Multiple forms of the catalytic centre, Cu_Z, in the enzyme nitrous oxide reductase from *Paracoccus pantotrophus*

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Nitrous oxide reductase catalyses the reduction of nitrous oxide to dinitrogen at a unique tetranuclear copper site, called Cu_Z, which has a central inorganic sulphide ligand. Limited incubation with oxygen during the preparation of nitrous oxide reductase from *Paracoccus pantotrophus* results in changed redox properties of the catalytic centre by comparison with anaerobic preparations. While the anaerobically purified enzyme has a catalytic centre which performs a single electron step at a midpoint potential of $E_m = +60$ mV versus the standard hydrogen electrode ($n = 1$), the altered centre shows no redox change under similar experimental conditions. Spectroscopic properties of this 'redox fixed' centre are similar to spectra of the reduced 'redox active' form of Cu_Z, although the positions and intensities of a number of transitions are changed in the optical spectrum. These observations are interpreted in terms of two forms of the catalytic centre, called Cu_Z and Cu_Z*. The structural relationship between these forms is unclear. EPR and magnetic circular dichroism spectra suggest that the basic Cu_4S structure is common to both. Curiously, steady-state activity of the aerobic enzyme preparation is slightly increased despite the fact the catalytic centre does not undergo detectable redox changes.

Key words: copper, denitrification, magnetic circular dichroism, oxygen sensitivity, redox titration.

INTRODUCTION

Denitrifying bacteria use oxidized nitrogen species as electron acceptors to survive under anaerobic conditions [1,2]. The last step of this process, the two-electron reduction of nitrous oxide to dinitrogen, is catalysed by the copper-containing enzyme nitrous oxide reductase (N_2OR) which is a functional homodimer with a molecular mass of 65 kDa per subunit. The X-ray crystal structure analysis shows that each subunit consists of two domains [3]. The first is a cupredoxin domain, containing a binuclear copper centre, designated Cu_A, and serving as an electron transfer group [4]. A similar centre is found in cytochrome *c* oxidases [5,6] and menaquinol NO reductase from *Bacillus azotoformans* [7]. The second domain is a seven bladed propeller of β-sheets which contains the catalytic site, a novel tetranuclear copper centre, designated Cu_Z [8]. Spectroscopy and biochemical data [9] together with a revised X-ray structure [10] revealed that Cu_Z is a unique copper–sulphide centre (Figure 1).

Attention is now turning, therefore, to the chemistry of Cu_Z in order to understand the redox cycle through which the centre travels during catalysis and also to discover whether the structure of this centre varies as a function of redox states. Such questions must be answered before the catalytic mechanisms can be understood. This paper addresses these issues using a combination of optical and magnetic spectroscopies of different redox states of the enzyme to resolve the properties of the Cu_Z centre from those of Cu_A. The spectral properties of Cu_A are now relatively well understood. It can exist in one of two oxidation states, the oxidized mixed valence form [$Cu^{1.5}$–$Cu^{1.5}$] which possesses one unpaired electron giving rise to a distinctive EPR spectrum with a partially resolved 7-line copper hyperfine structure centred at $g_\parallel$ [11–13]. The optical spectrum in the visible region is dominated by thiol-to-Cu(II) charge transfer (CT) bands plus intervalence copper absorption. This gives rise to an intense, temperature dependent magnetic circular dichroism (MCD) spectrum characterized by bi-signate bands at 475 and 530 nm plus a broad negative band at 750 nm [8,14]. On reduction by one electron, Cu_A becomes magnetically and optically silent. The metal core is [$Cu^{1.0}$–$Cu^{1.0}$] in which both 3d shells are filled ($3d^{10}$) and hence the cluster is diamagnetic and colourless. The electronic structure of the Cu_Z cluster is, by contrast, less well understood. A $[Cu_4S]^{n+}$ core can, in principle, adopt one of five different oxidation levels (see Figure 1) given that each copper ion adopts either the Cu(I) or Cu(II) oxidation state. With the contribution of one unpaired electron ($S = \frac{1}{2}$) for each Cu(II) the overall electron spin of each oxidation level can be written down straightforwardly, as shown in Figure 1. All redox levels, except the all-Cu(I) state, are expected to have strong optical absorption bands in the visible region arising from S^{2-} to Cu(II) CT plus intervalence transitions between Cu(I) and Cu(II). Only two of the states, namely, $[Cu_4S]^{3+}$ and $[Cu_4S]^{5+}$, are expected to give rise to $S = \frac{1}{2}$ EPR signals. Both could, in principle, undergo a one-electron oxidation to an even electron spin state that would be diamagnetic at cryogenic temperatures (provided the $S = 0$ component of the electronic ground state lies lower in energy). It is likely that these states would be EPR silent with weak, temperature-independent MCD spectra. It is not known which of these five oxidation states Cu_Z can adopt in the enzyme itself. However, by applying a combination of magnetic and optical

Abbreviations used: CT, charge transfer; E_m, midpoint potential; MCD, magnetic circular dichroism; N_2OR, nitrous oxide reductase; OTTLE cell, optically transparent thin-layer electrochemical cell; RR, resonance Raman; SHE, standard hydrogen electrode.

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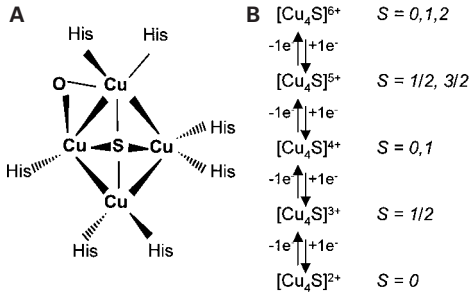

Figure 1 Structure of Cu_Z (A) and its possible redox and spin states (B)

spectroscopies the properties of the two clusters, Cu_A and Cu_Z, can be disentangled.

