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The copper site in nitrous oxide reductase

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Summary. The properties of the novel copper enzyme nitrous oxide reductase from denitrifying *Pseudomonas stutzeri* are described. Multifrequency electron paramagnetic resonance spectroscopy is used to characterize the various forms of the enzyme. The features observed at 2.4, 3.4, 4.5, 9.31 and 35 GHz are explained by a mixed-valence [Cu(1.5)...Cu(1.5)] $S = 1/2$ species with the unpaired electron delocalized between the two Cu nuclei. This site is also present in the catalytically inactive derivative of nitrous oxide reductase which was obtained from a transposon Tn*5*-induced mutant with defective chromophore biosynthesis. The resemblance of the low-frequency electron paramagnetic resonance spectra to the spectra for the so-called Cu_A of cytochrome *c* oxidase can be taken as a first indication that the Cu_A may have a structural and electronic arrangement similar to the electron-paramagnetic-resonance-detectable copper in nitrous oxide reductase. Results from oxidation/reduction experiments, and from a quantitative determination of sulfhydryl and disulfide residues in the various forms of nitrous oxide reductase, suggest the involvement of the redox-couple cysteine/cystine in the structural organization of the active site of nitrous oxide reductase.

Key words: Nitrous oxide reductase - Cytochrome *c* oxidase - Cu-Cu interaction - Mixed-valence complex - Denitrification

Introduction

Nitrous oxide reductase (N_2OR) has been isolated and purified to homogeneity from several denitrifying bacteria that utilize nitrous oxide as part of a respiratory pathway of energy conservation (Riester et al. 1989). N_2OR is involved in the conversion of nitrous oxide to dinitrogen. The N-O bond is cleaved upon transfer of $2e^-/2H^+$ forming 1 mol water, in analogy to the reduction of dioxygen ($4e^-/4H^+$ transfer) to 2 mol water catalyzed by cytochrome *c* oxidase (ferrocytochrome:O_2 oxidoreductase):

$$N_2O + 2H^+ + 2e^- \longrightarrow N_2 + H_2O.$$

Nitrous oxide is a linear asymmetrical molecule and rather unreactive as expected for 16-electron triatomic species. The N-N and N-O distances are short in agreement with the calculated bond orders of 2.73 (N,N) and 1.61 (N,O) (Jug 1978). N_2O is thermodynamically unstable. It decomposes above 600° C to N_2 and O_2 by fission of the weaker N-O bond. The activation energy of this process is around 250 kJ mol^{-1} (Jones 1975). Cupric and cuprous oxide seem to be effective catalysts of the cleavage of the N-O bond (Dell et al. 1953a; Dell et al. 1953b; Scholten and Kovalinka 1969). Nitrous oxide behaves as a rather poor ligand. There is spectral evidence for $[Ru(II)(NH_3)_5N_2O]^{2+}$; a microcrystalline solid, $[Ru(II)(NH_3)_5N_2O](BF_4)_2$ was isolated (Diamantis and Sparrow 1970). The linkage of N_2O (isoelectronic with cyanate, NCO^-) in these complexes is still a controversial issue. Molecular orbital calculations indicate that (a) N-linkage complexes are more stable than O-linkage complexes, and (b) that other transition-metal complexes of N_2O should be stable (Fu-Tai Tuan and Hoffmann 1985).

N_2OR activity is linked in all cases to the presence of Cu, and it is now generally accepted that N_2OR belongs to the class of multicopper enzymes (Riester et al. 1989). Unexpectedly, the N_2OR from the nondenitrifying bacterium *Wolinella succinogenes* has been shown to contain two heme *c* prosthetic groups in addition to six Cu atoms (Teraguchi and Hollocher 1989). The principal properties of N_2O reductases have been reviewed recently (Zumft and Kroneck 1990). All enzymes characterized so far are Cu-dependent; however, they do not follow a uniform molecular pattern. The enzymes from *Pseudomonas stutzeri* and *Paracoccus denitrificans* are dimeric proteins with approximately 8 Cu atoms/dimer. The nuclear mass of N_2OR from *Alcaligenes xylosoxidans* is close to that from *Pseudomonas stutzeri*, but only 5 Cu atoms/molecule are

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found (Matsubara and Sano 1985). The monomeric protein from *Rhodobacter sphaeroides* has approximately half the molecular mass and Cu content of the *Pseudomonas stutzeri* enzyme (Michalski et al. 1986).

