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A model of the copper centres of nitrous oxide reductase (*Pseudomonas stutzeri*)

Evidence from optical, EPR and MCD spectroscopy

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Nitrous oxide reductase (N_2OR), *Pseudomonas stutzeri*, catalyses the 2 electron reduction of nitrous oxide to di-nitrogen. The enzyme has 2 identical subunits (M_r ~ 70 000) of known amino acid sequence and contains ~ 4 Cu ions per subunit. By measurement of the optical absorption, electron paramagnetic resonance (EPR) and low-temperature magnetic circular dichroism (MCD) spectra of the oxidised state, a semi-reduced form and the fully reduced state of the enzyme it is shown that the enzyme contains 2 distinct copper centres of which one is assigned to an electron-transfer function, centre **A**, and the other to a catalytic site, centre **Z**. The latter is a binuclear copper centre with at least 1 cysteine ligand and cycles between oxidation levels Cu(II)/Cu(II) and Cu(II)/Cu(I) in the absence of substrate or inhibitors. The state Cu(II)/Cu(I) is enzymatically inactive. The MCD spectra provide evidence for a second form of centre Z, which may be enzymatically active, in the oxidised state of the enzyme. Centre **A** is structurally similar to that of Cu_A in bovine and bacterial cytochrome *c* oxidase and also contains copper ligated by cysteine. This centre may also be a binuclear copper complex.

Copper protein; EPR; MCD; Nitrous oxide reductase

1. INTRODUCTION

The denitrifying bacterium *Pseudomonas stutzeri* reduces nitrous oxide, N_2O, to di-nitrogen as part of a respiratory pathway of energy conservation which is coupled to ATP generation [1]. The enzyme responsible for catalysing this 2-electron reduction is nitrous oxide reductase (N_2OR), which is composed of 2 identical subunits (M_r ~70 000) of known amino acid sequence [2] and has a stoichiometry of about 4 copper ions per monomer [3]. A variety of spectroscopic methods have been applied to the study of the copper centres but no clear structural model has yet been proposed for them. Two oxidation levels of the enzyme have been observed previously [3,4]. The oxidised state, referred to as the 'purple' form, has EPR signals representing up to 30% of the total copper and absorption bands at 480 nm and 540 nm. On reduction with a variety of agents including dithionite, a second copper site is observed with an absorption band at 650 nm, a broad EPR signal with *g*-values close to 2.0 and a resonance Raman spectrum similar to that of type 1 copper sites in proteins [5]. This is known as the 'blue' form. The EPR spectrum of the purple form shows a distinctive 7-line hyperfine pattern which, it has been proposed, arises from a mixed-valence copper dimer in the oxidation state [Cu(+1.5)...Cu(+1.5)] with an unpaired electron delocalised between both copper nuclei (I_N = 3/2) [6]. Each copper ion in the dimer must therefore have an identical chemical environment. Evidence has been accumulating that this copper site is structurally identical to that of Cu_A in bacterial and eukaryotic cytochrome *c* oxidase [6,10,11]. In this letter we present ultra-low temperature magnetic circular dichroism (MCD) spectra together with optical and EPR spectra of 3 different oxidation levels of N_2OR. These results strongly suggest that there are 2 distinct copper centres in N_2OR, an electron transfer centre, **A**, and a catalytic centre, which we term **Z**. Results presented here show that the latter is a binuclear copper site and probably exists in resting enzyme in 2 forms. It remains to be definitively established that Cu_A is also binuclear.

2. MATERIALS AND METHODS

N_2OR was purified under anaerobic conditions by procedures previously described [7] and stored in liquid nitrogen until use. The copper content was determined by atomic absorbance spectroscopy and the

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protein by the method of Lowry et al. [8]. The preparation used for experiments reported here had an initial protein concentration of 602 μM and 3.32 Cu per subunit. The A_{540}/A_{480} ratio was 1.3.

EPR spectra were recorded with an ER-200D (Bruker) spectrometer using an Oxford Instruments ESR-9 flow cryostat. Integration of the EPR signals was carried out under non-saturating conditions using 1 mM Cu(II) EDTA as a standard and the method of Aasa and Vanngard [9]. MCD spectra were measured with a JASCO J-500D dichrograph, interfaced to an IBM AT-PC, with a split-coil, top-loading superconducting solenoid (SM4; Oxford Instruments) capable of generating magnetic fields up to 5 Tesla and giving sample temperature control between 1.5-200 K. MCD spectra are quoted in units of $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ (mM^{-1}·cm^{-1}) and are not normalised for magnetic field. The molarity refers to the concentration of electron spins as determined by integration of the EPR spectrum. All pH values quoted as pH* are the reading of pH meter when measuring a deuterated buffer.

