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The cupric site in nitrous oxide reductase contains a mixed-valence [Cu(II),Cu(I)] binuclear center: a multifrequency electron paramagnetic resonance investigation

Peter M.H. Kroneck, William A. Antholine*, Joachim Riester and Walter G. Zumft°

*Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-7750 Konstanz, FRG, *National Biomedical ESR Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA* and *°Lehrstuhl für Mikrobiologie, Universität Karlsruhe, Kaiserstr. 12, D-7500 Karlsruhe, FRG*

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Multifrequency electron paramagnetic resonance (EPR) spectra of the Cu(II) site in nitrous oxide reductase (N_2OR) from *Pseudomonas stutzeri* confirm the assignment of the low field g value at 2.18 consistent with the seven line pattern observed at 9.31 GHz, 10 K. S-band spectra at 20 K are better resolved than the X-band spectra recorded at 10 K. The features observed at 2.4, 3.4, 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 two equivalent Cu nuclei. The resemblance of the N_2OR S-band spectra to the spectra for the EPR-detectable Cu of cytochrome *c* oxidase suggests that the S-band spectrum for cytochrome *c* oxidase measured below 30 K may also contain hyperfine splittings from two approximately equivalent Cu nuclei.

Nitrous oxide reductase; Cytochrome *c*-oxidase; Mixed-valence Cu center; EPR, multifrequency

1. INTRODUCTION

N_2OR from the denitrifying bacterium *Pseudomonas stutzeri* is a multicopper enzyme (8 Cu/M_r 140 000) involved in the conversion of nitrous oxide to dinitrogen [1]. The N,O-bond which has double bond character [2] 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) [3]. Five spectroscopically distinct forms of N_2OR with different catalytic properties including the purple high-activity form I (isolated anaerobically) and the mutant form V (inactive, deficient in Cu chromophore biosynthesis) have been prepared [4–6]. EPR measurements at 9.32 GHz, 10 K, of N_2OR I reveal an axially symmetric signal of the Cu(II) site with $g_{II} = 2.18$ and $g_I = 2.03$. The low field part of the spectrum shows a seven line hyperfine pattern ($A_{II} = 3.83$ mT), the perpendicular region exhibited a narrower splitting of 2.8 mT [4]. The seven line EPR signal of N_2OR I has a variable contribution of a broad, feature-less signal. This signal, representing up to 30% of the total Cu determined by atomic absorption spectroscopy or chemical methods, is most clearly seen upon reduction of N_2OR I to the blue form, III. It is also present in samples of aerobically prepared N_2OR (pink form, II). Five hyperfine lines at g_{II} have been resolved in the EPR spectra of such probes. Double integration of the EPR signals of both N_2OR I and II and comparison to a $Cu(II)SO_4$ standard in $HCl/NaClO_4$ accounted for 20–40% of the total Cu whereas in N_2OR V approximately 50% was EPR detectable [6].

Both the physical and biochemical properties of

Correspondence address: P.M.H. Kroneck, Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-7750 Konstanz, FRG

Abbreviations: EPR, electron paramagnetic resonance; N_2OR, nitrous oxide reductase

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N_2OR obviously do not fit properly in the 'classical' scheme of type 1, 2 and 3 Cu proposed almost two decades ago [7]. The existence of a broad absorption maximum around 800 nm in oxidized N_2OR together with the position of g_{II}/A_{II} in the Peisach-Blumberg diagram [8] could be taken as a first indication that the EPR-detectable Cu in N_2OR I might have a structural and electronic arrangement similar to the Cu_A of beef heart cytochrome *c* oxidase.

The multifrequency EPR data described in the present communication provide new evidence for this intriguing assumption. Comparison of these data for the EPR detectable Cu(II) in N_2OR with the results reported for cytochrome *c* oxidase [9,10] suggests that the EPR signal observed at S- and L-band below 30 K arises from a mixed-valence [Cu(II),Cu(I)] $S = 1/2$ site.