In this article we summarize some recent results on N_2OR isolated from the marine organism *P. stutzeri*. A preliminary structural model for the active site of the multicopper protein is presented, and the structural relationship between the copper sites in N_2OR and cytochrome *c* oxidase will be discussed.

Molecular properties of N_2OR from *Pseudomonas stutzeri*

N_2OR was purified as a dimer consisting of two presumably identical subunits of M_r 74000. The protein exists in different forms with distinct spectroscopic properties (Table 1). By preparative isoelectric focusing of pure purple N_2OR (form I) two bands with pI values 4.98 and 5.04 are separated (Riester et al. 1989). The purple form I probably comes closest to the native protein, with the highest activity of the cathodic variant of N_2OR when purified under the strict exclusion of dioxygen throughout the purification process. Amino-acid analysis of N_2OR (form I) gives the composition reported earlier for the pink enzyme (form II), however, the values obtained for purple N_2OR were significantly higher for cysteine 12 vs 4) and tryptophan (9 vs 5) (Riester 1989; Riester et al. 1989). Genetic approaches finally led to the elucidation of the primary structure of the enzyme (Viebrock and Zumft 1988). Thus, per subunit, M_r 67280, there are 9 cysteine and tryptophan residues each, 23 histidines and 24 methionines (Viebrock and Zumft 1988). On this basis the Cu content determined for purple N_2OR I is 7.6 ± 0.7 atoms/molecule (obtained from our best four preparations). The Cu content of this form is the highest found among the different forms of N_2OR. None of the N_2OR samples from *P. stutzeri* showed substantial amounts of other transition metals, such as Mn, Fe, Co, Ni or Zn. For the protein isolated from the mutant MK402 (Form V), 2.1 ± 0.1 Cu atoms/molecule were determined (M_r 140000). As observed for the regenerated form IV, no N_2OR activity is found for N_2OR V. Attempts to incorporate further Cu atoms into the mutant protein remained unsuccessful. Several procedures were applied for the preparation and reconstitution of apo-N_2OR. So far, none of the reconstituted samples (form IV) prepared by dialysis against various Cu(I) and Cu(II) complexes in the absence or presence of dioxygen was active in our N_2OR test system (Coyle et al. 1985; Riester et al. 1989).

The most prominent features of the different forms of N_2OR from *P. stutzeri* are summarized in Table 1.

Table 1. Classification of various forms of nitrous oxide reductase from *P. stutzeri*

N_2OR	Molecular properties and observations
I (purple)	8 Cu atoms/140-kDa molecule; high activity; isolated anaerobically except for isoelectric focusing; 20–50% EPR-detectable Cu; $g_{II}=2.18$, $A_{II}=3.83$ mT (seven lines), $g_{\perp}=2.03$, $A_{\perp}=2.8$ mT (X-band)
Ia (cathodic)	variant of N_2OR I; high activity; focuses towards the cathode, pI = 5.04
II (pink)	7 Cu atoms/140-kDa molecule; low activity, isolated aerobically; 20–50% EPR-detectable Cu; $g_{II}=2.18$, $A_{II}=3.55$ mT, $g_{\perp}=2.03$, $A_{\perp}$ not resolved at X-band
III (blue)	obtained by anaerobic reduction of N_2OR I, Ia and II; catalytically inactive; 10–30% EPR-detectable Cu; broad featureless EPR signal at X- and Q-band, at S-band splitting of 2.4 mT; resonance Raman data indicate presence of type-1 Cu
IV (regenerated)	prepared by reconstitution of apo-N_2OR; catalytically inactive; EPR properties similar to those of N_2OR V
V (mutant)	2 Cu atoms/140-kDa molecule; isolated from mutant strain MK402 defective in chromophore biosynthesis; catalytically active; 50% EPR-detectable Cu; $g_{II}=2.18$, $A_{II}=2.55$ mT (at least five equidistant lines), $g_{\perp}=2.03$, $A_{\perp}$ not resolved at X-band; S-band spectra identical to those of N_2ORI