3. RESULTS

The absorption spectra of N_2OR as a function of the reduction by ascorbate are shown in Fig. 1. Excess ascorbate brings about only partial bleaching of the peak at 540 nm but complete removal of the shoulder at 480 nm. Further reduction is extremely slow. Addition of a trace of the mediator dye phenazine methosulphate, (PMS) (E_o = +80 mV), speeds up reduction generating a blue form absorbing at 650 nm. This is consistent with earlier reports that reduction of N_2OR with sodium dithionite proceeds in 2 kinetic steps, a fast phase followed by a slower phase [4]. The second phase of reduction, catalysed by PMS, gives rise to an isosbestic point at 624 nm in the absorption spectrum. Hence, over the last phase, only 2 coloured species are in equilibrium. The X-band EPR spectra have been recorded from the same sample of N_2OR in the oxidised, semi-reduced and fully reduced states (Fig. 2). The spectra of the oxidised and fully reduced forms agree well with those reported previously [3,4], the 7-line hyperfine structure of the oxidised state being clearly resolved. Integration of the signals shows that 22 and 10% of the total copper is paramagnetic in the oxidised and reduced forms, respectively. However, the EPR spectrum of the semi-reduced state is very weak, with only 0.45% of the total copper being paramagnetic. No other signals were detected from this state. This result shows that N_2OR can be reduced from an EPR-active form through an EPR-silent state to a second EPR-active form and further that the EPR-silent form corresponds to the completion of the fast phase of reduction by ascorbate.

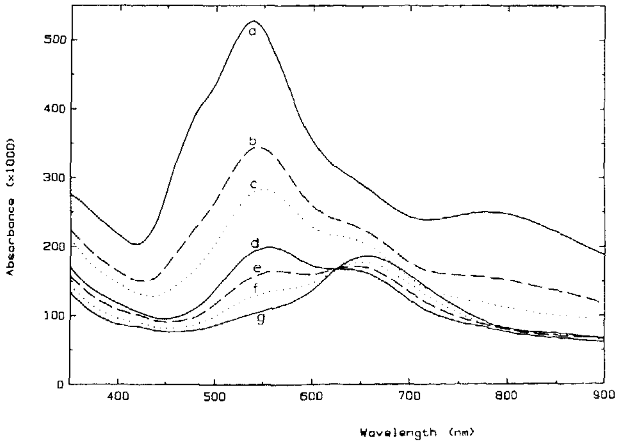


Fig. 1. Room temperature absorption spectra of anaerobic nitrous oxide reductase showing the effects of addition of sodium ascorbate and phenazine methosulphate. (a) N_2OR 602 μM in protein, 3.32 moles Cu/mole subunit in 100 mM TRIS/D_2O, pH* 7.5, 1 mm pathlength; (b,c) Spectra recorded 20 and 75 min after the addition of 5e$^-$ equivalents of sodium ascorbate; (d) Spectrum taken 40 min after the addition of a further 5e$^-$ equivalents of sodium ascorbate; (e,f,g) Spectra recorded 1, 15 and 45 min after the addition of a trace of PMS to sample at point (d).