2. EXPERIMENTAL

The various forms of N_2OR were prepared as described [4]. Samples for EPR measurements were transferred to quartz tubes that were frozen in liquid nitrogen and stored at 77 K. EPR spectra at different frequencies were obtained with either Varian or Bruker instruments located at the National Biomedical ESR Center in Milwaukee. EPR parameters were calculated from the spectra measuring the microwave frequency (EIP model 548 counter) and the magnetic field (Radiopan JTM 147 gaussmeter). The EPR-detectable Cu was determined with Cu(II)SO_4 in HCl/$NaClO_4$ as the standard [11]. At 9.3 and 35 GHz standard Varian rectangular or cylindrical cavities and standard microwave bridges were used. For lower frequencies the loop-gap resonator [12] and microwave bridges developed at the ESR center were used. Temperatures at approximately 125 and 20 K were maintained with gaseous nitrogen (Varian) or helium (Air Products) flow systems. For the measurements at 2.4, 3.4 and 35 GHz the resonator had to be placed into the quartz cold finger.

3. RESULTS

The various forms of N_2OR (I–V) including the high-pH form of I [4] have been investigated by EPR spectroscopy at several frequencies. Furthermore, the effect of the temperature in the range 5–125 K on the line shape and the spectral resolution has been studied. The EPR parameters are summarized in table 1, which also includes data for binuclear copper complexes and various copper proteins.

In fig.1, the EPR spectra of N_2OR I in Tris-HCl buffer, pH 7.5, 10–20 K, obtained at various frequencies over the range 2.4–35 GHz are compared. At 35 GHz, the spectra resemble dispersion spectra of spectra dominated by rapid passage. At 110 K, both N_2OR I and V show normal first derivative spectra with apparent *g* values at 2.16 and 1.99 [6]. There is an additional line centered at $g = 2.02$ in the spectrum of N_2OR I that is absent in the spectrum of the mutant, N_2OR V. For the

Table 1

Electron paramagnetic resonance parameters for Cu proteins and complexes with Cu–Cu interaction

Sample	Coupling $\Delta m = 1$	Coupling $\Delta m = 2$	Type of interaction	Ref.
	(mT)			
$Cu(II)_2$(carnosine)$_2$	9.3	7.2	[Cu(II)..Cu(II)]	[28]
Cu(II)(carnosine)$_4$	19.0		monomer	[28]
$Cu(II)_2$(glutathione disulfide)	not resolved[a]		[Cu(II)..Cu(II)]	[29]
Cu(II)(glutathione disulfide)	17.8	–	monomer	[29]
u-Acetato-Cu(II),Cu(I)/CH_3CN	4.2[b]	–	[Cu(1.5)..Cu(1.5)]	[20]
NO-hemocyanin	not resolved	6.6	[Cu(II)..Cu(II)]	[18]
Cysteamine-tyrosinase	6[c]	–	[Cu(1.5)..Cu(1.5)]	[15]
Nitrous oxide reductase (I)	3.8 (low field)			
	3.0 (high field)	–	[Cu(1.5)..Cu(1.5)]	this work
Cytochrome *c* oxidase	2.0–2.6	–	[Cu(1.5)..Cu(1.5)]?	[10]

[a] In frozen solution, pH 11.0; crystalline $Cu(II)_2$(glutathione disulfide)Na_4. $6H_2O$ does not show a $\Delta m = 2$ transition; $A_{zz} = 17.7$ mT [30].
[b] In aqueous acetonitrile, 298 K.
[c] From computer simulation

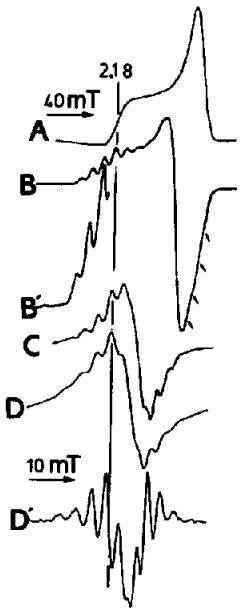

Fig.1. Multifrequency electron paramagnetic resonance spectra of nitrous oxide reductase from *Pseudomonas stutzeri* (form I), 0.27 mM in 25 mM Tris-HCl buffer, pH 7.5. (A) Microwave frequency 35.14 GHz, 10 K; (B) 9.315 GHz, 10 K; (B') 5-fold increase in gain; (C) 3.480 GHz, 20 K; (D) 2.393 GHz, 20 K; (D') second derivative spectrum.

dithionite-reduced enzyme, N_2OR III, only the broad line at $g = 2.02$ is observed at Q-band, 110 K [6]. Lines split by 3.8 mT and 3.0 mT for the S-band spectra recorded at 20 K are better resolved throughout the low and high field regions than the spectra recorded at 9.3 GHz (fig.1, spectra C,D). 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 (spectra not shown). 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.

