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COPPER AND MANGANESE ELECTRON SPIN RESONANCE STUDIES OF CYTOCHROME *c* OXIDASE FROM *PARACOCCUS DENITRIFICANS*

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The two-subunit cytochrome *c* oxidase from *Paracoccus denitrificans* contains two heme *a* groups and two copper atoms. However, when the enzyme is isolated from cells grown on a commonly employed medium, its electron paramagnetic resonance (EPR) spectrum reveals not only a Cu(II) powder pattern, but also a hyperfine pattern from tightly bound Mn(II). The pure Mn(II) spectrum is observed at −40°C; the pure Cu(II) spectrum can be seen with cytochrome *c* oxidase from *P. denitrificans* cells that had been grown in a Mn(II)-depleted medium. This Cu(II) spectrum is very similar to that of cytochrome *c* oxidase from yeast or bovine heart. Manganese is apparently not an essential component of *P. denitrificans* cytochrome *c* oxidase since it is present in substoichiometric amounts relative to copper or heme *a* and since the manganese-free enzyme retains essentially full activity in oxidizing ferrocytochrome *c*. However, the manganese is not removed by EDTA and its EPR spectrum responds to the oxidation state of the oxidase. In contrast, manganese added to the yeast oxidase or to the manganese-free *P. denitrificans* enzyme can be removed by EDTA and does not respond to the oxidation state of the enzyme. This suggests that the manganese normally associated with *P. denitrificans* cytochrome *c* oxidase is incorporated into one or more internal sites during the biogenesis of the enzyme.

Introduction

Beef heart cytochrome *c* oxidase (EC 1.9.3.1) contains two hemes (*a* and a_3) as well as two copper atoms, one of which is EPR visible (for comprehensive reviews, see Refs. 1 and 2). The association of the copper atoms with one of the seven subunits of the enzyme remains unclear [3]; however, sequence homology of subunit II with other copper-containing polypeptides [4] and experiments involving gentle dissociation of the enzyme [5] suggest that at least one of the copper atoms is bound to subunit II.

Recently, a cytochrome *c* oxidase was isolated from the bacterium *Paracoccus denitrificans* [6]. It contains only two subunits which resemble the two largest subunits of the seven-subunit mitochondrial enzymes; subunit II of *P. denitrificans*, yeast and bovine cytochrome *c* oxidase even show immunological cross-reactivity [7]. This bacterial enzyme rapidly oxidizes mammalian cytochrome *c* and has spectroscopic properties identical to those of the mitochondrial oxidases. It contains one copper atom for each of the two heme *a* groups [6]. This appears to conflict with reports that Cu(II) could not be detected by EPR in cytoplasmic membranes from *P. denitrificans* (see Ref. 8). Recently, however, such a Cu(II) signal was demonstrated in membranes and attributed to the oxidase [9].

Here we demonstrate that the copper of the purified two-subunit *P. denitrificans* oxidase gives rise to an EPR spectrum which is qualitatively identical to that of cytochrome *c* oxidase from yeast or bovine heart. However, the copper spectrum in the purified oxidase is obscured by an intense spectrum of tightly bound Mn(II). While this Mn(II) is not essential for the oxidation of ferrocytochrome *c*, it behaves as a 'reporter' for the oxidation state of the enzyme.

Materials and Methods

Paracoccus denitrificans (ATCC 13543) was grown aerobically in a synthetic medium in the presence of 50 μM $MnCl_2$ and succinate as the major carbon source [10]. When indicated, the concentration of $MnCl_2$ in the growth medium was lowered to 0.8 μM which did not affect growth parameters. (When $MnCl_2$ was strictly omitted from the culture, growth of cells was severely retarded or ceased all together). Cytochrome *c* oxidase was purified from cytoplasmic membranes and analyzed for purity and activity according to previous procedures [6,7]. Cytochrome *c* oxidase from yeast [11] and bovine heart [12] were isolated according to published procedures.

EPR spectroscopy was carried out with a standard Varian E-9 spectrometer operating at 9.3 GHz with a 100 kHz field modulation unit. Metal atom determinations were performed by atomic absorption spectroscopy. Enzyme concentrations and additions for recording EPR spectra are detailed in the figure legends.

