 5$, 1.78 and 1.69 (at 9 GHz), which were ascribed to a spin-coupled state involving cytochrome a_3 and Cu_B [18,19]. Recently, it was shown that Cu_B was able to bind reaction products of oxygen [5]. However, the precise geometry of the haem of cytochrome a_3 and Cu_B in the oxygen binding site is still unclear.

Under certain conditions it is possible to study the magnetic interactions between the haem iron of cytochrome a_3 and Cu_B by EPR. When cytochrome *c* oxidase was incubated with azide and nitric oxide, a cytochrome $a_3^{2+} \cdot NO\text{-}Cu_B^{2+}$ complex was formed with a characteristic EPR triplet signal due to the interaction of cytochrome $a_3^{2+} \cdot NO$ ($S = 1/2$) and Cu_B^{2+} ($S = 1/2$) [9,10]. As shown in Ref. 11, this complex is photodissociable and accurate triplet spectra could be obtained by subtracting the spectra before and after illumination.

Triplet EPR spectra were observed previously in the copper proteins hemocyanin, tyrosinase, ceruloplasmin and laccase treated with nitric oxide [20–24]. All these enzymes contain Cu-Cu metal pairs. Such EPR spectra could be simulated with a computer assuming that the Cu ions were coupled by a magnetic dipole-dipole interaction [21,24,25].

In this paper we report the EPR triplet spectra of cytochrome *c* oxidase, as measured at 9 GHz (with parallel and perpendicular microwave irradiation) and at 35 GHz. Furthermore, the temperature dependence of the triplet EPR signal was studied from 4.2 to 200 K. It is concluded that an exchange-interaction between cytochrome a_3 and Cu_B exists, but that a magnetic dipole-dipole interaction must explain the observed large anisotropy in the spectra. Computer simulations of these EPR spectra give information on the symmetry of the cytochrome a_3-Cu_B environment and yield a distance of 0.45 nm between the two metal atoms.

Methods

Cytochrome *c* oxidase was isolated by the method developed in our laboratory as described in Ref. 26 and dissolved in 50 mM potassium phosphate (pH 7.4)/0.5% Tween 80. Concentrations were determined optically with an absorbance coefficient (reduced minus oxidized) of 24 $mM^{-1} \cdot cm^{-1}$ at 605 nm [27]. Nitric oxide was purified as reported before [11]. Most experiments were carried out in anaerobic EPR tubes and anaerobiosis was achieved according to [11]. The samples for 35 GHz measurements and the samples for intensity measurements at 9 GHz were transferred via septa and gas-tight Hamilton syringes to the EPR tubes and immediately frozen.

Optical absorbance spectra were measured with a Cary-219 recording spectrophotometer. Most EPR spectra were obtained with a Varian E-9 spectrometer, coupled to a HP 2100 computer via an PDP 11/03 microcomputer. Spectra at temperatures above 77 K were recorded on a Varian E-3 spectrometer. For polarised 9 GHz measurements a Varian E-236 bimodal cavity was used. For the 35 GHz measurements a Varian E-110 microwave bridge was applied.

The helium flow units used for measurements from 4.2 to 90 K were as described in [28]. The temperature of the 9 GHz EPR system was measured with two calibrated carbon resistors placed in the EPR sample directly below and above the centre of the cavity, and a third carbon resistor directly under the EPR tube. The temperature at the sample was obtained by interpolation to the center of the cavity. During measurements with anaerobic EPR tubes and in the 35 GHz system only this third resistor was present.

Illumination was performed by irradiation through a light guide with a 150 W xenon lamp (Oriel). Wavelengths were changed by using differ-

ent interference filters (Balzers) and intensities were varied by a calibrated set of neutral density filters (Oriel). Light intensities were measured with a Photometer/Radiometer (E.G. and G. type 450). The temperature increase during illumination was checked to be less than 2 K.

Quantitation of low-spin haem EPR spectra was done by the method developed by Aasa and Vänngård [29] by integration of isolated low-field peaks.

Results

The addition of nitric oxide to cytochrome *c* oxidase in the presence of azide reveals an EPR spectrum characteristic for the triplet ($S = 1$) state [9–11]. Fig. 1 shows the formation of this complex as a function of the azide concentration. The results can be fitted to a hyperbolic binding curve with a dissociation constant K_d equal to 21.7 mM. The yield of this complex was independent of p_{NO} (0.1–100 kPa) and of pH in the range 6–9. The formed complex was sensitive to light, as shown previously [11]. Upon illumination of this complex in the 9 GHz spectrometer both the half-field signal at 150 mT and a broad signal from 250–400 mT disappeared indicating that both originate from the complex.

Fig. 2 shows the EPR spectra of cytochrome *c* oxidase in the presence of azide and NO, obtained before (Trace A) and after (Trace B) illumination. Apart from the disappearance of the triplet signal, a new EPR signal appeared with the same properties as pure nitric oxide in buffer (Fig. 3). The amount of NO observed by EPR was approximately the same as that of the triplet complex. After heating the sample to 77 K (Fig. 2, trace C) another EPR signal with *g*-values at 2.78, 2.2 and 1.75 appeared as reported before [11]. The formed EPR signal has the same EPR parameters as the two cytochrome $a_3^{3+} \cdot N_3^-$ complexes observed during partial reduction of cytochrome *c* oxidase in the presence of azide [6,30–32]. Crystal-field calculations, based on the formulae given in Ref. 33, showed no large differences in all three complexes (Table I). Integration of the EPR signal of the low-spin haem-azide complex obtained after heating gave an intensity of 0.8 spins per cytochrome *c* oxidase molecule indicating that at least 80% of the enzyme before illumination and heating was present as the complex showing the triplet

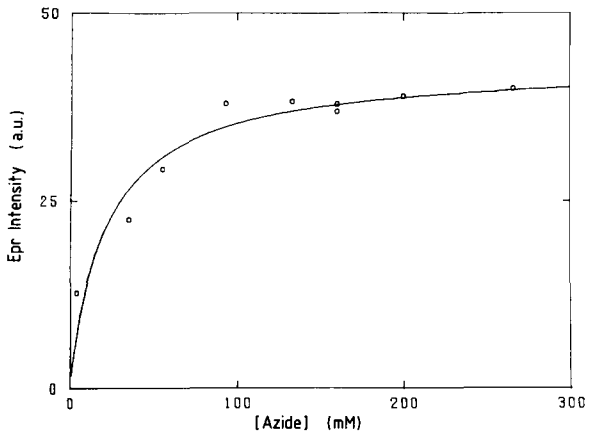


Fig. 1. The formation of the cytochrome $a_3^{2+} \cdot NO\text{-}Cu_B^{2+}$ complex of cytochrome *c* oxidase as a function of the azide concentration. The EPR intensity was obtained by double integration of the $\Delta m_s = 2$-signal. Conditions: 0.1–0.3 mM cytochrome *c* oxidase; 0.5% Tween 80, 50 mM potassium phosphate (pH = 7.4); p_{NO}, 60 kPa. Sodium azide was added to the sample before adding nitric oxide. The curve was obtained by least-square fitting of the data to a hyperbole.