At 9.3 GHz, 10 K, small differences between the signal of N_2OR I and its high-pH form (Ches buffer, pH 9.8 [4]) are detectable. This becomes more evident at 77 K using the second derivative spectrum, i.e. lines split by 1.37 mT are resolved in the g_1 region (spectra not shown).

No half-field signal resulting from a $\Delta m = 2$ transition at $g \sim 4$ in the EPR spectra recorded at 9.3, 3.4 or 2.4 GHz has been observed in the temperature range 10–125 K even after extensive signal averaging.

4. DISCUSSION

Our EPR experiments of the cupric site in N_2OR confirm the assignment of the low field g value at 2.18 determined at 9.3 GHz [4]. Consistent with the seven line hyperfine pattern observed for N_2OR at 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 were estimated for the various transitions at low and high magnetic field (vs 2.18, 2.02 and 1.99 for cytochrome *c* oxidase [13]). Conversion to the high-pH form by anaerobic dialysis of N_2OR I [4] seems to cause a structural rearrangement of the Cu coordination sphere as indicated by the resolution of additional lines in the perpendicular region of the X-band spectrum at 77 K. The magnitude of 1.37 mT might be assigned to a coordinated nitrogen but could also result from a second Cu center. Note that the X-band spectrum of N_2OR I (pH 7.5) exhibits a splitting of 2.8 mT at g_1 [4].

There is a striking similarity between the low frequency EPR signals of N_2OR I and beef heart cytochrome *c* oxidase [10]. For the EPR signal of the beef heart enzyme measured at S- and L-band, 10 K, Froncisz et al. reported newly resolved splittings ranging from 2.0 to 2.6 mT, and 5.6 to 8.1 mT around $g = 2.02$. These authors claim that both Cu hyperfine interaction plus some other (with cytochrome *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 110 K at 9.3 GHz [6]. Although up to the present time, S-band spectra were only recorded around 20 and 125 K, it seems that the resolution is also best in the lower temperature range. In analogy to the magnetic interaction between Cu_A and cytochrome *a* (?), or other nuclei, a metal–metal interaction for the EPR-detectable active site of N_2OR is proposed consistent with the loss of resolution at higher temperature. Both the hyperfine pattern observed at frequencies between 9.3 and 2.4 GHz and the magnitude of the splitting are in agreement with a mixed-valence species as

proposed for several Cu proteins and Cu model complexes (table 1). Following the notation of Solomon the mixed-valence $S = 1/2$ state detected below 110 K in frozen solutions of N_2OR I 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 [14]. Similar structures have been proposed for the thiol derivative of tyrosinase [15,16], or various derivatives of hemocyanin [17–19]. A well-resolved seven line EPR spectrum is observed for the u-acetato [Cu(1.5)..Cu(1.5)] complex in aqueous acetonitrile solution [20]. At 77 K, most likely because of structural rearrangements due to freezing, the main portion of the EPR signal is assigned to a mononuclear Cu(II) complex. Features from the mixed-valence species are still detectable in frozen solution at 77 K. In the case of N_2OR I an opposite temperature behavior is observed. This can be explained in at least two different ways: (i) above 110 K relaxation processes, because of the interaction between the various Cu nuclei, lead to significant line broadening. Note that for the multicopper enzymes laccase (4 Cu/M_r) [21] and ascorbate oxidase (8 Cu/M_r) (Kroneck, P.M.H., unpublished) the hyperfine structure at g_{II} is still observable at room temeprature; (ii) the redox potential of the various Cu sites in N_2OR I exhibit different temperature dependences leading to a class III [Cu(1.5)..Cu(1.5)] $S = 1/2$ state at lower temperature. Such differences in the temperature dependence of the redox potentials of the type 1 and type 3 Cu were found for ascorbate oxidase [22] and cytochrome *c* oxidase [23].

Until now, only Cu–heme interaction but not Cu–Cu interaction has been considered to be an important structural element of cytochrome *c* oxidase [24,25]. On the other hand, recent metal analysis data demonstrated the presence of three Cu atoms and two hemes [26]. 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 do both Cu centers become EPR detectable [27]. Thus, similar to the situation in N_2OR, a class III mixed-valence [Cu(1.5)..Cu(1.5)] state might be formed below 40 K in the active site of cytochrome *c* oxidase accounting for the multiline EPR signal at the S- and L-band [10].

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