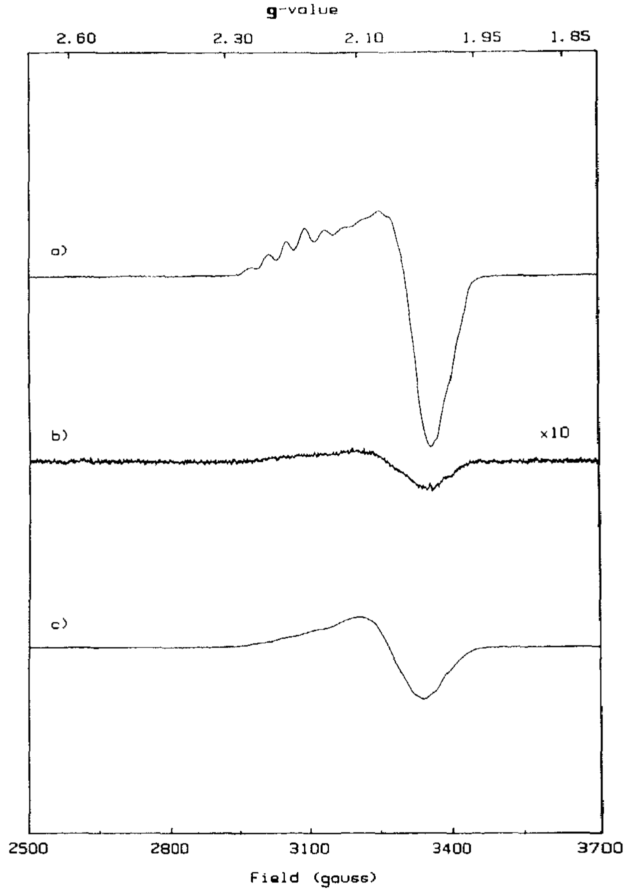


Fig. 2. X-band electron paramagnetic resonance spectra of nitrous oxide reductase. Frequency 9.39 GHz, power 0.2 mW, modulation amplitude 10 gauss, sample temperature 15 K. Protein conditions are as given in legend to Fig. 1. (a) Sample as in Fig. 1a. Relative signal gain = 1. Signal concentration = 890 μM, i.e. 22% of total copper; (b) Sample as in Fig. 1d. Relative signal gain = 10. Spin concentration = 0.45% of total copper; (c) Sample as in Fig. 1g. Relative signal gain = 1. Spin concentration = 10.1% of total copper.

The intensity of the MCD spectra of paramagnetic species is inversely proportional to the absolute temperature of the sample [12]. Hence at 4.2 K the spectra are invariably dominated by the contribution from the paramagnetic centres present. The MCD spectra of the oxidised and reduced forms of N_2OR have been measured in buffer/glycerol mixtures (Fig. 3). The EPR spec-

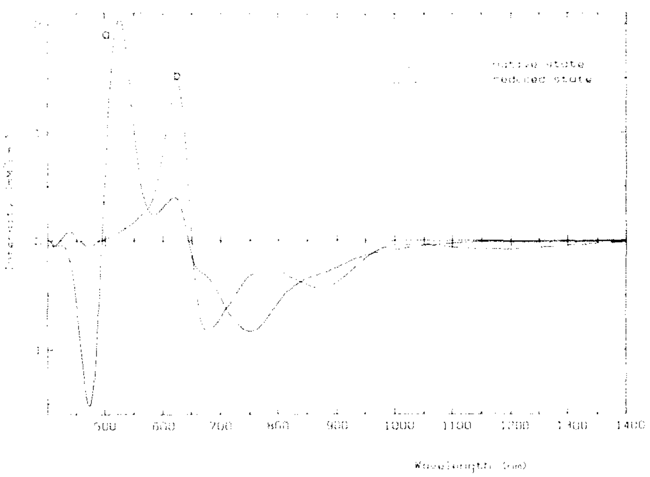


Fig. 3. Magnetic circular dichroism spectra of nitrous oxide reductase measured at 4.2 K and 5 Tesla. (a) Native sample 289 μM in protein dissolved in 50% (v/v) deuterated glycerol, TRIS/D_2O, pH* 7.5. $\Delta\varepsilon$ values for centre A, expressed in $mM^{-1}\cdot cm^{-1}$, are based on total spin integration less calculated contribution from centre **Z**. Centre A concentration = 0.325 μM. (b) Reduced sample. 201 μM protein in 50% (v/v) deuterated glycerol, 100 mM TRIS/D_2O, pH* 7.5. Reduced by sodium ascorbate plus PMS. $\Delta\varepsilon$, ($=\varepsilon_L-\varepsilon_R$, the molar extinction coefficients for left and right circularly polarised light), expressed in $mM^{-1}\cdot cm^{-1}$ based upon the spin molarity determined by integration of the EPR signal. Centre **Z** concentration = 0.135 μM.

tra are unchanged in this solvent and in aqueous buffer. MCD magnetisation curves [12] determined at all the major features in the spectra confirm that the spectra arise from a spin $S = \frac{1}{2}$ paramagnet with g values close to 2.0. Therefore the MCD spectra are dominated in both oxidation states by the EPR-active paramagnets. The MCD intensities, expressed as $\Delta\varepsilon$ ($mM^{-1}\cdot cm^{-1}$) and calculated on the basis of the electron spin concentrations, are similar for both oxidation states. However, the form and shape of the spectra are very different for the 2 oxidation states.