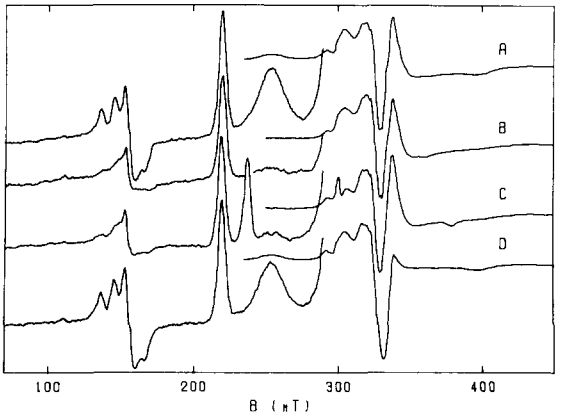


Fig. 2. Effects of illumination on the cytochrome $a_3^{2+} \cdot NO\text{-}Cu_B^{2+}\text{-}N_3^-$ complex of cytochrome *c* oxidase. A. EPR spectrum of cytochrome *c* oxidase plus azide after addition of nitric oxide. B. After illumination of A at 15 K. C. After heating B to 77 K. D. After thawing and freezing C. Conditions: 0.19 mM cytochrome *c* oxidase, 1% Tween 80; 100 mM potassium phosphate (pH 7.4); 90 mM sodium azide; p_{NO}, 60 kPa; 5 min incubation. EPR conditions: frequency, 9258 MHz; power, 30 dB; modulation amplitude, 1 mT; time constant, 1 s; scan rate, 50 mT·min^{-1}; temperature, 15 K.

EPR signal. Also some cytochrome $a_3^{2+} \cdot NO$ (less than 10%) was formed after heating. When the sample was allowed to warm further, it could be shown that at $-50°C$ the triplet complex was regenerated.

The photodissociation rates of light-sensitive heme-ligand complexes are proportional to the light intensity I [34]: $k = \varepsilon \Phi I$, where ε is the absorbance coefficient and Φ the quantum yield (i.e., the ratio of the number of dissociated molecules to the number of excited molecules). Since the cytochrome $a_3^{2+} \cdot NO$ complex can be photodissociated [11,35] we compared the dissociation rate, induced by illumination at 590 nm, of the triplet complex with the cytochrome $a_3^{2+} \cdot NO$ complex in fully reduced cytochrome c oxidase. Fig. 4 shows the dissociation rates of both complexes as a function of the light intensity. The slope observed for the triplet complex is only 0.10 compared to the slope of the fully reduced NO complex indicating that the complexes have different optical properties or a different quantum yield. The photodissociation rate of the triplet complex was also studied at different wavelengths. The high concentration of the EPR samples may cause that the light intensity in the centre of the EPR tube is considerably lower than at the surface. This results in a distribution of dissociation rates over the sample and a non-linear dissociation reaction (on a semi-logarithmic scale) will be observed. Therefore, the rate was obtained by measuring the initial rate of photodissociation.

Since the quantum yield of photodissociation reactions is nearly independent of the irradiation wavelength [36], it is possible to obtain the action spectrum for the photodissociation reaction, after correction for the different light intensities at each wavelength (Fig. 5). The spectrum is very similar to the action spectra in the visible region of cytochrome $a_3^{2+} \cdot NO$ in the fully reduced and mixed-valence cytochrome c oxidase [11]. There are, however, remarkable differences; in the ultraviolet a

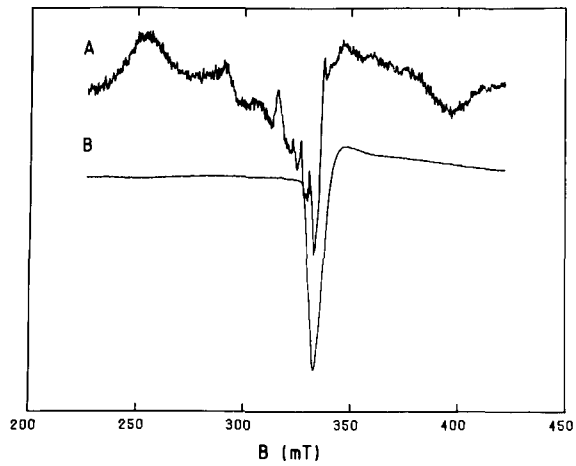


Fig. 3. EPR difference spectrum of the cytochrome c oxidase complex in the presence of azide and nitric oxide at 27 K and the EPR spectrum of nitric oxide in potassium phosphate buffer. The difference spectrum was obtained by subtracting the spectrum obtained after illumination from that before illumination. A. EPR difference spectrum of the complex. B. EPR spectrum of nitric oxide in buffer. Spectrum B was obtained with 1 mM NO in 50 mM potassium phosphate (pH 7.4). Conditions further as in Fig. 2. EPR conditions: frequency, 9258 MHz; power, 20 dB; modulation amplitude, 1 mT; scan rate 50 mT·min^{-1}; time constant, 1 s. Note that the EPR spectrum of nitric oxide (B) is drawn with a negative intensity, rescaled to the same concentration as the cytochrome c oxidase complex.

TABLE I

CRYSTAL-FIELD PARAMETERS DERIVED FROM THE g-VALUES OF CYTOCHROME a_3^{3+}-N_3^- COMPLEXES

The crystal-field parameters were calculated by an iterative method in the proper tetragonal axis system using the formulae given by Taylor [33].

Substance	g_x	g_y	g_z	A *	B *	C *	$A^2+B^2+C^2$	V/λ **	D/λ **	V/D **
$2e^-/aa_3/N_3^-$	1.647	2.210	2.896	0.971	0.237	0.107	1.010	2.09	4.42	0.47
$1e^-/aa_3/N_3^-$	1.745	2.197	2.779	0.979	0.203	0.089	1.007	2.48	5.26	0.47
$NO/N_3^-/aa_3$ (illuminated)	1.745	2.198	2.785	0.979	0.204	0.089	1.008	2.46	5.27	0.47

* A, B, C are the coefficients of the ground state Kramer's doublet.