Redox properties of N_2OR from *P. stutzeri*

Solutions of N_2OR I, II, and V, (Riester et al. 1989) show no further increase in absorbance over the range 400–800 nm on addition of a stoichiometric amount, or 10-fold excess, of ferricyanide. Solutions of N_2OR in Tris/HCl or phosphate pH 7.3–7.5, when kept at 2° C for more than 24 h under aerobic conditions, become enriched in N_2OR II as indicated by the decrease of the purity index A_{540}/A_{480} (Coyle et al. 1985). Irrespective of the nature of the reductant, the purple chromophore of N_2OR is bleached and a blue species is generated and persists under anaerobic conditions, with an absorption maximum at approximately 650 nm (N_2OR III). Cationic reductants (Cr(II) and Ru(II) complexes), anionic reductants (dithionite, L-ascorbate, thiolates, $Fe(II)EDTA^{2-}$) and photochemically generated intermediates of deazaflavin or hematoporphyrin are equally ineffective (Riester et al. 1989). The mutant protein, N_2OR V, reacts quite differently; at the end of the reduction, there is no absorbance in the region 400–800 nm, as is generally observed with other Cu proteins under reducing conditions. Reduction of N_2OR by a stoichiometric amount of dithionite ($1e^-$/Cu atom) produces the blue chromophore N_2OR III. Anaerobic reoxidation of N_2OR III (obtained from N_2OR I) by titration with ferricyanide results in the recovery of more than 95% of the original N_2OR I. In preparations of the pink form II the absorbance at 650 nm is higher in the oxidized sample (prepared aerobically) than in the reduced sample. By difference spectroscopy it is clearly demonstrated that no additional amount of the blue chromophore has been formed during the reduction of N_2OR II.

Once formed, the blue chromophore is rather inert

and can not be further reduced by a large variety of different types of reductants (Riester et al. 1989). Interestingly, after several cycles of photochemical reduction followed by reoxidation with ferricyanide not as much N_2OR III is produced per cycle as is observed in the first photochemical reduction of the protein by deazaflavin and oxalate. After two redox cycles N_2OR I is converted to a species with properties resembling N_2OR V. Presumably, a small amount of cyanide is liberated from ferricyanide or ferrocyanide during the second or third photochemical reduction, which then causes a partial but selective Cu depletion from the site responsible for the generation of the blue chromophore (Riester et al. 1989).

At 25° C the reaction of N_2OR I with a stoichiometric amount of dithionite proceeds in two phases. In the first, fast, phase (less than 30 s) almost half of the absorbance at 540 nm disappears, and the absorbance at 650 nm decreases simultaneously. In a second, slower phase (within minutes) a broad band with λ_{max} at about 650 nm appears and an isosbestic point at around 625 nm is observed. Subtraction of the spectrum recorded in the slow phase from the starting spectrum of N_2OR I clearly shows that, in the fast phase of the dithionite reaction, the Cu centers of N_2OR I become reduced, having ultraviolet/visible properties identical to those reported for N_2OR V. Upon titration of N_2OR I with dithionite a similar result is found (Riester et al. 1989).

Interaction of N_2OR I from *P. stutzeri* with exogenous ligands

We probed the Cu site of N_2OR by interaction with small ligands, among them azide, cyanate, thiocyanate, fluoride, nitrite and urea. The reactivity towards N_2O, CO, NO and acetylene was also investigated (Riester 1989; Riester et al. 1989). Nitrous oxide (26 mM, saturated solution) causes only a minor change in the electronic spectrum of N_2OR I after approximately 24 h. Addition of cyanate or thiocyanate (10-fold excess over protein) to N_2OR I causes, within 15 min, a decrease of A_{480}/A_{540} from 1.45 to 1.05; at the same time the absorbance at 640 nm increases. Addition of azide (10–100-fold excess over protein) causes partial bleaching of the absorbance in the region 400–800 nm. Anaerobic reduction of N_2OR, pretreated with azide by dialysis, completely bleaches the absorbance in the visible region.