Since the first isolation of N_2OR in 1982 [15] it has been known that the colour of the enzyme varies dramatically depending upon isolation conditions, especially the levels of exposure to oxygen. We show that these colour variations are due to changes at the Cu_Z site only, demonstrating that it can exist in different forms. Previous studies suggested that a small proportion of Cu_Z had different redox and spectroscopic properties, and this was designated Cu_Z* [8,16,17]. We show that the total Cu_Z content, Cu_Z^{total}, consists of two different forms which we term Cu_Z and Cu_Z*. N_2OR was isolated from *Paracoccus pantotrophus* (formerly *Thiosphaera pantotropha*) which had been incubated for a limited time during purification with oxygen and in other preparations kept strictly anaerobic. Our results show that these preparations differ in the relative proportions of Cu_Z* and Cu_Z. N_2OR from the anaerobic preparations had a low proportion of Cu_Z*, while a 12 h incubation with oxygen during the preparation resulted in a relatively high proportion of Cu_Z*. Using these enzyme forms prepared from *Pa. Pantotrophus*, which we term 'aerobic' and 'anaerobic' preparations, we have compared the properties of Cu_Z* with Cu_Z in detail.

EXPERIMENTAL PROCEDURES

Preparation of the different N_2OR forms

The preparation of N_2OR was adapted from previous methods [18,19]. The cell paste of a 200 litre anaerobic cell growth was re-suspended in 150 ml of 100 mM Tris/HCl, pH 8.0. Lysozyme (10 g, Sigma) in 50 ml of the same buffer was added. For the 'anaerobic' preparation the cell suspension was frozen in liquid nitrogen. The 'aerobic' preparation, however, was kept at −80 °C for 12 h exposed to air. Subsequent steps in the preparation were performed in both cases anaerobically in a cooled glove box. The cells were thawed and a soluble extract, obtained by centrifugation of the thawed cell suspension at 40000 ***g*** for 30 min, was loaded onto a 500 ml DEAE FF column (Pharmacia, Milton Keynes, U.K.) which had been equilibrated with 100 mM Tris/HCl, pH 8.0, and 20 % (v/v) glycerol. N_2OR was eluted with a 0 to 0.5 M NaCl gradient with a flow rate of 0.8 ml/min at approx. 0.35 M NaCl. The peak fractions, selected by optical spectroscopy, were mixed with $(NH_4)_2SO_4$ (22 % final concentration) and loaded onto a 500 ml phenyl Superose column (Pharmacia), equilibrated with 22 % $(NH_4)_2SO_4$ in 50 mM Hepes, pH 7.8. A 22 to 0 % $(NH_4)_2SO_4$ gradient was applied. The purified protein was concentrated and used for the experiments or frozen in liquid nitrogen until needed. Protein concentrations were determined with the bicinchoninic acid method [20] using BSA (Sigma) as a standard. The methyl viologen-linked N_2O-reductase activity was determined optically at 600 nm after incubation of N_2OR with ferricyanide, ascorbate or dithionite as described previously [21]. The inorganic sulphur content was determined by the Methylene Blue method [22] and the copper content was determined by inductively coupled plasma emission spectroscopy.

Spectroscopic methods

X-band EPR-spectra were measured on a Bruker ER200D spectrometer fitted with an Oxford Instruments ESR-9 flow cryostat at 15 K. The spin quantification was performed as described by Aasa and Vänngård [23] with Cu(II)EDTA as the standard. Low temperature MCD spectra were recorded on a Jasco J-500 in the 300 to 1000 nm region and with a J-730 in the 700 to 1400 nm region at 4.2 K and 5 T [24]. The samples contained 50 % glycerol. The glassing agent caused no changes in the absorption or EPR spectrum. CD spectra at 298 K were measured with the same spectrometers as the MCD spectra. Absorption spectra were recorded with a Hewlett Packard 8453 diode-array spectrometer. All absorption coefficients are based on the protein concentration of the monomer and in the case of the MCD spectra they are not normalized for the magnetic field. The samples were handled and poised in a glove box. All measurements were performed with closed cells to maintain anaerobic conditions.

Redox titration

N_2OR (1.3 mM) solutions in 50 mM Tris/HCl, pH 7.5, with 0.1 M KCl were titrated in a home-built optically transparent thin-layer electrochemical (OTTLE) cell at 15 °C. The cell was based on the design of Moss et al. [25] and had a pathlength of 70 μm (L. Male, S. J. George and J. N. Butt, unpublished work). In a three-electrode arrangement, a dithiodiethanol modified gold mesh was used as a working electrode with a platinum counter electrode and a home-made Ag/AgCl reference electrode. To obtain the potentials relative to the standard hydrogen electrode (SHE) from the experimental values, the midpoint potential of the reference electrode was determined with ferricyanide (E_m = +420 mV versus SHE). Redox mediators were added as follows: 5 μM methyl viologen, 5 μM benzyl viologen, 10 μM Neutral Red, 20 μM anthraquinone-2-sulphonate, 20 μM 9,10-anthraquinone-2,6-disuphonate, 10 μM indigotetrasulphonate, 100 μM menadione, 100 μM duroquinone, 100 μM phenazine methasulphate, 100 μM ruthenium(III)cloride, 5 μM 2,6 dichlorophenol indophenol, 100 μM *N*,*N*,*N*′,*N*′-tetramethyl-p-phenylenediamine, 100 μM ferricyanide and 100 μM ferrocene monocarboxylic acid.

RESULTS

Preparation of the different enzyme forms

Incubation of broken cells for 12 h with air, at −80 °C, results in an N_2OR form (called in this paper the 'aerobic' preparation) which shows the same chromatographic behaviour, during its isolation, as the strictly anaerobic form ('anaerobic' preparation; results not shown). However, the two forms are completely different as judged by optical absorption, EPR spin integration, and steady-state activity (see Tables 1 and 2). The 'aerobic' form has a higher steady-state activity than the 'anaerobic' form (Table 1).

