Low-Frequency EPR of the Copper in Particulate Methane Monooxygenase from *Methylomicrobium albus* BG8

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Coupled binuclear and trinuclear copper centers are formed in proteins including enzymes that catalyze the multielectron reduction of O_2 to H_2O and N_2O to N_2.[1] Recently Chan, Lidstrom, and co-workers proposed a model that involves dioxygen reduction and the conversion of methane to methanol by a trinuclear copper cluster in particulate methane monooxygenase (pMMO).[1,2] Zahn and DiSpirito present an alternative model in which the catalytic site involves both iron and copper, although they reserve the option of a single ferrous iron center or an iron−iron center.[3] Because of recent success in assigning the Cu_A site in nitrous oxide reductase and cytochrome *c* oxidase to a mixed valence binuclear copper [$Cu_A(1.5+)\cdots Cu_A(1.5+)$] site through interpretation of low-frequency EPR spectra,[4] it seemed appropriate to use these low-frequency EPR techniques on the EPR signal from pMMO. It is concluded that one of the EPR-detectable sites in pMMO is not a mixed valence trinuclear site, but arises from a type 2 site, in which cupric ion is bound to three or four nitrogen donor atoms.

The X-band (9 GHz) EPR spectrum (Figure 1A) from whole cells of *Methylomicrobium albus* BG8 is similar to the EPR spectrum obtained for pMMO from *Methylococcus capsulatus* (Bath)[2] (Table 1). Cells are used instead of membrane fractions or isolated protein to preserve the environment around the copper to avoid additional strains at the EPR-detectable site. The activity of pMMO in cells is determined routinely by the method of propylene oxidation. Cells are grown on ^{63}Cu to avoid a superposition of signals from ^{63}Cu (69% natural abundance) and ^{65}Cu (31%). One advantage of *M. albus* BG8 for these studies is that only copper-loaded pMMO is present and no soluble MMO with the oxo-bridged dinuclear iron center is present. A free radical signal at $g = 2.007$ is superimposed on the Cu(2+) signal in whole cells. This free radical signal is absent from membrane fractions under our conditions. The criteria for the assignment of the Cu(2+) EPR signal to pMMO are that (i) the concentration exceeds 500 μM in samples in which pMMO is the major membrane protein as determined by gel electrophoresis;[5] (ii) the EPR parameters $g_{\|}$ and $A_{\|}$ are similar to the values obtained from *M. capsulatus* (Bath);[2] and (iii) similar spectra for the copper (not shown) are found in membrane fractions.

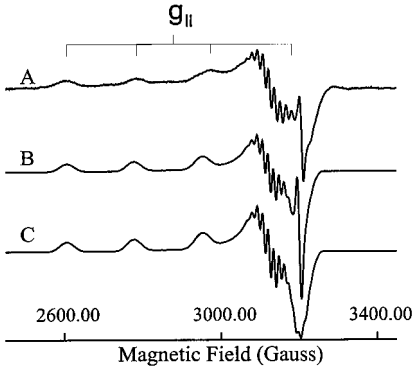

Figure 1. (A) X-band EPR spectra at 77 K of cupric site in *M. albus* grown on $^{63}Cu(2+)$. Experimental conditions: modulation frequency, 100 KHz; modulation amplitude, 5 G. (B) Simulation with $g_{\|} = 2.243$, $g_y = 2.061$, $g_x = 2.059$, and $A_{\|} = 194$ G plus a simulated free radical at $g = 2.007$. (C) Without the simulated free radical.

Table 1. EPR Parameters of Type 2 Cu(2+) in Proteins

proteins	$g_{\|}$	$A_{\|}^{a,b}$	$A_{\|}^N$	$A_{\perp}^N$
M. albus BG8	2.243	185 (194)	13	14
M. capsulatus (Bath)[2]	2.25	181		
peptide[c]	2.238	185 (200)	12	15
ascorbate oxidase[8]	2.242	190		
laccase[9]	2.245	182 (191)	12	15
monooxygenases[10]	2.277	145		
β-chain Hb[11]	2.210	193 (203)	15	15
serum albumin[12]	2.177	203 (213)	13	14

[a] Apparent hyperfine coupling (G) from the $M_I = -3/2$ and $-1/2$ lines on the low field side of the $g_{\|}$ region. [b] Numbers in parentheses are the parameters used in simulations. Note second order shifts result in a smaller apparent value as determined for $A_{\|}$ from the $M_I = -3/2$ and $M_I = -1/2$ lines in the $g_{\|}$ region. [c] Addition of Cu(2+) to a 15-amino acid synthetic peptide sequenced as AHGSVVKSEDYALPS.

The X-band spectrum in Figure 1A is consistent with the spectrum for a cupric ion in a square planar configuration in which Cu(2+) is bound to three or four nitrogen donor atoms. Simulations (Figure 1B,C) are consistent with, but not proof of, this assignment because the parameters used to simulate the spectra may not be unique. A simulated peak attributed to a free radical is added to the simulated type 2 copper spectrum to mimic the experimental spectrum. The suggestion that the structure in the $g_{\perp}$ region arises from three equivalent $I = 3/2$ copper nuclei to give a 10-line pattern[1,2] instead of a nine-line spectrum from one of the M_I lines of the unpaired electron spin on copper coupled to four nitrogen nuclei with $I = 1$ does not seem reasonable. First, the superhyperfine pattern assuming four equivalent nitrogen nuclei consists of four ($M_I = \pm 1/2$ and $\pm 3/2$) times nine ($I = 1$ for each of four nitrogens) or 36 lines plus overshoot lines plus lines from forbidden transitions. Although the $M_I = +1/2$ line is often more intense than the $-1/2$ and $\pm 3/2$ lines in the $g_{\perp}$ region, the pattern is expected to be more complicated than the nine-line pattern from only the line with $M_I = +1/2$. Second, the EPR parameters for $g_{\|} = 2.243$ and for an apparent $A_{\|}$ of 185 G are consistent with cupric ion bound to three or four approximately equivalent nitrogens as determined from Peisach−Blumberg plots.[6] Third, if one electron is delocalized over three approximately equivalent