Among the N,O compounds nitric oxide exhibits the highest reactivity towards the different forms of N_2OR (Riester 1989; Riester et al. 1989). In the presence of NO (50–250 μM), under the strict exclusion of dioxygen, N_2OR I is converted to N_2OR II within a few seconds at 0° C. N_2OR III also reacts with NO at low concentrations giving a species with the spectral characteristics of N_2OR II. The mutant protein N_2OR V does not react with NO. Concomitant with the spectral changes of N_2OR I with NO a new absorption maximum at 345 nm is detected which indicates the formation of nitrite. This is confirmed by direct reaction of the enzyme with NO_2^-.

By contrast to the fast reaction with NO, CO reacts very slowly in a comparable experiment. After approximately 15 h (1.5 mM CO) the absorbance at 540 nm has dropped by 50% and a clear maximum at 660 nm becomes visible, i.e. N_2OR II had been formed. This result is interesting in view of the fact that CO seems to enhance the catalytic activity of the enzyme (Riester et al. 1989). As observed for nitrous oxide, acetylene, which is a well-known inhibitor of nitrous oxide reduction (Knowles 1982), does not exhibit any significant reactivity towards the different forms of N_2OR (Riester 1989).

Sulfhydryl and disulfide groups in N_2OR from *P. stutzeri*

Our results from the reduction/oxidation experiments and from the experiments with apo-N_2OR and the regenerated N_2OR IV seem to indicate that the RSH-RSSR/Cu(II)-Cu(I) redox equilibrium (Hemmerich et al. 1966) may be involved in the organization of the active site of N_2OR and the formation of the blue chromophore of N_2OR III. Consequently, the number of 'free' sulfhydryl and disulfide groups in the different forms of N_2OR, including apo-N_2OR, were determined colorimetrically as described in the literature (Ellmann 1959; Riddles et al. 1983; Thannhauser et al. 1987). There are no free sulfhydryl residues in N_2OR I, in agreement with earlier experiment using *p*-hydroxymercuribenzoate or thallium compounds. Similar observations are reported for N_2OR from *Paracoccus denitrificans* (Snyder and Hollocher 1987). In the presence of guanidinium·HCl (GdnHCl, 5–6 M) 8 SH groups/dimer become accessible in N_2OR from *P. stutzeri* vs 11–12 in the presence of GdnHCl and EDTA. In N_2OR III (obtained from N_2OR I by photochemical reduction) these figures increase to 9 (GdnHCl) and 13–14 SH groups/dimer (GdnHCl, EDTA). Unexpectedly, apo-N_2OR does not exhibit any reactivity towards 5,5'-dithiobis(2-nitrobenzoic acid). After treatment with GdnHCl/EDTA 18–19 SH groups/dimer are determined, in good agreement with the number obtained from the sequence data (Viebrock and Zumft 1988). For the inactive forms N_2OR IV and N_2OR V, approximately 15 and 16 SH groups are observed in the presence of GdnHCl/EDTA.

Analysis of N_2OR I for disulfide residues (Thannhauser et al. 1987) gives a value of approximately 4 SS groups/dimer. No SS groups are detected for the other forms of N_2OR including apo-N_2OR.

Parallel to the quantitative determination of sulfhydryl and disulfide residues, we also investigated the fluorescence properties of the different forms of N_2OR. Upon excitation at 290 nm, N_2OR I shows an intense emission around 330 nm. The maximum shifts to 350 nm in N_2OR III accompanied by a significant loss in intensity. Reoxidation with ferricyanide leads to the original fluorescence spectrum observed for N_2OR I. Identical fluorescence properties are observed for apo-N_2OR.

By comparison with other multicopper proteins this behaviour is rather unexpected. Both laccase and ascorbate oxidase (Goldberg and Pecht 1974; Marchesini and Kroneck 1979) give an increase in intensity of the fluorescence emission upon reduction, and no shift of the emission maximum is observed.