Table 1 Steady-state activities of the 'anaerobic' and 'aerobic' forms of N_2OR from *Pa. pantotrophus*

Activities are given in μmol $N_2O \cdot min^{-1} \cdot mg^{-1}$ of protein ($\pm$S.D.).

N_2OR form	Pre-oxidized with ferricyanide	Pre-reduced with ascorbate	Pre-reduced with dithionite
'Anaerobic'	2.6 ± 0.5	1.6 ± 0.3	2.8 ± 0.5
'Aerobic'	8.5 ± 2.2	7.8 ± 0.5	8.8 ± 1.3

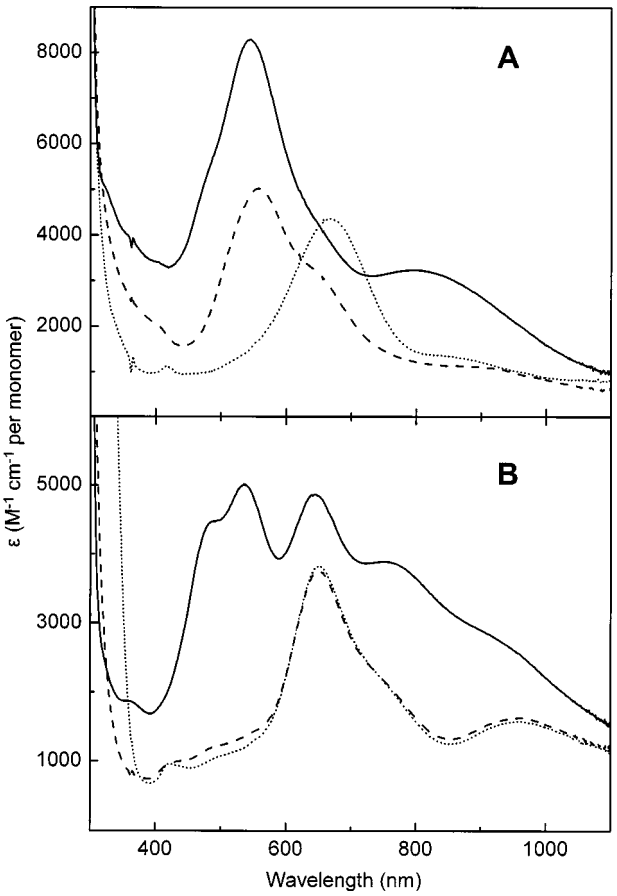

Figure 2 UV/Visible absorption spectra of the 'anaerobic' (A) and the 'aerobic' (B) preparations of N_2OR

Spectra of ferricyanide-oxidized (solid lines), ascorbate-reduced (dashed lines), and dithionite-reduced (dotted lines) samples are shown. ϵ is absorption coefficient.

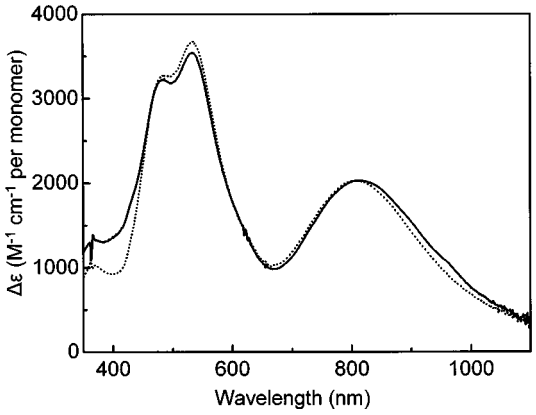

Figure 3 UV/Visible difference absorption spectra of ferricyanide-oxidized minus ascorbate-reduced samples of N_2OR

The 'anaerobic' preparation is shown as a solid line, the 'aerobic' preparation as a dotted line.

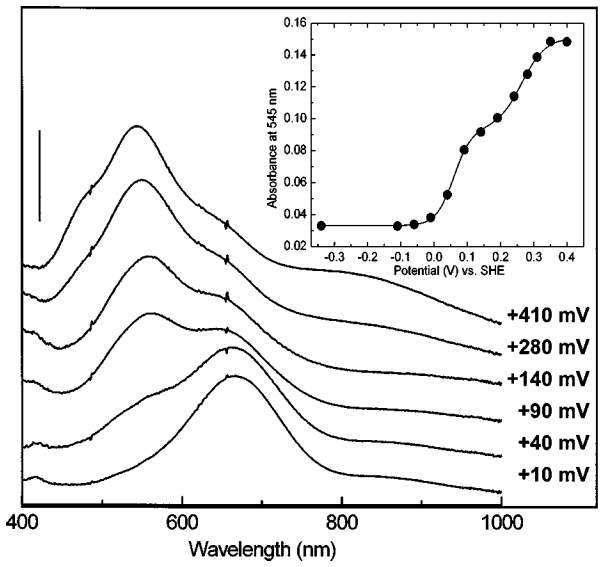

Figure 4 Optical redox titration of the 'anaerobic' preparation of N_2OR

Absorption spectra at different potentials are shown offset for clarity; the vertical bar marks an absorption change of $\Delta A = 0.05$. The inset shows the titration curve at 545 nm.

Absorption spectra of the different enzyme forms

Following the report of Farrar et al. [8] of three different redox levels for the holoenzyme, a comparison has been made of the absorption spectra of the two enzyme preparations, 'aerobic' and 'anaerobic', after treatment with ferricyanide, ascorbate and dithionite. These agents generate forms of the enzyme which are termed oxidized, semi-reduced and reduced.