Results and Discussion

The EPR-visible Cu(II) ion in cytochrome *c* oxidase from bovine heart gives rise to a unique EPR spectrum with an intense signal around $g \cong 2$ [13–18]. Yeast cytochrome *c* oxidase exhibits the same type of Cu(II) EPR spectrum (Fig. 1A). Both in beef and in yeast oxidase, the Cu(II) can be reduced reversibly to Cu(I) which no longer gives the signal at $g \cong 2$.

The Cu(II) signal is very temperature-sensitive. It can easily be measured at the temperature of liquid N_2, but not at around –40°C.

Fig. 1B shows the EPR spectrum of *P. denitrificans* cytochrome *c* oxidase recorded at –140°C. It has considerably more fine structure than the corresponding spectrum of the yeast enzyme, but a pronounced signal at $g \cong 2$, indicative of the presence of Cu(II) is still observable. At higher temperatures (Fig. 2), this Cu(II) resonance disappears and a relatively simple six-line pattern emerges, suggesting the presence of Mn(II). Elemental analyses confirmed this. As shown in Table I, roughly every fifth minimal unit of the *P. denitrificans* enzyme contains one Mn(II) ion.

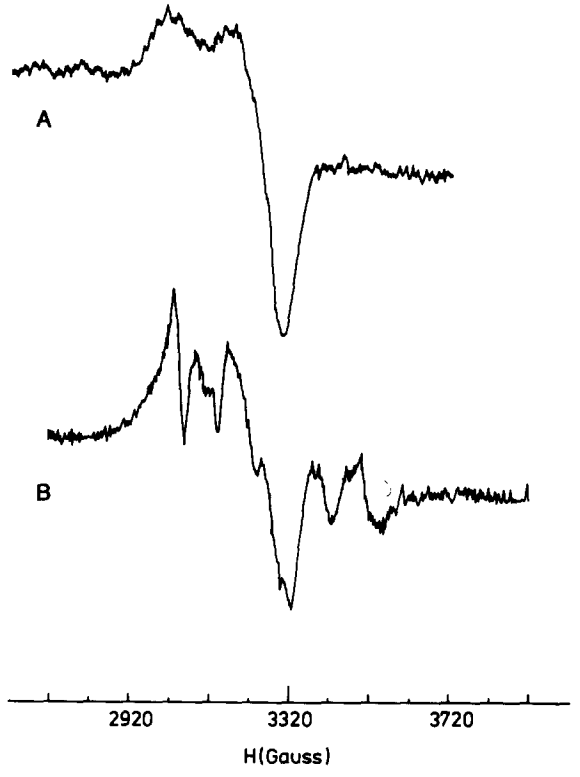

Fig. 1. The EPR spectra of cytochrome *c* oxidase isolated from yeast (A) and from *P. denitrificans* (B) at –140°C. Microwave power, 50 mW; frequency, 9.37 GHz; modulation, 4 Gauss. (A) 10.2 mg oxidase protein/ml (heme/protein = 11.4 nmol/mg); (B) 4.8 mg oxidase protein/ml (heme/protein = 27.3 nmol/mg) in 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.5. The growth medium for *P. denitrificans* contained 50 μM Mn(II).

Since the *P. denitrificans* cells had been grown on a synthetic medium containing 50 μM $MnCl_2$, it could be argued that the observed EPR signal simply reflects Mn(II) that had been adventitiously adsorbed to the enzyme during purification. This appears not to be the case. First, all solutions used during the enzyme purification contained 1 mM EDTA. Second, the enzyme-bound Mn(II) was not removed by overnight dialysis against 5 mM EDTA or by gel electrophoresis under non-denaturing conditions (Table I). The Mn(II) associated with the *P. denitrificans* enzyme is thus either bound extremely tightly or is inaccessible to EDTA. A relatively strong binding of Mn(II) to *P. denitrificans*