Electron paramagnetic resonance properties of N_2OR from *P. stutzeri*

We reported previously the unusual EPR spectrum of N_2OR I detectable at 9.32 GHz and 10 K (Coyle et al. 1985). Both the g_{II} and g_I region are resolved, with $A_{II}=3.82$ mT (seven equidistant lines) and $A_I=2.8$ mT (at least four lines). The shape and the number of hyperfine lines depend on the individual preparation of N_2OR, mainly because of the presence of a second rather broad EPR signal. This broad signal becomes clearly visible after reduction of N_2OR I and II by dithionite but is not observed with the regenerated form IV or the mutant protein N_2OR V. Highly concentrated samples of N_2OR have been carefully examined for the presence of a half-field signal at $g=4$, indicative of a weak magnetic interaction between Cu(II) centers (Solomon et al. 1983). So far, in the temperature range 5–120 K, and frequencies of 2.4, 3.5, 4.5, 9.3, 34 GHz, no significant EPR signal at $g=4$ has been detected. It seems that the component responsible for the broad and featureless signal at X-band, which is observed to some extent in N_2OR II and fully developed in N_2OR III, is missing from the mutant protein N_2OR V (approximately 2 Cu atoms/molecule). Furthermore, the array of Cu centers giving rise to the pattern of seven equidistant lines at an apparent g-value of 2.18 seems to be perturbed. To increase the resolution in terms of g-values, EPR spectra are recorded at 34 GHz, 110 K (Riester et al. 1989). N_2OR V gives a simple signal, as expected, with g_{II} at approximately 2.16, and g_I at approximately 1.99. In addition, there is a weaker third line around $g=2.02$, which is more intense in the spectra of N_2OR I and III. Both the features at $g=2.16$ and $g=1.99$ disappear in N_2OR III (obtained from N_2OR I/dithionite), whereas the line at $g=2.02$ remains almost unchanged. These EPR features are found in all N_2OR samples investigated at Q-band and 110 K.

The dependence of the EPR spectra at X-band on the microwave power and the temperature has been investigated. Over the range 10–77 K no unusual effects on the spectrum are detected. The signal intensity decreases as observed for ascorbate oxidase from *Cucurbita pepo medullosa* (Kroneck et al. 1982). A marked change of the EPR signal of N_2OR I occurs around 100 K, i.e. the resolution of the g_{II} region into 7 lines disappears. Similarly, the hyperfine structure at g_I is lost and a small splitting into at least three lines (approximately 1.3 mT) is observed at the minimum of the perpendicular transition. The EPR spectrum of N_2OR V does not show such a pronounced dependence on the sample temperature.

At 10–15 K, where all the EPR spectra have been recorded, the signal begins to saturate at 0.5–10 mW. We have previously made an estimate of the amount of EPR-detectable Cu in N_2OR (Coyle et al. 1985). This has been extended to several samples of the enzyme with different purity indices. The highest content (54%) is found in the high-pH form of N_2OR I (Coyle et al. 1985). In N_2OR V, pH 7.5, 47% of the total Cu is detected by EPR. The broad signal left after reduction of N_2OR I accounts for approximately 50% of the original signal intensity of N_2OR I. Thus, it would represent 10–30% of the total Cu, depending on the individual sample (Riester et al. 1989).

Of the exogenous ligands, NO and azide give the most interesting effects on the EPR spectra of N_2OR. The g_{II} region seems to undergo some broadening with most exogenous ligands and the hyperfine structure becomes less resolved at 9.32 GHz (Riester et al. 1989). In 0.10 mM azide, the shape of the EPR signal changes completely. Two new lines appear at lower field with a splitting of approximately 17 mT. Otherwise, the hyperfine structure observed for the EPR signal of N_2OR I has vanished. Exposure to CO leads to an EPR spectrum identical with the one reported for the reduced form N_2OR III. With NO, the hyperfine structure both in the g_{II} and in the g_I region is practically lost. As mentioned earlier, nitrite is formed during the reaction with NO. We investigated therefore, the effect of nitrite and nitrate on the EPR signals. Nitrate proves to be rather inert towards N_2OR I; the EPR spectrum of the nitrite-treated sample shows two extra lines around 270 mT with large splittings. Otherwise, the EPR spectrum resembles that of dithionite-reduced N_2OR (Riester et al. 1989).