Figure 2 compares the absorption spectra of the two enzyme preparations after treatment with these reagents, showing striking differences between them at all three oxidation levels. In ferricyanide-oxidized samples, the anaerobic preparation shows an intense peak at 550 nm ($\epsilon_{550} \approx 8000\ M^{-1}\ cm^{-1}$) and a weaker peak at 800 nm ($\epsilon_{500} \approx 3000\ M^{-1}\ cm^{-1}$), while the aerobic preparation has peaks at 480, 535 and 645 nm with almost the same intensity ($\epsilon \approx 5000\ M^{-1}\ cm^{-1}$) and two less intense shoulders at 760 and 920 nm (Figure 2, solid lines). The ascorbate-reduced form of the anaerobic preparation (Figure 2, dashed lines) has the most intense transition at 560 nm and the aerobic preparation has the most intense transition at 650 nm. However, the difference absorption spectra of the ferricyanide-oxidized minus ascorbate-reduced N_2OR, shown in Figure 3, are virtually identical to one another and to the absorption spectrum of isolated Cu_A, as seen in a mutant form of N_2OR, for example, which contains only the Cu_A centre [14]. The intensities of the difference spectra reflect a fully oxidized Cu_A centre quantity ($\epsilon_{500} \approx 3000\ M^{-1}\ cm^{-1}$). Hence, the effect of ascorbate treatment is to reduce only Cu_A in both N_2OR preparations. Therefore, the differences between the pairs of spectra of the aerobic and anaerobic preparation are entirely

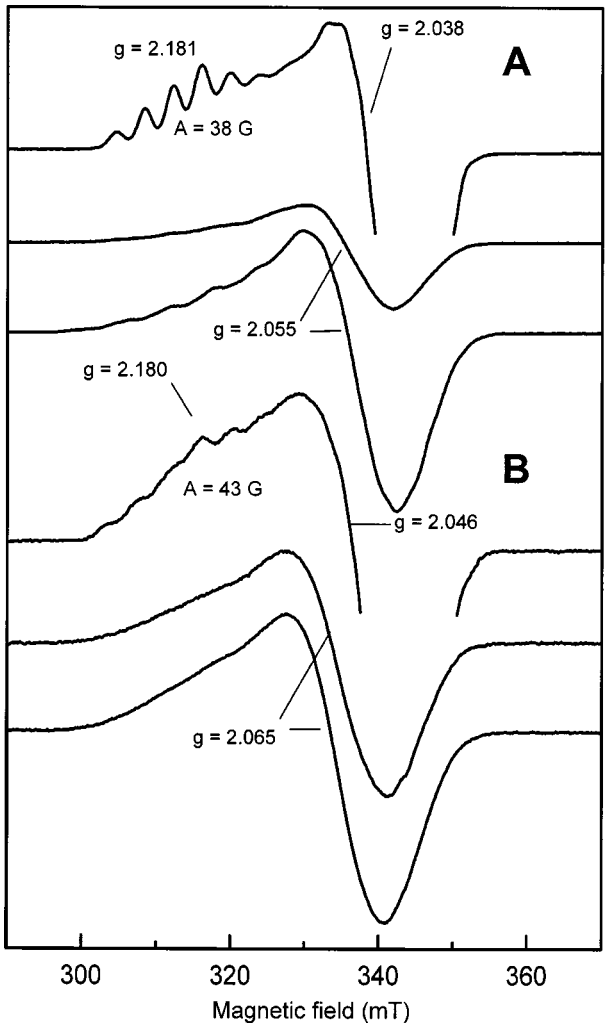

Figure 5 EPR spectra of the 'anaerobic' (A) and 'aerobic' (B) preparations of N_2OR

Spectra for ferricyanide-oxidized (top), ascorbate-reduced (middle), and dithionite-reduced (bottom) samples are shown, respectively (in **A** and **B**). The intensities are normalized with respect to spins per protein. The spectra were recorded at 15 K and 0.2 mW microwave power.

due to variations in the spectroscopic properties of Cu_Z, and not from any variation in the form of Cu_A.

Furthermore, there is no contribution from Cu_A to the absorption spectrum of either form over the 400 to 1000 nm range in either the ascorbate or the dithionite-reduced form of N_2OR. Figures 2(A) and 2(B) reveal a striking difference between the nature of Cu_Z in the two preparations. In the 'anaerobic' preparation, Figure 2(A), Cu_Z occurs in two forms which interconvert on reduction with dithionite. Thus, the oxidized form shows a peak at 560 nm ($\epsilon \approx 5000\ M^{-1}\ cm^{-1}$) and the reduced form has a peak at 670 nm ($\epsilon \approx 4400\ M^{-1}\ cm^{-1}$). This transformation, which has been followed previously spectrophotometrically [8], has a clear isosbestic point at 623 nm and is a reduction process. By contrast, the aerobic enzyme preparation shows no difference between the semi-reduced and dithionite reduced states (Figure 2B). Thus, no reductive conversion occurs. In addition, the absorption spectra are distinct from both Cu_Z species in the 'anaerobic' form. The absorption peak lies at 650 nm ($\epsilon \approx 3800\ M^{-1}\ cm^{-1}$) with a pronounced broad band at 960 nm ($\epsilon \approx 1600\ M^{-1}\ cm^{-1}$). A contribution from the spectral form present in dithionite samples can be seen by the presence of the shoulder at 650 nm in the absorption spectrum of the ascorbate-reduced form, Figure 2(A).

Redox titration of N_2OR

Since the catalytic centre of N_2OR prepared anaerobically can be reduced, a redox titration was carried out (Figure 4). The experiment was performed in the oxidative direction since N_2OR reduction at an electrode or using dithionite even in the presence of mediators is very slow. Fits of Nernst equations to absorption changes of our OTTLE cell experiments describe two components, one of which has a midpoint potential of approx. $E_{m,7.5} \approx +260$ mV with $n \approx 1$ and shows the spectroscopic features of Cu_A. This midpoint potential corresponds well with literature values of this centre in N_2OR [26,27]. Spectroscopic features of Cu_Z change with a midpoint potential of $E_{m,7.5} \approx +60$ mV and $n \approx 1$. This is the first reported midpoint potential for Cu_Z.