** V, D and λ are the constants of rhombic splitting, the tetragonal splitting and of spin orbit coupling respectively.

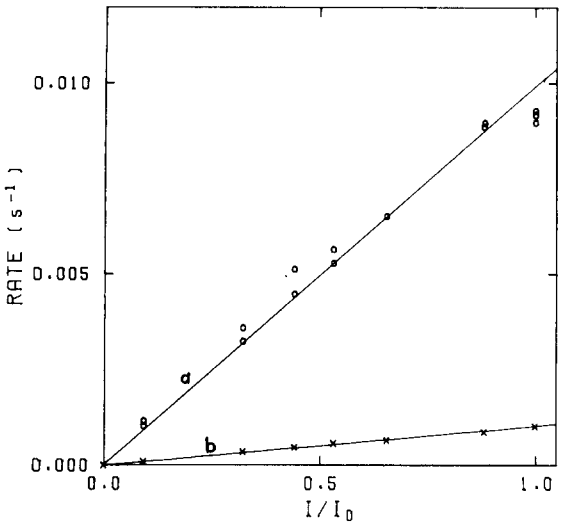


Fig. 4. Dependence of the dissociation rate of the cytochrome $a_3^{2+} \cdot NO$ complexes on the light intensities. The dissociation rates were calculated from the initial rates of the dissociation reactions. The irradiation wavelength was 590 nm. O, fully (dithionite-)reduced cytochrome *c* oxidase (sample a) x, cytochrome *c* oxidase complex in the presence of azide and nitric oxide (sample b). Conditions: 0.38 mM cytochrome *c* oxidase, 0.6% Tween 80, 75 mM potassium phosphate (pH 7.4), 100 mM sodium azide (sample b), p_{NO}, 20 kPa. EPR conditions: (a), frequency, 9259 MHz; power, 30 dB; modulation amplitude, 1 mT; field setting at 315.2 mT; temperature 25 K. (b), frequency, 9248 MHz; power, 0 dB; field setting, 255 mT; modulation amplitude, 1.25 mT; temperature, 20 K.

much higher absorbance for the triplet complex is observed and in addition, the intensity at 590 nm is only 0.10 of that of the normal cytochrome $a_3^{2+} \cdot NO$ complex. These differences are ascribed to the difference in valence state of Cu_B in the triplet complex compared to the other complexes, which contain Cu_B^{1+}.

The triplet signal observed by EPR is probably caused by magnetic interaction between two spins $S = 1/2$ [9,10]: cytochrome $a_3^{3+} \cdot NO$ and Cu_B^{2+} have both a spin $S = 1/2$. As discussed in the Appendix such an interaction can stem from isotropic and anisotropic exchange interactions or from a strong dipole-dipole interaction. Since an isotropic exchange interaction between two spin $S = 1/2$ systems leads to the formation of singlet and triplet levels, the temperature dependence of the normalised integrated area A of the EPR signal should follow [37] ($J \gg D$):

$$AT = \frac{3e^{-J/kT}}{1+3e^{-J/kT}}$$

where J is the energy difference between the resulting singlet and triplet levels. Fig. 6 shows the temperature dependence of the half-field EPR triplet signal. From 77 to 200 K the EPR signal showed normal Curie behaviour. The temperature dependence can be fitted to an isotropic exchange interaction $J = -7.1\ cm^{-1}$, showing that the complex has a triplet ground state and a singlet excited state.

However, the observed broad EPR spectrum from 250–400 mT of the triplet complex cannot be explained by isotropic exchange interaction. The large anisotropy of the spectrum could be caused by a large zero-field splitting within the triplet levels, due to anisotropic exchange or magnetic dipole-dipole interaction, or even to a large *g*-anisotropy. To study this EPR signal in more detail we have measured EPR difference spectra before-minus-after illumination of the complex, by irradiation with microwaves polarized parallel and perpendicular to the magnetic field.

Fig. 7 shows that the half-field transitions (Δm_s

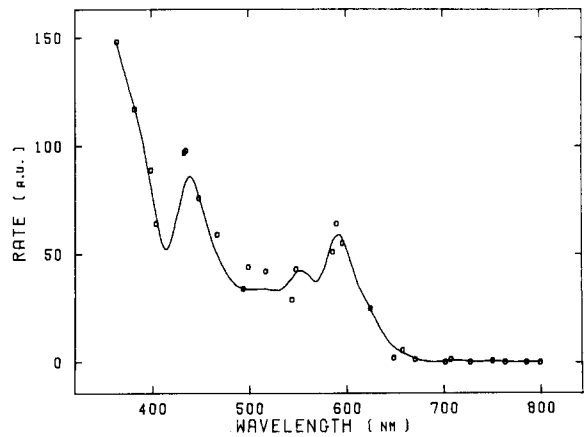


Fig. 5. Action spectrum of the photodissociation reaction of the cytochrome *c* oxidase complex in the presence of azide and nitric oxide. Wavelengths were varied with interference filters. The dissociation rates were corrected for the differences in transmission of the different interference filters. The dissociation rates were calculated as in Fig. 4. Conditions and EPR conditions were as in Fig. 4.

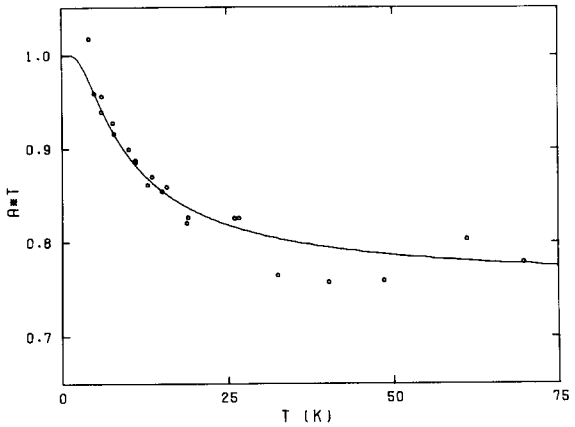


Fig. 6. The temperature dependence of the EPR intensity of the cytochrome *c* oxidase-triplet complex in the presence of azide and nitric oxide. The intensities, *A*, were obtained by double integration of the $\Delta m_s = 2$ transition and multiplied by the temperature, *T*, to correct for the Curie behaviour. The curve was obtained by least-square fitting of the data to the expression of the temperature dependence of a triplet EPR signal. Conditions: 0.4 mM cytochrome *c* oxidase; 0.5% Tween 80; 50 mM potassium phosphate (pH 7.4); 100 mM sodium azide; p_{NO}, 50 kPa, EPR conditions: frequency, 9249 MHz; power, 6–18 dB; modulation amplitude, 1 mT; time constant, 0.3–3 s.