The various forms of N_2OR (I–V) were also investigated at 2.4, 3.5 and 35 GHz, at 10–20 K (Kroneck et al. 1988). AT 35 GHz, the spectra resemble dispersion spectra of spectra dominated by rapid passage. Lines split by 3.8 mT and 3.0 mT in the S-band spectra are better resolved throughout the low- and high-field regions than the spectra recorded at 9.32 GHz (Coyle et al. 1985; Riester et al. 1989). For the reduced species, N_2OR III, some resolution in the central part of the EPR signal at 2.4 GHz is achieved at 20 K. From the second derivative spectrum, a splitting of approximately 2.4 mT is derived. This coupling is somewhat large for a coordinated nitrogen attributed to the interaction with a Cu nucleus.

The nature of the cupric site in N_2OR and of Cu_A in cytochrome *c* oxidase

From the very beginning of the spectroscopic characterization of N_2OR (Coyle et al. 1985), it was evident that the features of this bacterial copper enzyme were not readily described within the 'classical' scheme of three types (Malkin and Malmström 1970). There is now compelling evidence that the EPR-active Cu site of N_2OR has properties of the Cu_A site of cytochrome *c* oxidase (Kroneck et al. 1989; Li et al. 1989). From a so-called Peisach-Blumberg plot of A_{II} vs g_{II} (Peisach

and Blumberg 1974), using the parameters obtained from both the EPR spectra at 9.32 GHz and 34 GHz, we conclude that the EPR-detectable centers in N_2OR I and V might have an electronic environment similar to that found for Cu_A of cytochrome *c* oxidase (Riester et al. 1989). This site has Cu ligated by two cysteine and two histidine residues (Li et al. 1987). The primary structure of N_2OR reveals at its carboxy terminus a highly conserved consensus sequence with that of subunit II of cytochrome *c* oxidase, containing three or four of the Cu ligands (Viebrock and Zumft 1988). The electron spin echo (ESE) envelope modulation frequencies and the modulation depth of N_2OR are strikingly similar to that of cytochrome *c* oxidase (Jin et al. 1989). All frequencies are also observed in the spectrum of N_2OR. The ESE data specifically suggest for N_2OR a highly covalent Cu(II) site with imidazole ligation. The magnetic circular dichroic spectrum of N_2OR again is different from other copper proteins but very similar to cytochrome *c* oxidase reflecting similar electronic structures of the corresponding Cu sites (Scott et al. 1989). Resonance Raman spectroscopy led to the proposal of a $Cu(II)S_2(Cys)N(His)$ site for N_2OR (Dooley et al. 1987). Recent extended X-ray absorption fine structure (EXAFS) measurements of N_2OR are fully consistent with such a model (Scott et al. 1989). Cytochrome *c* oxidase and N_2OR give a rather small edge shift upon reduction, which is taken as indirect evidence for sulfur ligation. Although the interpretation of the EXAFS data is problematic due to the multiple sites in N_2OR, acceptable fits require a Cu–(N,O) interaction at approximately 0.20 nm, a Cu–(S,Cl) interaction at 0.227 nm for the oxidized enzyme as compared to the average Cu–(S,Cl) distance of 0.228 nm in cytochrome *c* oxidase (Li et al. 1987). A long Cu–(S,Cl) interaction of unknown origin, of approximately 0.26 nm, in the oxidized N_2OR and 0.228 nm in the reduced protein, is also found in cytochrome *c* oxidase (Scott et al. 1989).