EPR spectra

The EPR spectra of the 'aerobic' and 'anaerobic' preparations in the three oxidation states are compared in Figure 5. Spin quantifications are given in Table 2. The spectrum of the anaerobic preparation, Figure 5(A), is dominated by signals from oxidized Cu_A showing in the ferricyanide-oxidized level the 7-line copper hyperfine $g_\parallel$, characteristic of the mixed valence Cu(I)/Cu(II) state. However, the spin quantification of 1.23 electron spins per monomer reveals the presence of a second $S = \frac{1}{2}$ paramagnetic species. Reduction with ascorbate causes loss of the signal of oxidized Cu_A. The 7-line hyperfine structure is lost and the g value at $g_\perp$ shifts from 2.038 to 2.055. The spin integration after ascorbate reduction is 0.27 electron spins per monomer, in excellent agreement with the integration of the ferricyanide-oxidized form, if it is assumed that 1 electron spin accounts for Cu_A in that form. Further reduction with dithionite gives an increase in the EPR signal intensity which enhances the spin integration to 0.78. This is consistent with results from the absorption spectrum, Figure 2(A), which shows Cu_Z being reduced. The g values remain the same as for the species detected in the ascorbate-reduced state but some copper hyperfine structure becomes evident. There appear to be four lines with $A \approx 60$ G in the g region resolved. The EPR spectra of the three redox levels of the aerobic preparation, Figure 5(B), show broadly similar qualitative results. However, the spin quantifications are significantly different (Table 2). The spin quantification in the ferricyanide-oxidized form is 1.51, showing that the catalytic centre contributes about 0.51 spin. As a consequence, the hyperfine structure in the g region is much less distinct. On reduction with ascorbate, the spin intensity falls to 0.57, again in close agreement with the expectation of Cu_A being completely reduced. Further reduction with dithionite causes a small increase in unpaired electrons to 0.77 as the redox active form of the catalytic centre is reduced to an EPR active state.

MCD spectra

The MCD spectra of the 'anaerobic' and 'aerobic' preparations measured at 4.2 K and 5 T are compared in Figure 6. Only paramagnetic species will make contributions to the spectra at this temperature. This is confirmed by the temperature dependence of the spectra (not shown). In the ferricyanide-oxidized states of both 'aerobic' and 'anaerobic' preparations the MCD

Table 2 Quantification of copper centres in N_2OR

Amounts of copper centres Cu_A, Cu_Z and Cu_Z* in N_2OR from *Pa. pantotrophus* are determined from EPR and MCD quantifications and from elemental analysis. n.d., not determined.

	EPR spin quantification (spins/monomer)			Content (atoms/dimer)		MCD intensity at 620 nm (M^{-1} cm^{-1}/monomer)		
N_2OR form	Ferricyanide oxidized	Ascorbate reduced	Dithionite reduced	Copper	Inorganic sulphur	Ferricyanide oxidized	Ascorbate reduced	Dithionite reduced
'Anaerobic'								
Experimental value	1.23	0.27	0.78	10.5 ± 2.6	1.7 ± 0.4	300	340	1090
Expected value†			1	12	2			
Assignment of Intensities								
Cu_A‡	1			4				
Cu_Z		0.05	0.55				40	790
Cu_Z*	0.23	0.23	0.23			300	300	300
Cu_Z^{total}			0.78	6.5	1.7			1090
Normalized Cu_Z^{total}/monomer			**0.78**	**0.81**	**0.85**			
Ratio Cu_Z*/Cu_Z^{total}			**0.29**					**0.28**
'Aerobic'								
Experimental value	1.51	0.57	0.77	8.4 ± 0.5	1.5 ± 0.2	950	n.d.	1230
Expected value†			1	12	2			
Assignment of Intensities								
Cu_A‡	1			4				
Cu_Z		0.06	0.26					
Cu_Z*	0.51	0.51	0.51			950		
Cu_Z^{total}			0.77	4.4	1.5			1230
Normalized Cu_Z^{total}/monomer			**0.77**	**0.55**	**0.75**			
Ratio Cu_Z*/Cu_Z^{total}			**0.66**					**0.77**

† For fully occupied centres.
‡ Assuming that no Cu_A is lost.

features of oxidized Cu_A are clearly visible at 475, 530 and 750 nm. In the dithionite-reduced samples, the main feature centres at 650 nm of the 'anaerobic' and 'aerobic' forms (caused by perpendicular polarised CT) are similar. Peaks at higher and lower energy are shifted and changes in intensities suggest variation of electronic structure between Cu_Z and Cu_Z*. Signals are visible from about 30 % of oxidized Cu_A in the spectrum of the ascorbate-reduced sample of the 'anaerobic' preparation. Interestingly, the signals from the catalytic site at about 700 and 1100 nm can only be explained by those from dithionite-reduced samples of the 'aerobic' but not the 'anaerobic' preparation (Figure 6C). This confirms that only Cu_Z* is reduced in the ascorbate-treated sample. These signals are still visible in the ferricyanide-oxidized sample.