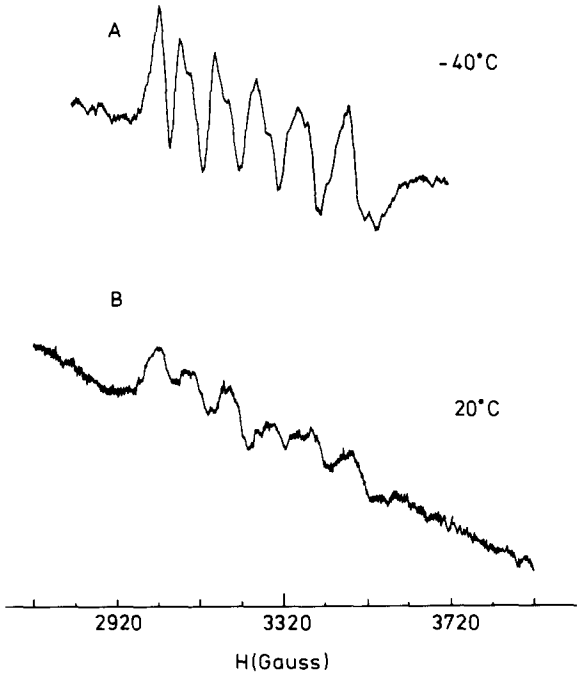

Fig. 2. Temperature dependence of the EPR spectra of *P. denitrificans* cytochrome *c* oxidase. Same sample as Fig. 1B. At the temperatures employed, the Cu(II) signal is broadened beyond detection. Furthermore, the line broadening and decrease in signal amplitude by raising the temperature from −40 to +20°C is typical for Mn(II) bound to a slowly tumbling macromolecule [19,20].

cytochrome *c* oxidase is also suggested by the temperature dependence of the EPR spectra shown in Fig. 2. With increasing temperature the lines broaden, which is characteristic for bound Mn(II), whereas the signal of free $Mn(H_2O)_6^{2+}$ exhibits the opposite temperature dependence [19].

Mn(II) can be released from *P. denitrificans* cytochrome *c* oxidase by acidification with a few drops of concentrated HCl; the resulting EPR spectrum (Fig. 3) is identical to that of free Mn(II). Since the bands are 5-fold more intense than those observed before acidification, the spectra of the enzyme-bound Mn(II) probably reveal mainly the $-1/2 \rightarrow 1/2$ fine structure band [20]. The broadening of the other transitions ($m_s \neq 1/2$) beyond detection suggests furthermore that Mn(II) in the native cytochrome *c* oxidase is bound in an asymmetric environment. Amazingly similar spectra have been reported for Mn(II) bound to lettuce chloroplasts and for Mn(II) released from chloroplasts by acidification [20].

The intense EPR signal of the tightly bound Mn(II) almost obscures the EPR spectrum of the Cu(II) in the *P. denitrificans* oxidase. This probably explains why the Cu(II) signal of *P. denitrificans* membranes was missed in earlier studies (cf. Ref. 8).

TABLE I

CHEMICAL COMPOSITION AND ENZYMATIC ACTIVITY OF DIFFERENT PREPARATIONS OF *PARACOCCUS DENITRIFICANS* CYTOCHROME *c* OXIDASE

See Materials and Methods for experimental details.

Cytochrome *c* oxidase preparation	Concentration (nmol/mg protein)				Molar activity (s^{-1})
	Cu	Mn	Fe	Heme *a*	
(A) From cells grown in full medium [6,10]	30.6	2.4	26.6	26.3	77 *
(B) Same as (A), but after additional purification by gel electrophoresis as described in Ref. 6	29.1	2.1	23.6	28.2	
(C) From cells grown on a Mn(II)-deficient medium	28.8	0.13	26.8	26.3	49 **

* Mean of five determinations on four different preparations.
** Mean of two determinations on two different preparations.

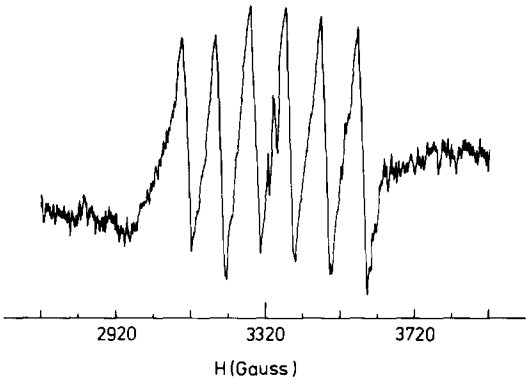

Fig. 3. EPR spectrum of *P. denitrificans* cytochrome *c* oxidase acidified with concentrated HCl at room temperature. Same sample as Fig. 1B.