Our EPR experiments of the cupric site in N_2OR confirm the assignment of the low-field *g*-value at 2.18 determined at 9.32 GHz. Consistent with the seven-line hyperfine pattern observed for N_2OR at the X band, 10 K, the fourth line in the S-band spectrum corresponds to the *g*-value at 2.18. From the Q-band spectra taken at 110 K, 2.16, 2.02 and 1.99 are estimated for the transitions at low and high field [vs 2.18, 2.02 and 1.99 for cytochrome *c* oxidase (Aasa et al. 1976)]. There is a striking similarity between the low-frequency EPR signals of N_2OR and beef heart cytochrome *c* oxidase (Fig. 1) (Kroneck et al. 1989). For the EPR signal of the beef heart enzyme measured at S- and L-band, 10 K, newly resolved splittings were reported ranging over 2.0–2.6 mT and 5.6–8.1 mT around $g = 2.02$ (Froncisz et al. 1979). It is proposed that both Cu hyperfine interaction plus some other (with heme *a*?) magnetic interaction could account for the features observed in the low-frequency spectra below 30 K. The loss of resolution above 40 K is similar in character to that observed for the Mo-Fe-S interaction in xanthine oxidase. In the case of N_2OR the seven-line pattern is not observed above approximately 100 K (Riester et al. 1989). In analogy to the magnetic interaction between Cu_A and heme *a* (?), or other nuclei, a metal–metal interaction for the EPR-detectable Cu site of N_2OR is proposed consistent with the loss of resolution at higher temperatures. The EPR features are in agreement with a mixed-valence species proposed for several Cu proteins and Cu model complexes (Kroneck et al. 1988). The mixed-valence $S = 1/2$ state observed below 110 K belongs to the class III, i.e. a [Cu(1.5)...Cu(1.5)] site with strong delocalization of the unpaired spin between the metal centers (Solomon et al. 1983).

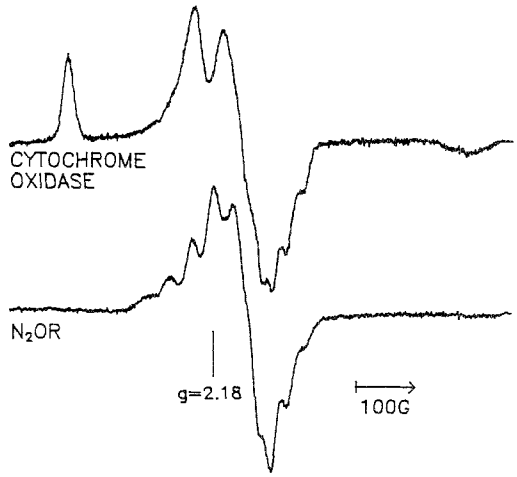

Fig. 1. S-band first-derivative EPR spectra of cytochrome *c* oxidase and nitrous reductase. Microwave frequency 2.792 GHz; modulation frequency 100 kHz; modulation amplitude 0.3 mT (100 G ≡ 10 mT); microwave power 10 dB; temperature 20 K. Taken from Kroneck et al. (1989) with permission

In the case of cytochrome *c* oxidase Cu–heme interaction but not Cu–Cu interaction has been considered to be an important structural element to explain the features observed in the low-frequency EPR spectra (Froncisz et al. 1979). On the other hand, metal analysis data demonstrate the presence of three Cu atoms and two hemes (per monomeric unit) in cytochrome *c* oxidase (Steffens et al. 1987). Only two of the Cu atoms seem to participate in catalysis. Note that less than 40% of the catalytic Cu centers is accessible to EPR in native cytochrome *c* oxidase. Only after denaturation in the presence of mercurials both Cu centers become EPR-detectable. Thus, similar to the situation in N_2OR, a class-III mixed-valence [Cu(1.5)...Cu(1.5)] state may be formed below 40 K in the active site of cytochrome *c* oxidase accounting for the multiline EPR signal at S- and L-band.