Quantification of Cu_Z* in the enzyme preparations

The differences between the redox and spectroscopic properties of N_2OR prepared 'aerobically' and 'anaerobically' can be interpreted in terms of the form of the catalytic centre, designated Cu_Z* and Cu_Z. We can quantify the amount of each form as well as the total content (Cu_Z^{total}) of catalytic centres (Table 2). Cu_Z as well as Cu_Z* are present as the reduced, EPR-active oxidation state in dithionite-reduced samples. Therefore, an estimation of 1 spin per monomer is expected, representing the Cu_Z^{total}. However, the lower experimental values of approx. 0.8 spin indicates that either some of the catalytic centre is lost or some is further reduced to an EPR-silent oxidation state. Elemental-copper and inorganic-sulphur analysis support the former explanation. Both preparations appear to have lost about the same amount of the catalytic centre. The difference between the 'anaerobic' and the 'aerobic' preparation is the relative content of Cu_Z*. Since Cu_Z* stays in the EPR-active oxidation state, whichever oxidant or reductant is added, the spin quantification of the ascorbate-reduced sample, with reduced Cu_A and oxidized Cu_Z being EPR-silent, is a direct measure of the Cu_Z* content. For the 'anaerobic' preparation, with 0.23 spin per monomer, this is much less than for the 'aerobic' preparation, with 0.51 spin per monomer. Therefore, 29 % of the catalytic centre in the 'anaerobic' preparation is in the Cu_Z* form and 66 % in the 'aerobic' preparation. This is given in Table 2 as the ratio Cu_Z*/Cu_Z^{total}. These values are independently confirmed by a quantification of the MCD signal at 620 nm. At this wavelength, Cu_Z* and Cu_Z have about the same $\Delta\epsilon$-values, as judged by the MCD spectra of the dithionite reduced samples. Since this transition is not disturbed by any Cu_A signals, the MCD quantification shows more directly than any other technique that the quantity of paramagnetic Cu_Z* does not change in the ferricyanide-oxidized samples. It must be emphasized, that the Cu_Z* proportions determined in this work only characterize our N_2OR preparations. Depending on the pretreatment of the preparation, the proportions of Cu_Z* will be variable.

CD spectra

Apart from the main peak at 720 nm in dithionite-reduced samples, the CD spectrum of the 'aerobic' preparation shows additional peaks at 480, 570 and 800 nm not seen in the 'anaerobic' preparation (Figure 7). It is similar to the CD spectrum of the dithionite-reduced 'pink' N_2OR form of *P. stutzeri* [28]. At the low energy edge a positive signal appears at 1100 nm, as discussed by Dooley et al. [29], while reduced Cu_Z has only negative signals in this region. In the spectrum of the oxidized 'anaerobic' preparation, the strong negative signal of the oxidized Cu_Z centred at 570 nm overlays the negative signal of Cu_A at 530 nm. This spectrum corresponds not to CD spectra of N_2OR from *Pseudomonas aeruginosa* [30] but from *P. stutzeri* [29,31].

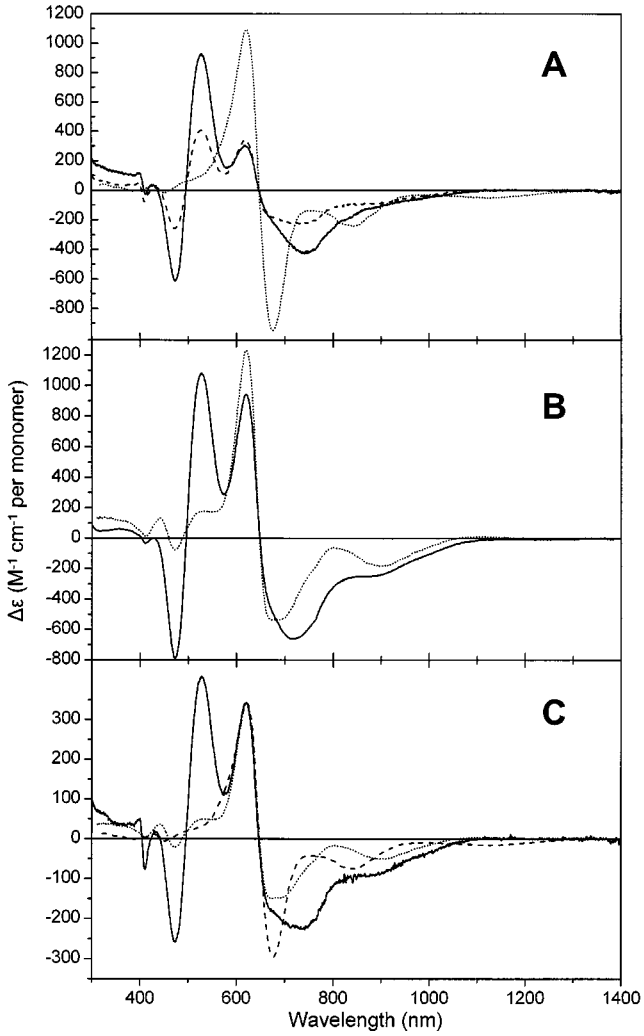

Figure 6 MCD spectra of the 'anaerobic' (A) and of the 'aerobic' (B) preparation of N_2OR

Spectra of ferricyanide-oxidized (solid lines), dithionite-reduced (dotted lines), and ascorbate-reduced (dashed lines) samples are shown for the 'anaerobic' preparation, for the 'aerobic' preparation only the first two are shown. In (**C**), the spectrum of the ascorbate-reduced 'anaerobic' preparation (solid lines) is compared with the spectra of the dithionite-reduced 'anaerobic' (dashed lines) and 'aerobic' (dotted lines) preparations (normalized at 620 nm). Spectra were recorded at 4.2 K and a magnetic field of 5 T.