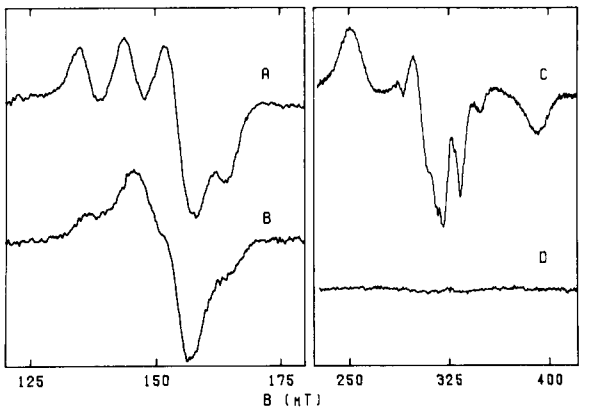


Fig. 7. EPR spectra of the cytochrome *c* oxidase complex in the presence of azide and nitric oxide at 4.2 K. The difference spectra were obtained by subtracting the spectra obtained after illumination from those before illumination. A and C, perpendicular mode of microwave irradiation; B and D, parallel mode. EPR conditions: frequency, 9150 MHz; power, 18 dB; gain, $1.25 \cdot 10^4$ for $\Delta m_2 = 1$ and $2.5 \cdot 10^4$ for $\Delta m_s = 2$; modulation amplitude, 1 mT; time constant, 1 s; scan rate 12.5 mT·min^{-1}; temperature, 4.2 K. Conditions as Fig. 6. Illumination was done by rectangular irradiation into the bimodal cavity. The parallel mode experiment, measured at 9094 MHz was recalculated to 9150 MHz.

$= 2$) in both modes have intensities of the same order of magnitude, while the $\Delta m_s = 1$ transitions are observed only with perpendicular polarized microwaves. This behaviour of the EPR spectra can be explained by the existence of a triplet $S = 1$ system, with a large zero-field splitting. The hyperfine splitting present in the $\Delta m_s = 2$-spectra is due to interaction of the spins in the triplet with the nuclear spin on Cu_B^{2+} ($I = 3/2$). From the hyperfine splitting of $98 \cdot 10^{-4}$ T (Fig. 7A) and an average *g*-value of 2.11, for the triplet complex in the same direction as used in the simulations of the EPR spectra, a hyperfine interaction constant was calculated of $193 \cdot 10^{-4}$ cm^{-1}.

The interpretation of the $\Delta m_s = 1$-spectrum is more complicated. The signals at 255 and 396 mT belong to the triplet complex. The signal at 325 mT is greatly affected when the difference spectrum was obtained at other temperatures (compare Fig. 3 with Fig. 7) and is partly arising from dissociated NO. The peaks at 296 and 365 mT also seem to belong to the triplet spectrum as well as the peaks at 312 and 352 mT. Several assignments of the triplet spectrum are possible. It is conceivable that the transitions at $h\nu/g\beta \pm D'$ (see Appendix) occur at 255 and 396 mT, while the transitions at 296 and 365 mT are from the $h\nu/g\beta \pm \frac{1}{2}D'$ transitions of the triplet system. However, it is also possible that the rhombic zero-field splitting is very large: $|D| = 3|E|$. Then two resonances will be observed at $h\nu/g\beta \pm D'$ and one at $h\nu/g\beta$, in line with the assumption of Brudvig et al. [10] in their interpretation of the triplet EPR spectrum. The calculated axial zero-field splitting parameter *D* of the triplet state for both assignments is then 0.067 cm^{-1}. A third possibility is that the signals at 255 and 396 mT are due to $h\nu/g\beta \pm \frac{1}{2}D'$ and the transitions at $h\nu/g\beta \pm D'$ are too weak to observe. The calculated zero-field splitting parameter *D* is then 0.134 cm^{-1}. Finally, a part of the observed large anisotropy may be explained by *g*-anisotropy.

It is possible to settle the origin of these and the other signals appearing in the difference spectra by measuring at other microwave frequencies. Therefore, we also recorded 35 GHz EPR spectra. Fig. 8 shows the difference EPR spectra obtained by subtracting the spectra before and after il-

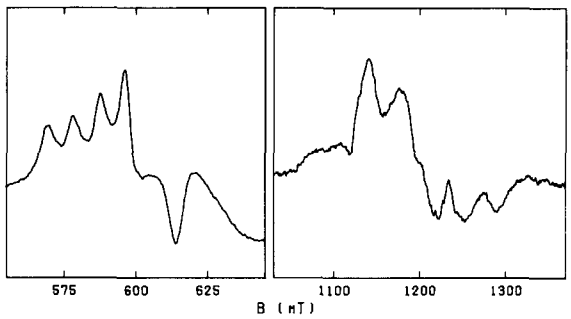


Fig. 8. EPR difference spectra of the triplet-complex of cytochrome *c* oxidase measured at 35 GHz. Conditions: preparation of the sample as in Fig. 6. The sample was transferred anaerobically into a 35 GHz EPR tube. Illumination was done at 77 K outside the 35 GHz cavity. EPR conditions: frequency, 34.65 GHz; power, 6 dB; gain $6.3 \cdot 10^1$ for $\Delta m_s = 1$ and $1.25 \cdot 10^3$ for $\Delta m_s = 2$; modulation amplitude, 2 mT; time constant, 0.33; scan rate, 50 mT·min^{-1}, the spectra are the average of 16 scans.

lumination. The $\Delta m_s = 1$-spectrum had the same width of 145 mT at 35 GHz and at 9 GHz: some structure within the spectrum is different, possibly due to *g*-anisotropy. The spectrum can be assigned in the following way: the resonances at 1090 and 1290 mT correspond to $h\nu/g\beta \pm D'$, while the resonances at 1140, 1190, 1250 and 1290 mT arise from the transitions at $h\nu/g\beta \pm \frac{1}{2}(D' \pm 3E')$. The axial and rhombic zero-field splitting parameters D and E are then 0.091 and 0.007 cm^{-1}. The $\Delta m_s = 2$-spectrum shows the same hyperfine interaction of $193 \cdot 10^{-4}$ cm^{-1} as at 9 GHz, but some *g*-anisotropy becomes visible. Thus, the anisotropy in the $\Delta m_s = 1$ spectrum of 145 mT at both frequencies is mainly caused by a zero-field splitting constant. The deviation in estimated zero-field splitting parameters is due to the fact that the approximate assignments are only correct assuming $D \ll h\nu$.