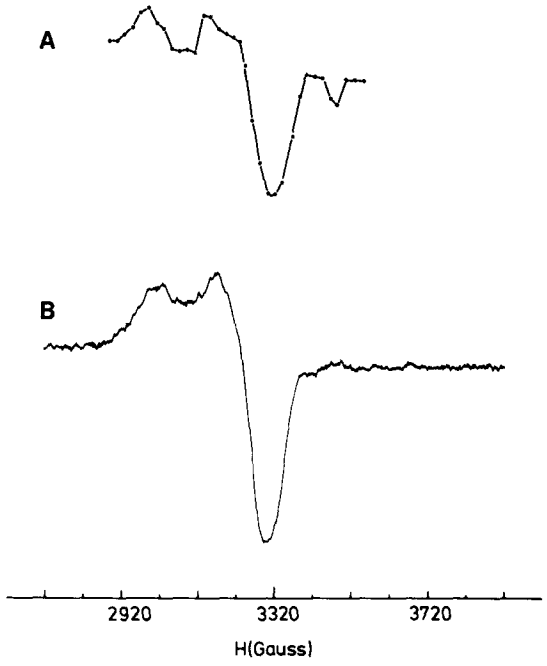

Fig. 4. Pure Cu(II) EPR spectra of cytochrome *c* oxidase of *P. denitrificans*. (A) Numerical subtraction of Fig. 2A (pure Mn(II) EPR spectrum) from Fig. 1B. (B) Cytochrome oxidase isolated from *P. denitrificans* grown on a Mn(II)-deficient medium (see Materials and Methods).

One possibility to visualize the pure Cu(II) EPR spectrum is to first record the pure Mn(II) spectrum at –40°C (Fig. 2A) and then subtract this spectrum from the complex spectrum recorded at –140°C (Fig. 1B). The resulting difference spectrum is given in Fig. 4A. This method assumes that the Mn(II) spectrum is essentially unchanged between –40°C and –140°C. This assumption appears to be essentially valid since the computed difference spectrum closely resembles a typical Cu(II) spectrum with only a few small additional peaks (Fig. 1A). Another way to eliminate the interfering Mn(II) spectrum is mild denaturation of the enzyme by repeated oxidation-reduction cycles at +4°C.

The most direct way to show the pure Cu(II) EPR spectrum of an active enzyme is to grow *P. denitrificans* in a medium which contains about 60-times less Mn than the usual medium. Cytochrome *c* oxidase purified from these cells was essentially free of Mn as established by elemental analysis (Table I), yet it was nearly as active as the Mn(II)-containing enzyme. Since enzymic activity measurements are generally less reproducible than elemental analysis (cf. Ref. 6), the activity data given in Table I are the means of several determinations on different enzyme preparations. The EPR spectrum of the Mn-free oxidase (Fig. 4B) was identical to the EPR spectra of bovine heart- and yeast cytochrome *c* oxidase.

Fig. 4 unambiguously demonstrates that oxidized cytochrome *c* oxidase from *P. denitrificans* has a Cu(II) redox center in the same environment as the mitochondrial cytochrome *c* oxidases. The spectrum has little resemblance to that of the presently known synthetic copper complexes but appears to be unique to heme aa_3-type cytochrome *c* oxidases regardless of their origin and subunit composition. As discussed in detail elsewhere [13–18], this spectrum exhibits an anisotropic and almost axially symmetric *g*-tensor ($g_\parallel \simeq 2.20$; $g_\perp \simeq 2.02$) and lacks hyperfine structure, indicating a highly covalent bonding of the copper atom [21]. Surprisingly, the EPR spectrum of Mn(II) associated with the *P. denitrificans* oxidase responds to the oxidation state of the enzyme. This is readily seen by comparing Fig. 1B (oxidized enzyme) with Fig. 5 (reduced enzyme). Apart from the loss of the Cu(II) signal at $g \cong 2$, the most obvious change in the reduced state is sharpening of the EPR signals from an average peak-to-peak linewidth of $\Delta H \simeq 30$ G for the oxidized form to $\Delta H \simeq 20$ G in the reduced form, leading to a considerably better resolved

