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# Multifrequency EPR evidence for a bimetallic center at the $Cu_A$ site in cytochrome *c* oxidase

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Multifrequency electron paramagnetic resonance (EPR) spectra of the Cu(II) site in bovine heart cytochrome *c* oxidase (COX) and nitrous oxide reductase (N2OR) from *Pseudomonas stutzeri* confirm the existence of Cu-Cu interaction in both enzymes. C-band (4.5 GHz) proves to be a particularly good frequency complementing the spectra of COX and N2OR recorded at 2.4 and 3.5 GHz. Both the high and low field region of the EPR spectra show the presence of a well-resolved 7-line pattern consistent with the idea of a binuclear Cu center in COX and N2OR. Based on this assumption consistent g-values are calculated for $g_z$ and $g_x$ at four frequencies. No consistent g-values are obtained with the assumption of a 4-line pattern indicative for a mononuclear Cu site.

Cytochrome c oxidase; $Copper_A$; Bimetallic center; Copper-copper interaction; Nitrous oxide reductase; Electron paramagnetic resonance, multifrequency

## 1. INTRODUCTION

Recently it has been argued that the EPR spectrum generally ascribed to $Cu_A$ in cytochrome *c* oxidase (COX) may contain a Cu–Cu coupling pattern that is inconsistent with the current structural picture of a mononuclear $Cu_A$ site [1-3]. Evidence for the similarity between the EPR-detectable copper in nitrous oxide reductase (N2OR) and $Cu_A$ in COX arose from EPR spectroscopy [4], electron spin echo envelope modulation data [5], MCD and EXAFS spectroscopy [6]. Furthermore, comparison of the N2OR sequence, determined by translating the structural *NosZ* gene, with COX subunit II sequences from several sources indicated that a Gly-Xaa-Xaa-Xaa-Xaa-Xaa-Cys-Ser-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-His stretch was highly conserved [7]. Similarities between the ESR spectra on the high field side at 2.7 GHz (S-band) for N20R and the $Cu_A$ site in COX led to the hypothesis that the seven line pattern in the EPR spectra could be attributed to a mixed-valence binnuclear copper center, [Cu(1.5)...Cu(1.5)] [1]. The pattern at 2.62 and 3.78 GHz in COX has previously been attributed to a hyperfine interaction between copper and another yet unknown paramagnet [8].

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*Abbreviations:* COX, cytochrome *c* oxidase; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; MCD, magnetic circular dichroism; N2OR, nitrous oxide reductase

## 2. MATERIALS AND METHODS

Nitrous oxide reductase from *P. stutzeri* [9] and bovine heart cytochrome *c* oxidase [10] were prepared according to literature methods. EPR spectra were recorded and evaluated as described [1].

## 3. RESULTS AND DISCUSSION

EPR spectra recorded at 4.5 GHz from a new C-band spectrometer operating with a loop-gap resonator [11], together with earlier data obtained at S-, X- and Q-band [1,4], are used to determine a g-value from the apparent 7-line pattern on the high field side of the EPR signal for the $Cu_A$ site in COX. Again, the spectrum from N20R, for which the 7-line pattern is also resolved on the high field side as well as in the low field $g_z$ region, helps to identify the 7-line pattern in the $Cu_A$ site of COX. The 7-line spectrum at X- and S-band frequencies for the EPR-detectable Cu site in N20R [1,4] and, now, C-band is consistent with a 7-line pattern in the low field $g_z$ region (Fig. 1, spectrum 3). The $g_z$ region of the X-band spectrum reveals the entire 7-line pattern while the $g_z$ region of the S-band spectrum has only 4 lines of the 7-line pattern separated from a superposition of lines in the $g_x$ and $g_y$ region [1,4]. The center of the pattern is the fourth line with a g-value of 2.18 (Table I). At C-band, 5 of the 7 lines are resolved in the $g_z$ region and at least 3 lines, possibly 4 lines, on the high field side (Fig. 1, spectrum 3). Assuming a 7-line pattern, a value of 2.02 is calculated for $g_x$ at all 3 frequencies. No consistent g-value is calculated for either the $g_z$ or $g_x$ region with the assumption of a 4-line pattern (Table I). Simulations with a perturbation program



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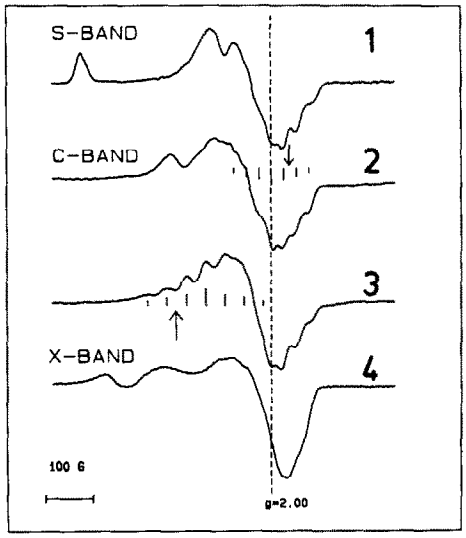