Another complicating factor in the assignment of the resonances in the triplet system is the assumption that the *g*-tensor and the spin-spin interaction *D*-tensor diagonalise in the same coordinate system [38]. When the *D*-tensor is not parallel to the *g*-tensor of the triplet system (which is possible for example, when the spin-spin interaction is mainly due to a magnetic dipole-dipole interaction in a direction not parallel to one of the main axes of the *g*-tensor) the half-field transitions in a parallel-polarised microwave measurement will show up

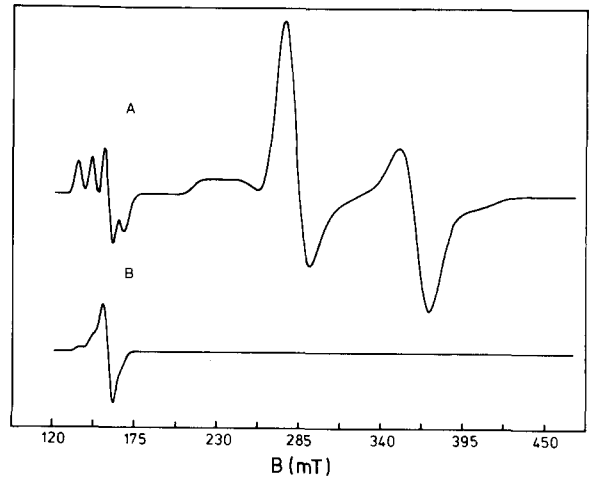


Fig. 9. Simulated X-band EPR spectra of the cytochrome $a_3^{2+} \cdot$NO-Cu_B^{2+} complex. The calculations included dipole-dipole interaction only and were done with the program DISSYM (see Appendix). (A), perpendicular-mode irradiation; (B), parallel-mode irradiation; frequency, 9150 MHz. Further parameters in Table II A.

nuclear hyperfine interaction (on the assumption that the hyperfine tensor and the *g*-tensor are parallel). As can be observed in the half-field spectra in Fig. 7 some hyperfine structure is visible in the parallel mode. Thus, the direction of the anisotropic spin-spin interaction is not completely

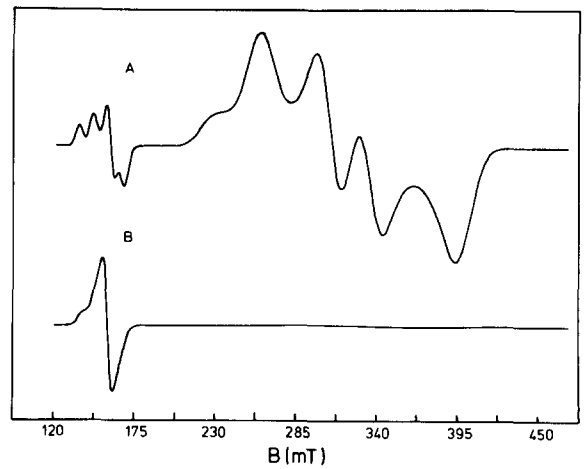


Fig. 10. Simulated X-band EPR spectra of the cytochrome $a_3^{2+} \cdot$NO-Cu_B^{2+} complex. The calculations included both dipole-dipole interaction and pseudodipolar exchange and were done with the program DIAGON (see Appendix). A, perpendicular mode irradiation; B, parallel-mode irradiation; frequency, 9150 MHz. Further parameters in Table II B.

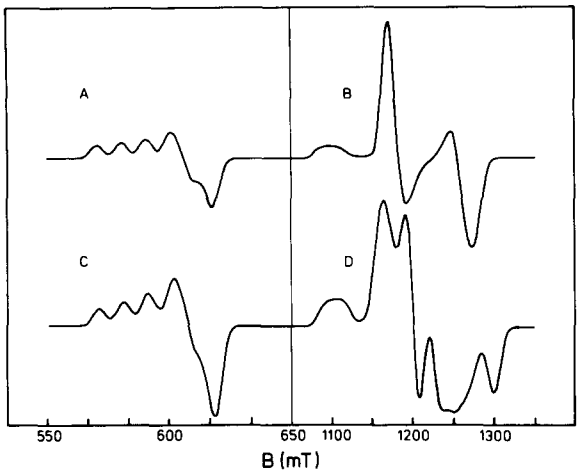


Fig. 11. Simulated Q-band EPR spectra of the cytochrome a_3^{2+}·NO-Cu_B^{2+} complex. A and B were calculated with the program DISSYM (see Appendix) assuming dipole-dipole interaction only (Table IIA); C and D were calculated with the program DIAGON and assumed both dipole-dipole interaction and pseudo-dipolar exchange (Table II B); frequency, 34.65 GHz. It should be noted that A is expanded 20× with respect to B and C 40× with respect to D.

parallel to one of the main axes of the Cu-hyperfine tensor. This complicates the above assignment much more and to interpret the observed EPR spectra we used the computer programs based on the theory outlined in the Appendix and treated in detail by Schoot Uiterkamp et al. [20] to simulate all triplet spectra. To calculate the spectra we assumed that the triplet is due to an axial copper ion ($S = 1/2$) which has a magnetic interaction with the NO radical in cytochrome a_3^{2+} · NO ($S = 1/2$). The interaction was assumed to be due to isotropic exchange and a magnetic dipole-dipole interaction. The simulation parameters were g values (g_x, g_y, g_z), linewidths (W_x, W_y, W_z) and nuclear hyperfine interaction (I, A_x, A_y, A_z) for both spins, orientation of the spins relative to each other (α, β, γ), orientation of the interaction (ε, η) and distance (r) between the spins. Several hundreds of simulations were performed with the programs DISSYM and DIAGON (see Appendix) varying distance, orientation and anisotropy. The shape of the simulated 9 and 35 GHz spectra ($\Delta m_s = 2$) depended very critically on g-anisotropy and direction of the dipolar interaction. The 9 GHz spectra of the $\Delta m_s = 2$-transition in the parallel mode were very sensitive to the orientation parameters. The width of the $\Delta m_s = 1$-spectrum and the intensities of the $\Delta m_s = 2$-spectra changed strongly with the distance between the spins. The half-field EPR spectra could be simulated reasonably well assuming this simple spin-spin interaction. Figs. 9 and 11 (A and B) show the results of simulation by the DISSYM program assuming that the only anisotropic interaction is a magnetic dipole-dipole interaction. However, to obtain