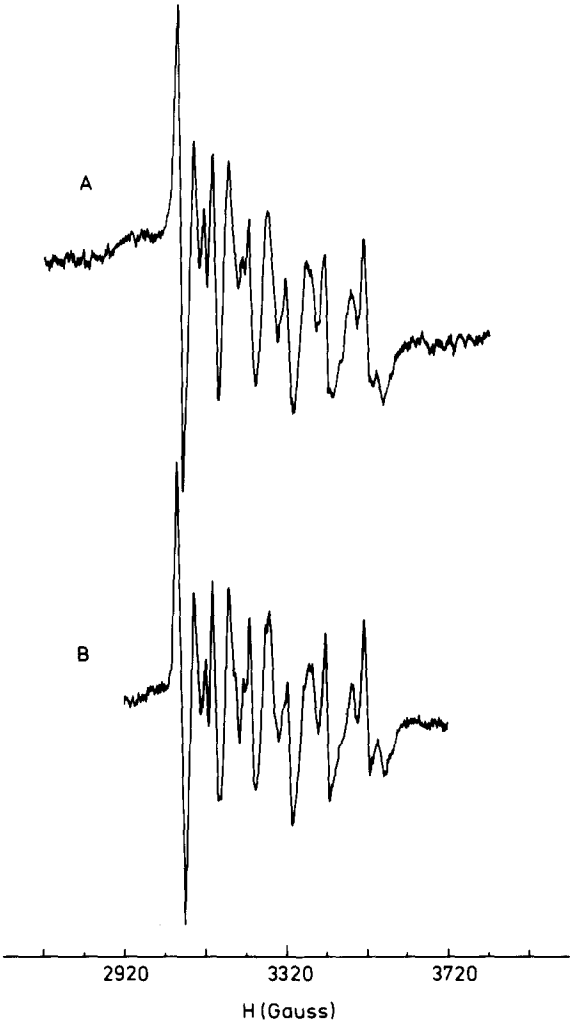

Fig. 5. EPR spectra of reduced cytochrome *c* oxidase isolated from *P. denitrificans* grown on a Mn(II)-containing medium. Measuring temperature was −140°C. The Cu(II) EPR signal is eliminated by reduction and only the Mn(II) signal is detected. (A) Reduction with dithionite. (B) Reduction with ferrocytochrome *c*. Cytochrome *c* was reduced by ascorbate and freed from excess reductant by passage through a Sephadex G-25 column; 2 mg of the ferrocytochrome *c* were added to an anaerobic mixture of 1 mg of *P. denitrificans* cytochrome *c* oxidase and 0.5 mg of sonicated asolectin in a total volume of 0.5 ml.

fine structure in the Mn(II) 'powder-type' spectrum. Fig. 5 further demonstrates that these spectral changes are independent of the reducing agent used. Both dithionite (Fig. 5A) and reduced cytochrome *c* (Fig. 5B) elicit the same spectral changes. These changes cannot be explained by an oxidation or reduction of Mn(II) itself since the redox potential of Mn(II) (1.5 V) is too high for this. Rather, reduction of cytochrome *c* oxidase appears to trigger a conformational change within the enzyme which is sensed by the bound Mn(II). The sharpening of the resonances is indicative of a more symmetric electronic environment of Mn(II) in the reduced enzyme than in the oxidized one.

When Mn(II) was added to Mn(II)-free *P. denitrificans* cytochrome *c* oxidase at a ratio of 1 Mn(II) per 5 molecules of enzyme complex, the resulting EPR spectrum was very similar to that shown in Fig. 1B. However, the Mn(II) could easily be removed by complexation with EDTA, as monitored by the disappearance of the Mn(II) EPR spectrum. Furthermore, the EPR spectrum was found to be completely insensitive to the oxidation state of the enzyme. Externally added Mn(II) is thus bound to the oxidase but differently from the intrinsically bound one. Identical results were obtained when Mn(II) was added to yeast cytochrome *c* oxidase.

What is the role of Mn(II) in *P. denitrificans* cytochrome *c* oxidase? Our data argue against a catalytic function in electron transfer. However, its presence in what appears to be a rather specific environment may well reflect an evolutionary relationship to Mn(II)-containing photosystems which catalyze the splitting, rather than the formation, of water.

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