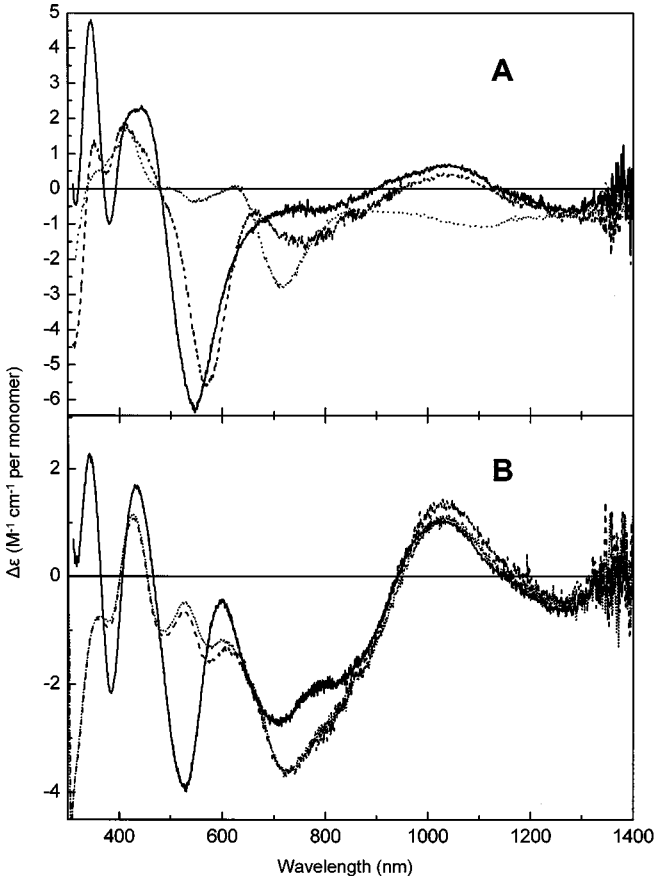

Figure 7 CD spectra of the 'anaerobic' (A) and the 'aerobic' (B) preparations of N_2OR at room temperature

Spectra of ferricyanide-oxidized (solid lines), ascorbate-reduced (dashed lines), and dithionite-reduced (dotted lines) samples are shown.

DISCUSSION

The different forms of N_2OR after exposure to oxygen

The first isolation of N_2OR was performed under aerobic conditions [15] but it became clear that N_2OR has a higher steady-state activity and different spectroscopic properties, if isolated and maintained anaerobically [32]. The aerobic form was called 'pink' N_2OR or N_2OR II while the anaerobic form was designated as the 'purple' form or N_2OR I [2,33]. Both forms can be reduced with dithionite to a 'blue' form, or N_2OR III. N_2OR IV, reconstituted from the apo-enzyme, and N_2OR V, isolated from a Cu_Z centre assembly mutant [33], are both inactive since they contain only Cu_A but no catalytic centre Cu_Z.

The reaction of N_2OR with oxygen apparently depends on a number of factors. Inactivation of N_2OR by oxygen is faster in the crude extract than in solutions of the purified enzyme [26]. A low activity form is obtained during aerobic chromatography of crude extract at the migration front [32]. An acceleration of inactivation is found if the enzyme is turning over on exposure to oxygen [34]. On the other hand, N_2OR from *Pa. pantotrophus* has proved to be active under aerobic conditions [35]. This sensitivity to oxygen appears to depend on the source from which N_2OR has been purified. N_2OR from *Achromobacter cycloclastes* and *Pseudomonas nautica* showed high activities even though they were isolated aerobically [27,36].

Depending on whether the preparations were anaerobic or aerobic, we obtained N_2OR with different properties of the catalytic centre. However, our aerobic preparation is different from the 'pink' form. If reduced with dithionite, the 'pink' form from *P. stutzeri* has an absorption coefficient of only $3.4\ mM^{-1}\ cm^{-1}$ per dimer at 650 nm in comparison to $6.1\ mM^{-1}\ cm^{-1}$ in the 'purple' form [26,28,33]. The lower absorption coefficients at 650 nm in the dithionite-reduced state, as well as the lower copper content, point to loss of Cu_Z^{total} in the 'pink' form. It can be estimated from the absorption coefficients of the 'pink' and 'purple' forms from *P. stutzeri* reduced with dithionite that the 'pink' form must have lost about half of Cu_Z^{total}. Consequently, the activity of this form is decreased. In contrast, our aerobic preparation, when exposed to only a limited incubation with oxygen, is not 'pink' but 'blue' in colour

Table 3 Comparison of Cu_Z and Cu_Z*

	Cu_Z^{total}	
	Cu_Z	Cu_Z*
Midpoint potential	$E_m \approx +60$ mV	$E_m > +400$ mV†
X-ray crystal structure	–	From *P. nautica* [3,10,27]
Preparation	Anaerobic	Incubation with oxygen, CO, SCN^-, NCO^-, site-directed mutagenesis
Examples from literature	N_2OR I from *P. stutzeri* [26,33]	(N_2OR II from *P. stutzeri*)‡ [26,33]
	From *Pa. denitrificans* [34]	Incubation of purified N_2OR from *R. sphaeroides* with O_2 for 3 days [37]
	From *R. sphaeroides* [37]	N_2OR from mutants C622D and H583G [38]
	From *A. xylosoxidans* [43]	Incubation with CO, SCN^-, and NCO^- [33]

† Concluded from the failure of oxidation with e.g. ferricyanide.
‡ Cu_Z^{total} content is significantly lower.

due to the higher absorption coefficient at 650 nm. It also has about the same Cu_Z^{total} as the anaerobic preparation, and is highly active. Sato et al. [37] discovered that aerobic incubation of purified *Rhodobacter sphaeroides* N_2OR for 3 days resulted in a form with spectroscopic properties between N_2OR I and II. Aerobic preparations of N_2OR from *Ps. nautica* is active and 'blue' [27]. This form, therefore, fits neither the classification of N_2OR I nor of N_2OR II.

Our results, put together with these data from the literature, establish that the catalytic centre, Cu_Z, of N_2OR can react with oxygen in two ways. First, Cu_Z can be altered to a form with drastically changed redox properties, called Cu_Z*, which, under our experimental conditions, is fixed in one redox state (Table 3). Cu_Z* however still seems to be capable of catalysis as indicated by the high steady-state activity. Completely aerobic N_2OR preparations often result in a 'pink' form of the enzyme which has most likely lost a greater amount of Cu_Z. Oxygen incubation can therefore cause both the transformation of Cu_Z to Cu_Z* as well as the overall loss of the catalytic site.