According to Q-band EPR studies at 110 K of the oxidised form of this enzyme 2 paramagnetic, $S = \frac{1}{2}$ species are present [18]. In addition to the major species with g values of 2.16 and 1.99 there is a weaker line at $g = 2.02$. This raises the question of which bands in the MCD spectrum belong to these 2 species. There is a form of the enzyme, N_2OV, isolated from a mutant strain, MK 402, which is catalytically inactive and contains only 2 Cu atoms per dimer. The EPR spectrum of the oxidised form of N_2OV lacks the signal at $g = 2.02$. The MCD spectrum of this form of the enzyme is almost identical to that of the MCD of the native state protein except that the peak at 625 nm and the trough at 660 nm are absent. Hence the MCD bands at 525, 473 and 750 nm are associated with the major paramagnetic species. We note that the MCD spectrum of the second species in the native protein (with bands at 625 and 660 nm) is similar in form to that of the paramagnet observed in the dithionite-reduced state of the enzyme suggesting a close structural relationship between them. However the observation that on reduction with ascorbate the enzyme is rendered virtually EPR-silent shows that both paramagnetic centres present in the oxidised state of the enzyme can be reduced to diamagnetic states.

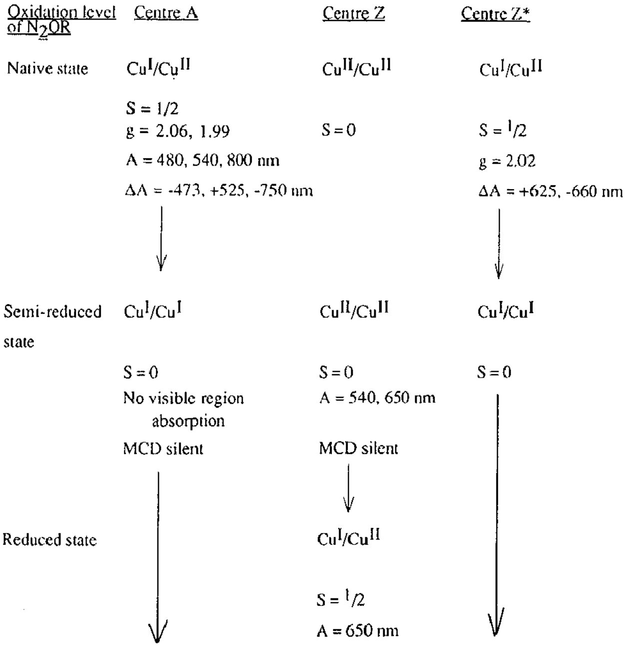
The semi-reduced state of the enzyme is virtually EPR-silent and therefore contains the reduced diamagnetic form of centre **A**. The optical absorption spectrum of the semi-reduced state shows intense absorption bands at 540 and 650 nm ($\varepsilon \sim 2000\ M^{-1}\cdot cm^{-1}$ based on the concentration of protein monomer). These bands must belong to a second centre, which we term **Z**. This centre is readily reduced by ascorbate only in the presence of the mediator PMS. The absorption spectra show, as a function of further reduction, an isosbestic point at ~624 nm. Hence 2 species are being interconverted by addition of 1 or more electrons. This further reduction step leads to the generation of a new EPR-active paramagnetic state with electron spin $S = \frac{1}{2}$ and intense temperature-dependent MCD transitions. Further reduction, even by powerful reductants such as sodium dithionite, does not take place [4]. The presence of intense absorption bands arising from centre **Z** when oxidised suggests the presence of ligand-to-Cu(II) charge-transfer bands and therefore gives evidence for the presence of copper(II) ions. Reduction of this centre generates an EPR-detectable copper(II) centre with spin $S = \frac{1}{2}$. This behaviour can be explained if the copper(II) ion, in the oxidised state, is rendered EPR-silent by being exchange-coupled to a neighbouring paramagnetic species. If the species were an organic radical [13] it is difficult to understand why further reduction of centre **Z** with a strong reductant is impossible. This explanation also fails to account for all copper ions in the enzyme. A straightforward interpretation is that centre **Z** is a binuclear copper site which can cycle between oxidation states Cu(II)/Cu(II) and Cu(II)/Cu(I) in the absence of substrate or inhibitors. If the 2 metal ions were strongly coupled in the oxidised state this would yield a ground state spin $S = 0$. However, optical absorption due to ligand-to-metal charge-transfer bands would still be present. In the reduced state, a Cu(II)/Cu(I) pair will generate a spin $S = \frac{1}{2}$ paramagnet with intense absorption bands. Further reduction would be prevented by the low potential of the pair.