TABLE II

EPR PARAMETERS OF THE DIPOLAR-COUPLED CYTOCHROME (a_3-Cu_B) PAIR

All values are measured in 10^{-4} cm^{-1}. D is the pseudodipolar exchange, defined along the z-axis. α, β, γ define the Euler transformation of the coordinates of spin 2 to the coordinates of spin 1; ε, the angle between r and the z-axis of spin 1; η, the angle between the projection of r in the xy-plane of spin 1 and the x-axis of spin 1; r, the distance between spin 1 and spin 2.

	g_x	g_y	g_z	A_x	A_y	A_z	W_x	W_y	W_z
Cu^{2+}	2.00	2.00	2.22	10	10	195	35	35	35
							70	70	70
Cytochrome a_3^{2+}·NO	2.08	2.00	2.00	–	–	–	35	35	35
							70	70	70

A. Dipolar interaction
- $J = -7.06$ cm^{-1}
- $D = 0$ cm^{-1}
- $\alpha = \beta = \gamma = 0$
- $\varepsilon = 25$
- $\eta = 0$
- $r = 0.325$ nm

B. Dipolar interaction plus pseudodipolar exchange
- $J = -7.06$ cm^{-1}
- $D = 0.04$ cm^{-1}
- $\alpha = \beta = \gamma = 0$
- $\varepsilon = 30$
- $\eta = 0$
- $r = 0.35$ nm

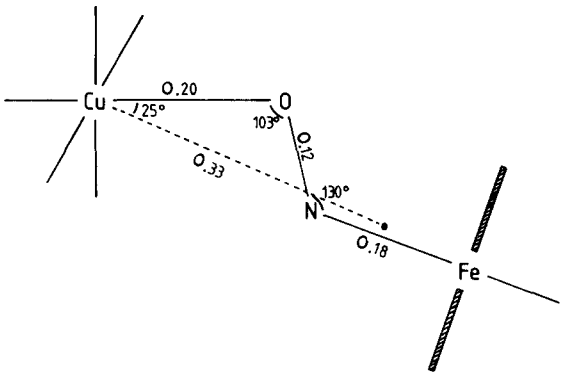


Fig. 12. Model of the cytochrome a_3^{2+}-NO-Cu_B^{2+} complex. The copper atom is proposed to have a tetragonal environment and has its x- and z-axis in the plane formed by Fe-N-O. The z-axis points to the oxygen atom of nitric oxide. The model drawn corresponds to a copper-iron distance of 0.42 nm. The black dot represents the assumed position of the free electron.

genuine $\Delta m_s = 1$-spectra, large line-widths had to be assumed. Therefore a second anisotropic interaction, the pseudodipolar exchange, was added to the magnetic dipole-dipole interaction. It was assumed that this second interaction had a different orientation. Figs. 10 and 11 (C and D) show the resulting spectra, which were simulated with the program DIAGON. The simulation parameters of the computed spectra in Figs. 9–11 are given in Table II. The distance parameters correspond with an axial zero-field splitting $D \approx 0.10$ cm^{-1} and a rhombic splitting $E \approx 0.002$ cm^{-1} and explain most of the observed anisotropy.

Discussion

Simulations of the EPR spectra show that the observed zero-field splitting in the cytochrome c oxidase-azide-NO-complex can be attributed mainly to a magnetic dipole-dipole interaction between the electron spin on cytochrome $a_3^{2+} \cdot$ NO and the electron spin on a closely located copper ion as assumed by Brudvig et al. [10]. However, part of the observed anisotropy can be due to pseudodipolar and antisymmetric exchange [37–39] that arises from the combined effect of spin-orbit coupling and exchange. The contribution of the pseudodipolar exchange (D_{pseudo}) to the observed anisotropy ($D \approx 0.10$ cm^{-1}) can be estimated from the deviation of the g-values from 2.0023, which is related to the spin-orbit interaction and to the exchange interaction in the excited state [37]:

$$g \approx g_e\left(1+\frac{\lambda}{\Delta}\right)$$

$$D_{pseudo} \approx \left(\frac{\lambda}{\Delta}\right)^2 J$$

where λ is the spin-orbit coupling constant and Δ the energy difference between the ground and excited states. With $g = 2.11$ and $J = -7.1$ cm^{-1} a maximal contribution of 0.02 cm^{-1} for the pseudodipolar interaction can be calculated, assuming that J is equal in the ground and excited states. Also the antisymmetric exchange may contribute to the observed anisotropy in this asymmetric spin system. The antisymmetric exchange(d) couples the single and triplet states but not within the triplet states [39] and gives a contribution to the zero field-splitting [38] of:

$$D_{antisymmetric} \approx \frac{d}{J},\ d = \left(\frac{\lambda}{\Delta}\right)^2 J$$

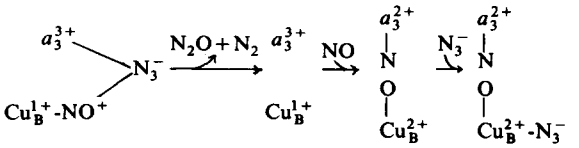
which is of the same order as the pseudodipolar exchange. Thus both interactions together could explain an anisotropy of 0.04 cm^{-1} in the $\Delta m_s =$ 1-spectrum. However, it can be argued that the contributions are largely overestimated. In a large number of Cu-metal pairs [25] it was found that up to values of $|J| = 30$ cm^{-1}, the pseudodipolar exchange did not contribute significantly to the zero-field splitting. Also the exchange parameters for the excited state can be smaller by an order of magnitude than the ground state value [37]. Finally, the exchange coupling between cytochrome $a_3^{2+} \cdot$ NO and Cu_B^{2+} is likely to occur via NO which is bound on the 6th ligand binding position of the iron in cytochrome a_3. However, the spin-orbit coupling of the cytochrome $a_3^{2+} \cdot$ NO complex is very small, especially in the direction (z) of this ligand, as estimated from the g-values: $g_x = 2.09$, $g_y = 2.00$ and $g_z = 2.005$ [40].

Therefore, it is reasonable to assume that the observed zero-field splitting is largely due to a magnetic dipole-dipole interaction. This hypothesis is supported by our calculations which included the pseudodipolar exchange. From the EPR simulations then a distance of 0.33 ± 0.02 nm was

determined between the electron spin on cytochrome $a_3^{2+} \cdot NO$ and Cu_B^{2+} when a point-dipole approximation was used. It is generally accepted that such an assumption may give errors of approx. 10% in the value of the distance [40]. It should further be emphasized that the location of the free electron in the cytochrome $a_3^{2+} \cdot NO$ complex does not coincide with the iron ion as is indicated already by the different hyperfine splitting constants of the electron with the nitrogens from the axial histidine ($A = 6.3 \cdot 10^{-4}$ cm^{-1}) and NO ($A = 19.0 \cdot 10^{-4}$ cm^{-1}) [40]. The unpaired electron density for the β subunit of nitrosyl hemoglobin, which has similar EPR parameters as cytochrome $a_3^{2+} \cdot NO$, was found to be 0.6 on the nitrogen of NO and about 0.4 on the iron [41]. In a recent ENDOR study on cytochrome $a_3^{2+} \cdot NO$ [42] the unpaired electron spin was found to be 0.7 on the iron and about 0.2 on the nitrogen of NO. As a model the average position of the unpaired electron is assumed to be almost in the middle of the Fe-N bond.