Spectroscopic differences between Cu_Z and Cu_Z*

Our strictly 'anaerobic' preparation, which is equivalent to N_2OR I, already contains some Cu_Z* and, furthermore, some of the catalytic centre is completely lost. Despite the obvious heterogeneity of our preparations, we could characterize spectroscopic properties of Cu_Z* in comparison to Cu_Z. There are alterations in the spectroscopic properties of Cu_Z* though the overall features are clearly similar to the spectra of the dithionite-reduced Cu_Z. These alterations, especially in the MCD spectra, show that only Cu_Z* and not Cu_Z is reduced in ferricyanide- and ascorbate-reduced samples. Absorption spectra of Cu_Z* have an additional peak at 930 nm and a shoulder at 770 nm. Interestingly, Cu_Z of the mutant C622D from *P. stutzeri* has similar properties [38]. In this mutant, one cysteine residue coordinating the Cu_A centre, is changed to an aspartate and consequently it has no Cu_A centre. If a Cu_A-coordinating histidine residue is mutated (mutant H583G [38]), the Cu_A site is not lost but the intense peak at 650 nm, as well as the shoulder at 930 nm, show a high Cu_Z* proportion. Obviously, alterations at the Cu_A site can effect the catalytic site on the other subunit. As for Cu_Z*, the catalytic centre in N_2OR of the C622D mutant is not able to change the redox state [38].

The CD spectrum of the 'aerobic' form has in general more features than the spectrum of the 'anaerobic' form, suggesting that Cu_Z* may have a lower symmetry than Cu_Z. Small changes in the resonance Raman spectra of Cu_Z* and Cu_Z were interpreted as minor structural differences [17]. The differences of the MCD spectra suggest some changes in the electronic structure of Cu_Z*.

Redox behaviour of Cu_Z in the different enzyme forms

Cu_A has a midpoint potential of $E_{m,7.5} \approx +260$ mV with $n \approx 1$ while Cu_Z has a midpoint potential of $E_{m,7.5} \approx +60$ mV ($n \approx 1$). We could not detect any redox changes of Cu_Z*, neither oxidation nor reduction. Since Cu_Z* has spectroscopic features of the reduced Cu_Z and no oxidation occurs with ferricyanide, the midpoint potential appears to be positively shifted by more than 400 mV.

The reduction of Cu_A with dithionite takes only seconds, whereas Cu_Z is reduced in about 1 h [33,37]. There must be a change in the reduction kinetic of Cu_Z during turnover for an efficient catalysis. Changes in the Cu_A domain can trigger the conversion of Cu_Z into Cu_Z*, as observed in N_2OR from the mutants C622D and H583G of *P. stutzeri*. Furthermore, the hyperfine splitting constant of Cu_A in our aerobic preparation is slightly changed. Alterations of the hyperfine splitting have been related to the influence of axial ligands at the Cu_A site, which effects the redox potential as well as the electron transfer rate [39–41]. Distinct structural changes in the Cu_A domain could be fine-tuning the electron flow to the catalytic site [5,42]. A structural change of the Cu_A domain, gating electron transfer to the active site, is a possible explanation for the obvious differences of the reduction kinetics between non-turnover and turnover experiments.

Reactions with oxygen

The chemical reaction with oxygen causing the change to Cu_Z* has not been investigated. Cu_Z seems to react similarly but faster with H_2O_2, as within seconds a blue absorption peak, presumably the 'aerobic' form, appears, followed by a loss of absorption [33]. Oxidation of the central sulphur atom to sulphoxide would explain the strong positive shift of the redox potential but seems unlikely since no sulphoxide group has been detected in the crystal structure. Furthermore, more substantial spectroscopic changes are expected to accompany the oxidation of the sulphide ligand to sulphoxide. If an amino acid in the binding pocket of Cu_Z were oxidized, it would introduce a negative charge and the midpoint potential might be expected to shift negatively. Site-directed mutants C622D and H583G show similar effects to oxygen in forming a Cu_Z* centre, as does reaction of cyanate, thiocyanate and CO in producing a Cu_Z*-rich form [33]. In the

case of CO, an increased activity is reported. These reactions are unlikely to involve the direct oxidation of the inorganic sulphur or an amino acid causing a change to Cu_Z*. It is more likely that a secondary structural change may determine, for example, a hydrogen bound switch to the inorganic sulphur or the solvent accessibility, causing a drastic change of the midpoint potential. Similar structural changes could have a functional role during turnover.

The aerobic preparation of N_2OR from *P. nautica* was similar to our 'aerobic' preparation, since the redox titration showed no redox change of Cu_Z, but the intensity of the blue peak was higher than described for the 'pink' form [27]. The co-purified assembly factor might protect N_2OR from losing Cu_Z during the completely aerobic preparation. The reported crystal structure of Cu_Z [10] is therefore that of Cu_Z*. A structure of Cu_Z is not yet available. A comparison of the structure of both forms may uncover reasons underlying the transformation to Cu_Z*.

This work has failed to correlate the relative amount of the two variants of the catalytic site, Cu_Z and Cu_Z*, with levels of catalytic activity. There is the curious paradox that the redox inactive form has the same or higher steady-state activity than the redox active form. This suggests that the EPR active oxidation state of Cu_Z may not be involved in the catalytic cycle of the enzyme under turnover conditions. It might be expected that the catalytic centre of N_2OR can undergo a 2-electron redox change to effect reduction of N_2O to N_2. It remains a possibility that Cu_Z can perform a 2-electron oxidation between, for example, the all Cu(I) state, $[Cu_4S]^{2+}$ and $[Cu_4S]^{4+}$, rather than undergoing separate 1-electron processes up and down the ladder of states, as shown in Figure 1(B).

We thank Ms Louise Male for developing the OTTLE cell and helpful discussions and Mr David A. Clarke for cell growth. This work was supported by BBSRC grants 83/B08413 to A.J.T. and B.C.B., 83/B111958 to the CMSB, and Wellcome Trust Grant (050709) to J.N.B.

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Received 8 January 2002/6 March 2002; accepted 11 April 2002