Fig. 1. S-band (2.760 GHz, No. 1), C-band (4.530 GHz, No. 2) and X-band (9.130 GHz, No. 4) EPR spectra of cytochrome *c* oxidase, and C-band EPR spectrum of nitrous oxide reductase (4.670 GHz, No. 3). Modulation frequency 100 kHz, modulation amplitude 0.3 mT, temperature 20K. Nitrous oxide reductase from *Pseudomonas stutzeri*, 0.27 mM in 50 mM Tris-HCl, pH 7.5, was prepared as described [9]. Cytochrome *c* oxidase was obtained from bovine heart [10]. The metal content determined by ICP-AES was 2.94 Cu, 1.96 Fe, 1.07 Zn and 1.03 Mg. The protein was 68 mg/ml 0.5% Tween 80, 50 mM Tris-HCl, pH 7.4. Note that the spectrum for N20R (No. 3) is shifted so that g = 2.02 for N20R is superimposed on g = 2.00 for COX. The seven line pattern for a Cu–Cu coupling is indicated by the stick diagram. An arrow indicates the center of a 4-line pattern for a mononuclear Cu site.

modified to include hyperfine splittings from two equivalent cupric nuclei [12], are in agreement with, but not necessarily proof of, the 7-line pattern (data not shown). A similar g-value determination is made for the multifrequency EPR data for the $Cu_A$ site in cytochrome *c* oxidase. The X- and S-band spectra in Fig. 1 are consistent with previously published spectra [2,8]. Thus, slight differences in sample preparation seem not to affect the data. The spectrum from C-band is crucial to the argument because no lines are as yet resolved at X-bands (Fig. 1). Compared to the EPR spectra obtained for N20R the resolution in the $g_z$ region is not as good at all 3 frequencies. We have attributed this to the overlap of the $g_{mid}$ line of heme *a* and the interaction with ferric sites in COX. The lines in the $g_x$ and $g_y$ region are resolved in the C-band spectrum (Fig. 1, spectrum 2). The microwave frequency for C-band is just high enough relative to the S-band microwave frequency to discriminate between the expected g-value for a seven line pattern or a 4-line pattern (Table I). A $g_x$-value of 2.00 for a 7-line pattern is consistent with spectra from all 3 frequencies (Table I). For a 4-line pattern indicative for a mononuclear Cu center, no consistent $g_x$-value is obtained (Table I).

Table I
g values[a] calculated from either a 7-line pattern ($g^{Cu-Cu}$) or a 4-line pattern ($g^{Cu}$)[b]

| Sample | Frequency (GHz) | $g_z^{Cu-Cu}$ | $g_z^{Cu}$ | $g_x^{Cu-Cu}$ | $g_x^{Cu}$ |
|---|---|---|---|---|---|
| COX | 9.130 (X-band) | – | – | 2.00[c] | – |
| COX | 4.530 (C-band) | – | – | 2.00 | 1.96 |
| COX | 2.760 (S-band) | – | – | 2.00 | 1.94 |
| N20R | 9.130 (X-band) | 2.18 | 2.22 | 2.02 | 1.98 |
| N20R | 4.670 (C-band) | 2.18 | 2.26 | 2.02 | 1.97 |
| N20R | 3.480 (S-band) | 2.18 | 2.29 | 2.02 | 1.95 |
| N20R | 2.397 (S-band) | 2.18 | 2.34 | 2.02 | 1.92 |

[a]Where two or more calculations are averaged, the range is $< \pm$, 0.006. All values were rounded off to 0.01 [b]Position of $g^{Cu-Cu}$ and $g^{Cu}$ is given in Fig. 1 [c]Value is only from simulated data

That cytochrome *c* oxidase contains a 7-line pattern has been difficult to determine because only 3 of the high field lines are resolved at low frequencies without overlapping of the $g_x$ region with $g_y$ and $g_z$. C-band is a particularly good frequency to complement the S- and X-bands data because 5 of the 7 lines in the $g_z$ region of the EPR detectable site in nitrous oxide reductase are easily observed. Furthermore, 3 lines in the $g_x$ region for both nitrous oxide reductase and cutochrome *c* oxidase are observed. While a 7-line pattern could be constructed from other combinations, the magnitude of the coupling, $A_z = 38$ G and $A_x = 29$ G for $N_2OR$ and $A_x = 32$ G for COX is most compatible with half the coupling from a type-1 Cu site, which implies that both copper atoms of the binuclear center may be type-1 [13]. Even though the parameters and the relaxation properties for the EPR-detectable copper site in N20R are not identical to the parameters for the $Cu_A$ site in COX, the parameters are close enough to suggest structurally similar, if not identical sites. The unusual relaxation properties that prevent saturation of the EPR-detectable sites in COX and N20R above 20 K, an unusually low $g_x$-value of 2.00 for the $Cu_A$ site, and a 7-line pattern are difficult to explain using a mononuclear center $Cu(II)S_2(Cys)N_2(His)$ with $S = 1/2$ [14]. These data may argue for a more complex structure, i.e. a binuclear Cu center with $S = 1/2$, or even a binuclear center coupled to another paramagnet giving a state with $S > 1$ in which a fictitious $S = 1/2$ spin state is detected [15].