The nitric oxide must be bound at the same side of the haem plane as Cu_B, otherwise the Fe-Cu distance would be unreasonable small. Flash-photolytic experiments of cytochrome $a_3^{2+} \cdot NO$ [11,35] and cytochrome $a_3^{2+} \cdot CO$ [8] in the fully reduced enzyme show that the ligand was bound to Cu_B after photolysis. This is also in agreement with the idea that the ligand binding site and Cu_B are at one side of the haem plane. With a bond length for Fe-N between NO and cytochrome a_3^{2+} of 0.175 nm, the distance between Fe and Cu will be 0.40–0.50 nm depending on the geometry of the complex and location of the electron spin. Assuming that the NO molecule is bound in a staggered bridge the metal-metal distance is 0.45 nm. The observed distance is then in good agreement with the value (0.375 nm) calculated from Cu and Fe EXAFS data of oxidized cytochrome *c* oxidase. [7]. Fig. 12 gives a schematic presentation of the geometry of the NO binding site in the 'triplet' complex. Cu_B has the EPR parameters (cf. Table II) of a copper ion with a tetragonal environment [24] as proposed originally by Brudvig et al. [43]. This is in good agreement with the observed reactivity of Cu_B. A similar tetragonal environment is also observed in the EPR spectra of other proteins, containing type III copper, when they are treated with nitric oxide [21].

We have strong indications in line with Refs. 12 and 43 that the above mentioned (cytochrome a_3-Cu_B) pair can bind two ligands simultaneously. The EPR experiments show that the complex formed in the presence of azide and nitric oxide still contains azide: after illumination and heating to 77 K an EPR signal was formed with the same EPR parameters as other cytochrome *c* oxidase-azide complexes [6,30–32]. On the other hand also evidence is present that the complex contains NO, as was already assumed in the discussion of the triplet EPR signal: the optical absorbance spectrum of this complex and the action spectrum had maxima at the same wavelengths (595, 560 and 430 nm) as cytochrome $a_3^{2+} \cdot NO$ [9,11]. Furthermore, after flash photolysis of this complex NO is released as was observed in the EPR spectra (cf. Fig. 3). The presence of two ligand binding sites in the (a_3-Cu_B) pair also explains the rapid formation of the triplet complex. When NO is added to oxidized cytochrome *c* oxidase, NO binds to Cu_B forming a Cu_B^{1+}-NO^+ complex [9,11]. This complex can also be formed when cyanide is bound to cytochrome a_3^{3+} [12,43]. The cyanide molecule is suggested to bridge between cytochrome a_3^{3+} and Cu_B [12,44]. A similar complex may be present, when azide and NO are added to oxidized cytochrome *c* oxidase. Since nitrosyl-azide is very unstable forming N_2O and N_2 [10,45], a one-electron reduction of Cu_B^{2+} results. By binding again NO and N_3^- the 'triplet' complex is formed:



In the 'triplet' complex NO forms a bridge between cytochrome a_3 and Cu_B as proposed by Stevens et al. [9]. This ligand bridge is responsible for the isotropic exchange interaction of -7.1 cm^{-1} between both spins in the triplet complex.

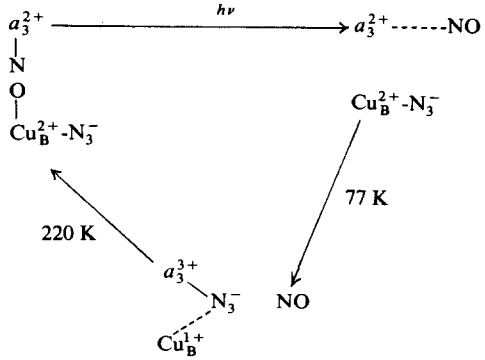
The presence of two ligand binding sites very close to each other may have implications on the mechanism of the oxygen reduction reaction of cytochrome *c* oxidase. The intermediates of the oxygen reduction, like O_2^- and O_2^{2-}, which are formed directly after the binding of oxygen to the

reduced cytochrome (a_3-Cu_B) pair [3], can bridge between the iron of cytochrome a_3 and Cu_B, as suggested by several authors [13–16] and are thus structurally related to the 'triplet' complex. The direction of the dipolar interaction in the 'triplet' complex points to a NO molecule bonded in a staggered bridge, which is also the preferred structure for superoxo and peroxo bridges [45].

The estimated distance between Cu and Fe of 0.45 nm in the 'triplet' complex is close to the expected value in these oxygen intermediates.

The second binding site which in the 'triplet' complex is occupied by azide is of importance in the further reduction of peroxide: the reduction is accompanied by bond breaking of the peroxide and formation of two reduced oxygen atoms [4]. One of the oxygen atoms could be bound in a μ-oxo bridge between the ferryl iron of cytochrome a_3 and Cu_B as proposed by Peisach et al. [15] or is only bound to cytochrome a_3 as suggested by Karlsson et al. [17]. The other oxygen atom is bound then as hydroxide to Cu_B [5]. During further reduction of the cytochrome (a_3-Cu_B) pair these ligands dissociate and a new oxygen molecule is bound and reduced.