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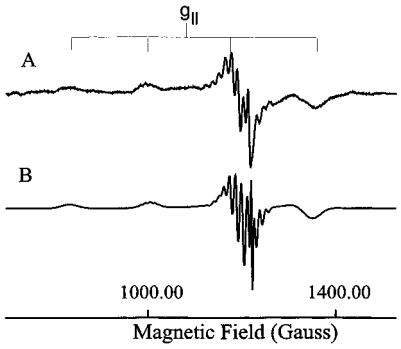

Figure 2. S-band (3.444 GHz) EPR spectra. (A) *M. albus* cells grown with ^{63}Cu(2+). Experimental conditions: temperature, 123 K; modulation amplitude, 5 G. (B) Simulation with $g_{||} = 2.243$, $g_y = 2.061$, $g_x = 2.059$, and $A_{||} = 194$ G plus a simulated free radical at $g = 2.007$.

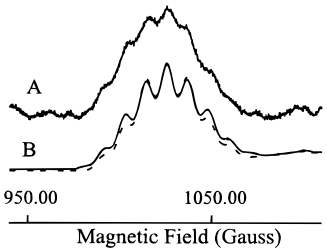

Figure 3. Expansion of $M_I = -1/2$ line in the $g_{||}$ region of the S-band spectrum. (A) Sample and experimental conditions are the same as in Figure 2A. (B) Simulation with four equivalent nitrogen atoms (solid line) and three equivalent nitrogen atoms (dashed line).

coppers, a 10-line pattern with $^1/_3$ the coupling expected from monomeric copper should be observed in the $g_{||}$ region as well as the $g_\perp$ region, whereas, experimentally, only a four-line pattern is obtained (Figure 1A).[2] Fourth, cupric ion with these EPR parameters is most likely bound to nitrogen donor atoms, which would contribute to the superhyperfine pattern. Thus, the hyperfine and superhyperfine patterns are consistent with monomeric cupric ion and nitrogen ligation.

Low-frequency S-band (3.4 GHz) EPR lines from samples of *M. albus* BG8 grown on ^{63}Cu with $g_{||} = 2.243$ and an apparent $A_{||}$ of 185 G are well resolved in the $g_{||}$ region as well as in the $g_\perp$ region (Figure 2A). Contributions from a minor signal are less than 10% as determined from the peak height of the $M_I = -1/2$ line in the X-band spectrum. The superhyperfine structure in Figure 2 is attributed to the dominant signal. At least 10 lines and a free radical signal are resolved in the $g_\perp$ region.

On expansion of the $M_I = -1/2$ line in the $g_{||}$ region and extensive signal averaging, at least seven lines with a coupling of 36 MHz (13 G) are resolved (Figure 3A). Secondary signals from the sample are also evident in the baseline. It is difficult to distinguish between a seven-line pattern with relative intensities of 1:3:6:7:6:3:1 for three approximately equivalent nitrogens and a nine-line pattern with relative intensities of 1:4:10:16:19:16:10:4:1 for four equivalent nitrogens (Figure 3B), especially when the outer lines with intensity one are buried in the noise. One difference between an ordinary square planar type 2 copper and the type 2 site in pMMO is $P_{1/2}$, the power for which the signal is $^1/_2$ the signal expected in the absence of saturation. $P_{1/2}$ for the type 2 site in pMMO is 70 mW at 77 K, while $P_{1/2}$ for an ordinary type 2 cupric ion is 30 mW. Since $P_{1/2}$ can increase if a fast relaxing paramagnetic species is nearby or if pairs, trimers, or aggregates are formed,[7] it is assumed that another paramagnetic site is nearby.

There are only 12 possible histidines in the sequence of *M. capsulatus* (Bath)[13] that could bind to copper. A simple interpretation for the coordination of the type 2 site in pMMO is that nitrogen donor atoms from a primary amine, usually the N-terminal amine, a nitrogen or nitrogens from the imidazoles of histidines, and peptide nitrogen(s) comprise the square planar configuration. EPR parameters for this type of site are given in Table 1. These sites often contain a histidine residue either as the second or the third residue in the N-terminal sequence.[11,12] The N-terminal sequence of pMMO-B has a histidine as the first amino acid.[13] The terminal amine and nitrogen from the imidazole are expected to provide a strong bidentate chelator, but extensive folding would be needed to add one or two additional nitrogen donor atoms.

The EPR parameters for pMMO are similar to the EPR parameters for the type 2 site in ascorbate oxidase and laccase (Table 1). The EPR-detectable cupric ion in laccase is most often bound to two nitrogen atoms,[14] but under certain conditions, is bound to three nitrogen donor atoms.[9] After treatment of the pMMO sample with ferricyanide or nitric oxide, it has been argued that a trimeric configuration, i.e., Cu(2+)- - -Cu-(2+)- - -Cu(2+), NO−Cu(1+)- - -NO−Cu(1+)- - -NO−Cu-(1+), or a Fe(3+)- - -Cu(2+) pair exists.[1−3] However, the cupric EPR signal obtained as isolated or upon reduction of pMMO is not a signal from a mixed valence delocalized copper trimer as previously described.

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