Multifrequency data obtained for N20R from a mutant defective in chromophore biosynthesis [4] confirm the results described here. This form of the enzyme contains approximately 2–3 Cu atoms/$M_r$ [4]. It shows the characteristic multiline EPR signals at S-, C- and X-band observed for native N20R which is consistent with the presence of the mixed-valence $S = 1/2$ [Cu(1.5)...Cu(1.5)] center as the main Cu site in the mutant enzyme. The existence of a trinuclear Cu site in the enzyme ascorbate oxidase (8 Cu/$M_r$) was demonstrated by high-resolution X-ray crystallography most recently [16]. Note that both N20R and COX are multicopper proteins with 4 Cu/subunit in the case of N20R (8 Cu/$M_r$ [4] and 3 Cu, 2 Fe/catalytically active unit in the case of COX [10]. For COX the existence of the third

copper atom was just confirmed most recently [17]; however, its functional role remains unclear. It is proposed to exist in the Cu(I) redox state, i.e. does not participate in the reduction of dioxygen [18,19].

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## REFERENCES

[1] Kroneck, P.M.H., Antholine, W.E., Riester, J. and Zumft, W.G. (1988) FEBS Lett. 242, 70–74.
[2] Li, P.M., Malmstroem, B.G. and Chan, S.I. (1989) FEBS Lett. 248, 210–211.
[3] Kroneck, P.H.M., Antholine, W.E., Riester, J. and Zumft, W.G. (1989) FEBS Lett. 248, 212–213.
[4] Riester, J., Zumft, W.G. and Kroneck, P.M.H. (1989) Eur. J. Biochem. 178, 751–762.
[5] Jin, H., Thomann, H., Coyle, C.L., and Zumft, W.G. (1989) J. Am. Chem. Soc. 111, 4262–4269.
[6] Scott, R.A., Zumft, W.G., Coyle, C.L., and Dooley, D.M. (1989) Proc. Natl. Acad. Sci. USA 86, 4082–4086.
[7] Viebrock, A. and Zumft, W.G. (1988) J. Bacteriol. 170, 4658–4668.
[8] Froncisz, W., Scholes, C.P., Hyde, J.S., Wei, Y.-H., King, T.E., Shaw, R.W. and Beinert, H. (1979) J. Biol. Chem. 254, 7482–7484.
[9] Coyle C.L., Zumft, W.G., Kroneck, P.M.H., Koerner, H. and Jakob, W. (1985) Eur. J. Biochem. 153, 459–467.
[10] Steffens, G.C., Bienwald, K. and Buse, G. (1987) Eur. J. Biochem. 164, 295–300.
[11] Froncisz, W. and Hyde, J.S. (1982) J. Magn. Reson. 47, 515–521.
[12] Rakhit, G., Antholine, W.E., Froncisz, W., Hyde, J.S., Pilbrow, J.R., Sinclair, G.R., and Sarkar, B. (1985) J. Inorg. Biochem. 25, 217–224.
[13] Malkin, R. and Malmstroem, B.G. (1970) Adv. Enzymol. 33, 177–244.
[14] Li, P.M., Gelles, J., Chan, S.I., Sullivan, R.J. and Scott, R.A. (1987) Biochemistry 26, 2091–2095.
[15] Coffmann, R.E. and Pezeshk, A. (1985) J. Magn. Reson. 65, 62–81.
[16] Messerschmidt, A., Rossi, A., Ladenstein, R., Huber, R., Bolognesi, M., Gatti, A., Marchesini, A., Petruzelli, R. and Finazzi-Agro, A. (1989) J. Mol. Biol. 206, 513–529.
[17] Oeplad, M., Selin, E., Malmstroem, B., Strid, L., Aaasa, R. and Malmstroem, B.G. (1989) Biochim. Biophys. Acta 975, 267–270.
[18] Malmstroem, B.G. (1989) FEBS lett. 250, 9–21.
[19] Chan, S.I. and Li, P.M. (1990) Biochemistry, 29, 1–12.