This suggestion was questioned in favour of a mononuclear Cu_A center (Li et al. 1989). On the other hand, the finding of a third Cu atom in cytochrome *c* oxidase which seems to be in the Cu(I) state, and the reported presence of only one cysteine in the ba_3 oxidase from *Thermus thermophilus* (which has a typical Cu_A) has cast doubt on the accepted picture of the Cu_A site (Kroneck et al. 1989). So far, neither a four-line pattern for a mononuclear site nor a seven-line pattern for a binuclear site has been fully resolved in the g_{II} region. Possibly the g_{II} region for cytochrome *c* oxidase is not

resolved because: (a) the g_{mid} component of the heme of cytochrome *a* is superimposed onto the g_{II} region for the S- and X-band data; (b) the interaction of the ferric sites in cytochrome *c* oxidase but not in N_2OR affects the resolution of the lines; and (c) the lines in the Q-band spectra are broad and the hyperfine structure, irrespective of whether taken under passage conditions, is not resolved. Since the presence of a [Cu(II)...Cu(I)] site implies the existence of a lower oxidation state, it appears more reasonable to suggest that the reduced hyperfine coupling reflects delocalization of the electron from the cupric site. Most likely, delocalization of the spin density is accomplished by Cu–Cu interaction in N_2OR; in the case of cytochrome *c* oxidase further studies are needed to answer this question. At present, EPR investigations at 4.5 GHz (C-band) are in progress which will allow a better analysis of the signals of cytochrome *c* oxidase and of N_2OR. The C-band is a particularly good frequency to complement the S- and X-band data because five of the seven lines in the g_z region of the EPR-detectable site in N_2OR are easily observed. The unusual relaxation properties of the EPR-detectable sites for both 'Cu_A' sites in cytochrome *c* oxidase and N_2OR above 20 K, an unusually low *g*-value of approximately 2.00 for the Cu_A site, and a seven-line pattern are difficult to explain using a mononuclear site $Cu(II)S_2(Cys)N_2(His)$ with $S = 1/2$.

Structural model for the active site of N_2OR from *P. stutzeri*

On the basis of the biochemical and physical properties, we propose a preliminary structural model for the active site of the multicopper enzyme N_2OR. This model is based on the following premises: (a) N_2OR consists of two identical subunits each containing 4 Cu atoms, similar to the situation of the plant copper enzyme ascorbate oxidase (Messerschmidt et al. 1989); (b) the cysteine(CySH)/cystine(CySSCy) redox couple is involved in the structural organization of the active site; (c) each Cu center is coordinated on the average to at least one sulfur ligand (Scott et al. 1989). For native N_2OR (form I, oxidized) two distinct Cu sites have been characterized (centers A and C). The mononuclear center A is best described by $Cu(II)S_2(Cys)N_2(His)$ as proposed for the 'Cu_A' site of cytochrome *c* oxidase. In view of its reduced EPR activity, magnetic interaction with another paramagnet is likely. Center A is relatively labile towards dioxygen and changes in the chemical environment (Riester et al. 1989). One of the cysteine ligands is irreversibly oxidized (giving center A*) which would account for the low activity of N_2OR purified in the presence of dioxygen.

By contrast, center C is binuclear, and is represented by the mixed-valence $S = 1/2$ species [Cu(1.5)...Cu(1.5)] with the unpaired electron delocalized between two equivalent Cu nuclei. Again it is assumed that each Cu of center C is coordinated by at least one N(His) and one S(Cys) residue.

Center B is formed by the reduction of native N_2OR yielding the blue form N_2OR III. This center shows rather unusual properties for a 'reduced' Cu species, i.e. it exhibits a blue chromophore and a broad featureless EPR signal at X-band. Its resistance towards further reduction, and its spectral properties together with the results from our quantitative determination of sulfhydryl and disulfide groups, leads to the conclusion that reduction of a proximal disulfide residue may contribute to the stabilization of center B (Riester 1989).

Both centers A and C react very rapidly with a large variety of reducing agents (Riester et al. 1989). On the other hand, the formation of center B proceeds rather slowly, thus seems to be of no physiological importance. Center A may be the entrance point for reducing equivalents after reaction of N_2OR with the (so far unknown) physiological electron donor. This would leave the binuclear mixed-valence center C as the binding site of the substrate N_2O, or other small exogenous ligands.

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