The MCD spectra of both centre A and Z in N_2OR are intense, $\Delta\varepsilon > 1400\ M^{-1}\cdot cm^{-1}$ at 4.2 K and 5 Tesla, and there are 2 bands of opposite sign under the absorption band. The high intensity, together with 2 bands of opposite sign, indicates 2 charge-transfer transitions with components perpendicular to each other. The peak and trough in the MCD at 473 and 525 nm of centre **A** and at 678 and 625 nm of centre **Z** show the presence of 2 CT transitions. The only ligand known to give such transitions with copper(II) ions in this spectral region is cysteine. Hence the MCD spectra give evidence of cysteine–copper coordination in both centres **A** and **Z**. Furthermore, in each centre there must be 2 thiolate-copper(II) CT transitions. If the centre contained only a single copper(II) ion, a minimum of 2 cysteine ligands would be required. However, if the centre contains 2 copper ions in the oxidation state Cu(II)/Cu(II) or Cu(II)/Cu(I) a single thiolate group bridging both metal ions could generate 2 perpendicularly polarized CT transitions.

We return to the identity of the second paramagnetic species detected in the oxidised enzyme by Q-band EPR [18] and by MCD spectroscopy. The form of the MCD spectrum suggests it is similar in structure to the dithionite-reduced form of centre **Z**, namely a Cu(I)/Cu(II) dimer. However, it is reduced by ascorbate to an EPR-silent form. If centre **Z** is the catalytic site it will have 1 or both copper ions in a state reactive towards ligands. Therefore it is to be expected that centre **Z** may have a number of forms, one of which could be open to react with substrate and another with other groups such as OH^-, or carboxylate, occupying the site. These forms will have different redox potentials. It is, we suggest, the presence of multiple forms of the catalytic site **Z** which has led to the difficulty in understanding the EPR spectra of this enzyme. The low EPR spin integrations of centre **Z** bear this out.

4. DISCUSSION

By using a combination of EPR and low temperature MCD spectroscopy of N_2OR in several oxidation levels we have provided evidence for the presence of 2 distinct copper centres, which we propose to label **A** and **Z** (Scheme 1). The model of 2 centres, 1 to transfer electrons and 1 to bind substrate and effect catalysis, suggested by this work contrasts with some other proposals. An alternative model, recently reviewed [18], identifies 3 centres in N_2OR, labelled **A**, **B**, and **C**, 2 of which are suggested to be monomeric, namely **A** and **B**, and the third, centre **C**, a binuclear class III mixed-valence copper pair but with cysteine coordination and thought to be the binding site of substrate. The centre **A** is described as mononuclear possibly with magnetic interaction with another paramagnet and similar to the Cu_A site of cytochrome oxidase. Centre **C**, the catalytic site, is assigned to a mixed valence binuclear site associated with the 7-line hyperfine structure in the EPR spectra.



Scheme 1. Centre **A** is shown here as a dimer [6] but this has yet to be proved. Two forms of centre **Z** are shown, indicated by **Z** and **Z***, which must differ in their respective redox potentials. A, maxima in the absorption spectrum; ΔA, maxima (+) and minima (−) in the MCD spectrum.

In our model the centre labelled **A** is shown to be clearly similar in structure to Cu_A in bovine cytochrome *c* oxidase [15,16]. Although the form of **A** is controversial, the 7-line hyperfine structure in the EPR spectrum of this centre has found an explanation so far only in terms of a binuclear mixed-valence metal dimer [6]. The MCD spectra and resonance Raman spectra require that this centre is liganded by cysteine ligands and, as suggested by sequence data comparison, 2 cysteine ligands are required. This centre acts as an electron carrier in cytochrome *c* oxidase and presumably carries out the same function in N_2OR.

Centre **Z**, assigned as the catalytic site, exists in at least 2 forms, which probably correspond to an active and an inactive or blocked form. This centre also has ligation by cysteine. The mixed-valence state Cu(I)/Cu(II) of **Z** produced by dithionite reduction has no enzymatic acitivity. Re-oxidation of this state with ferricyanide ion will restore activity to the enzyme [3]. It is therefore possible that the active state of **Z** can be regenerated from the inactive form. It seems that in the absence of substrate or inhibitor, such as azide ion, some of **Z** can be reduced to this inactive mixed-valence state. This suggests that under these circumstances there is an interaction between both copper ions preventing substrate binding and also stopping further reduction. This could occur if a bridging ligand, such as OH^-, or an endogenous amino acid side-chain, such as carboxylate, were to enter the substrate site. This would also change the redox potential of the dimer and hence account for the difference between the redox properties of the 2 forms of **Z**.

Because the enzyme carries out a 2 electron reduction of substrate it might be expected that the binding site of substrate, N_2O, would have the capacity to store 2 electrons. There is evidence that in the presence of the inhibitor azide the enzyme can be reduced to an EPR-silent and colourless state [4]. In this state of the enzyme

Z has been converted into a single form and has accepted 2 electrons to give Cu(I)/Cu(I) spins.

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