Upon illumination of the triplet complex two processes occur: photodissociation and redox reactions. The major process is the photodissociation of cytochrome $a_2^{2+} \cdot NO$, which results in the disappearance of the triplet signal and the appearance of broad signals part of which is due to free NO. It has been observed before [35] that upon photodissociation of cytochrome $a_3^{2+} \cdot NO$ in fully reduced cytochrome *c* oxidase at low temperature a broad signal appeared, which was assigned to the formation of a Cu_B^{1+}-NO complex ($S = 1/2$). Also Cu_B^{2+} is able to bind NO as can be concluded from EPR studies on oxidised cytochrome *c* oxidase [9,11,12,43]. In the 'triplet' complex the released nitric oxide after photodissociation, seems not to bind to Cu_B^{2+}, possibly because the binding site for NO on Cu_B^{2+} is occupied by azide. Upon warming this photodissociated complex to 77 K, an EPR signal of the cytochrome $a_3^{3+} \cdot N_3^-$ complex appeared. Two cytochrome $a_3^{3+} \cdot N_3^-$ complexes are described in the literature: one with a characteristic $g = 2.9$ signal and the other with a $g = 2.77$ signal [6,30]. While the signal at $g = 2.9$ usually arises during reductive titrations [6], the signal at $g = 2.77$ can be seen in oxidative titrations [32] and in the first phase of reductive titrations [31]; this may imply that the azide-complex with the EPR signal at $g = 2.77$ is due to a molecule of cytochrome *c* oxidase reduced by one electron. The azide-complex observed in our experiments after photodissociation of the triplet-complex has the same *g*-values as this one-electron reduced cytochrome *c* oxidase. When this complex is heated to $-50°C$, the triplet complex is slowly formed again. The reaction cycle can be summarised as follows:



The reaction cycle shows that a reversible electron transfer is possible between cytochrome a_3 and Cu_B.

A minor process which occurs during illumination of the triplet complex is an irreversible reduction of Cu_B^{2+}: this is responsible for the appearance of some cytochrome $a_3^{2+} \cdot NO$ (less than 10%) with EPR signals at $g = 2$. This reduction might be due to direct photoreduction of Cu_B^{2+}, similarly to the photoreduction of type III copper in laccase and ceruloplasmin [46] or photoreduction of cytochrome a^{3+} [47] followed by electron transfer to Cu_B^{2+}. Some reversible intensity changes in the cytochrome a^{3+} intensity at $g = 3.03$ were observed during the cycles: after illumination the peak at $g = 3.03$ has diminished by 5–10% due to reduction of cytochrome a^{3+} by cytochrome a_3^{2+}. Similar electron transfer processes were observed before after photodissociation of mixed-valence NO-cytochrome *c* oxidase [11]. However, after binding of N_3^- the cytochrome a^{3+} intensity was restored completely, possibly due to reduction of Cu_B. Thus, the midpoint potential of Cu_B is higher than that of cytochrome *a* and a_3 under these conditions.

The observed action spectrum of the disappearance of the triplet complex is very similar to the optical absorbance and action spectra of cytochrome $a_3^{2+} \cdot NO$ in the fully reduced and mixed-valence cytochrome *c* oxidase NO complexes [11], having absorbance maxima at 595, 560 and 430 nm. There is, however, an additional band at a wavelenth smaller than 360 nm. This is possibly due to an absorbance band of Cu_B^{2+} in the 'triplet' pair, similar to the 320–340 nm bands of Type III copper in laccase [48], hemocyanin [49] and ceruloplasmin [50]. Another difference is the decrease in quantum yield of the disappearance of this complex, compared to cytochrome $a_3^{2+} \cdot NO$ in the fully reduced enzyme. The main difference between both complexes is the valence state of Cu_B, indicating that the redox state of this atom affects the properties of the haem in cytochrome a_3. The same conclusion was drawn from optical studies in which the absorbance spectrum of cytochrome $a_3^{2+} \cdot NO$ was investigated and was found to be affected by the redox state of Cu_B [51].

Another interaction between cytochrome $a_3^{2+} \cdot NO$ and Cu_B^{2+} is the observed isotropic exchange interaction. Similar exchange interactions were thought to exist in oxidised cytochrome *c* oxidase [6,52] and the cyanide complex of cytochrome *c* oxidase [44] because of the absence of simple EPR signals in these systems attributable to cytochrome a_3^{3+} or Cu_B^{2+}. In the oxidized enzyme high-spin Fe^{3+} and Cu_B^{2+} are suggested to couple to an $S = 2$ system, while in the cyanide complex low-spin $Fe^{3+} \cdot CN^-$ and Cu_B^{2+} couple to a $S = 1$ system [44]. Another explanation for the absence of EPR signals could be that cytochrome a_3 contains high-spin Fe^{4+} [53,54] which is converted to low-spin upon addition of cyanide [39] and that the redox state of Cu_B is unchanged in most redox experiments. Our experiments clearly show that magnetic interactions can exist between cytochrome a_3 and Cu_B, though the observed isotropic exchange interaction ($J = -7.1$ cm^{-1}) is much weaker than the value used to explain the EPR properties of oxidised cytochrome *c* oxidase [52,55] ($J \geqslant 400$ cm^{-1}). The difference in exchange value may be due to the presence of different bridging ligands in these complexes. On the other hand, for the explanation of the EPR spectrum of the reaction intermediate of fully reduced cytochrome *c* oxidase with oxygen an exchange interaction of about 10 cm^{-1} was used [5]. Thus the 'triplet' complex is a good model complex for the oxygen binding site of cytochrome *c* oxidase.

The cytochrome (a_3-Cu_B) pair is very similar in magnetic behaviour to the type III copper-pair in hemocyanin. In this latter protein a large isotropic exchange interaction ($J \geqslant 550$ cm^{-1}) exists between both Cu^{2+} ions in the oxy state [56]. Upon treatment with nitric oxide (in the presence of traces of oxygen) [57] a triplet EPR signal arises; the exchange interaction diminishes to $|J| \leqslant 14$ cm^{-1} and the triplet state arises due to a dipole-dipole interaction between the two ions [21]. This change in magnetisation is related to a change in distance between both copper ions: from 0.37 nm in the oxy-state [7] to 0.6 nm in the dipolar-coupled state [21].

Also type III copper of ceruloplasmin [22] and laccase [23] show triplet signals upon treatment with nitric oxide under appropriate conditions, whereas in the oxidised state large exchange interactions are present [56,58]. Thus the dipole-dipole interaction between two metal ions after treatment with nitric oxide seems a common feature of the oxygen-binding and reducing enzymes.

In the presence of other ligands the distance between copper ions in the dipolar-coupled state in hemocyanin can be varied to 0.3 nm (with Br^-) and 0.5 nm (with N_3^-) [24]. These experiments show the flexibility of multicentre metalloproteins. A similar flexibility is observed in the cytochrome (a_3-Cu_B) pair of cytochrome *c* oxidase. The ion-copper distance changes from 0.375 nm in the resting state [7], to 0.45 nm in the triplet state and about 0.5 nm in cyano-cytochrome *c* oxidase [12,59]. The same flexibility can be observed in the various conformations of the cytochrome (a_3-Cu_B) pair in the oxidised enzyme [43].

Conclusively, it is stated that our experiments show that cytochrome a_3 and Cu_B are closely located to each other, presenting a flexible cage where oxygen easily binds as a bidentate during the reduction cycle.

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