Electron Spin-Echo Envelope Modulation Studies on Copper Site of Particulate Methane Monooxygenase from *Methylosinus trichosporium* OB3b

Masayuki Takeguchi, Kouichi Fukui,† Hiroaki Ohya,† and Ichiro Okura*
Department of Bioengineering, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226-8501
†*Institute for Life Support Technology, Yamagata Technopolis Foundation, 2-2-1 Matsuei, Yamagata 990-2473*

(Received March 5, 1999; CL-990148)

The electron spin-echo envelope modulation (ESEEM) effect was studied on the type II copper site in the particulate methane monooxygenase (pMMO) from *Methylosinus trichosporium* OB3b. From comparing the ESEEM spectra of the membranes treated with ethylenediaminetetraacetic acid and some model compounds, it was clarified that there were at least two imidazole ligands bound copper (II) in pMMO.

pMMO from *Methylococcus capsulatus* (Bath) and *M. trichosporium* OB3b have been purified and it is reported that the enzyme contains both copper and iron.[1,2] On the basis of the dependence of pMMO activity on copper concentration and the ESR spectrum it is clarified that the active site of pMMO is a copper cluster.[3] The characteristics of the copper binding site in pMMO have been studied by continuous-wave (CW) ESR spectroscopy,[1-5] which shows that pMMO is a typical type II copper protein. The CW-ESR measurements, however, did not clearly identify copper sites with respect to their ligands and symmetries. Since knowledge of the ligands at the copper site is important for understanding the active site of pMMO, the electron spin-echo envelope modulation (ESEEM) spectroscopy was used to characterize the magnetic interactions between copper (II) and weakly coupled magnetic nuclei, and in this way to identify the ligands on the copper site in pMMO from *M. trichosporium* OB3b.

Figure 1 shows the X-band ESR spectra of the membranes treated with ethylenediaminetetraacetic acid (EDTA) and the purified pMMO from *M. trichosporium* OB3b. The ESR spectrum of the EDTA-treated membranes (Figure 1 A) shows a type II copper signal ($g_{//} = 2.24$, $A_{//} = 18.4$ mT, $g = 2.06$) with a multiple hyperfine structure ($A = 1.45$ mT) at the g region, being almost identical to the spectrum of the purified pMMO (Figure 1 B, C). In this study the EDTA-treated membranes containing copper site(s) in the active site of pMMO was used.

ESEEM spectroscopy is a useful tool for identification and characterization of nitrogenous ligands in copper complexes and copper-proteins.[8,9] To gain a better understanding of the active site of pMMO, we performed ESEEM measurements for the copper site in the EDTA-treated membranes. The EDTA-treated membranes were frozen to liquid nitrogen temperature, and immediately lyophilized at ambient temperature for 12 h just prior to ESEEM measurements. ESEEM measurements

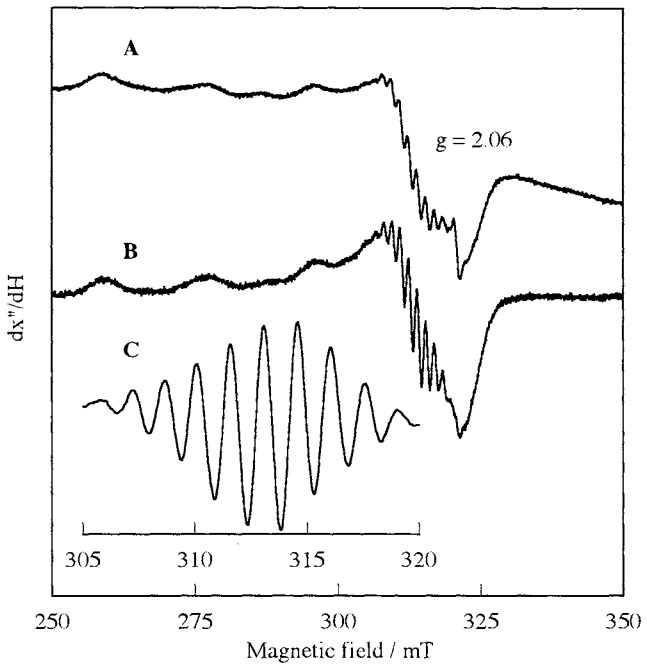

Figure 1. X-band ESR spectra of the EDTA-treated membranes (A), the purified pMMO (B) from *M. trichosporium* OB3b, and second derivative of the absorption at g = 2.06 in trace B. The spectra were recorded at 90 K with 1.0 mW of microwave power, modulation amplitude of 1.0 mT, modulation frequency of 100 kHz, time constant of 0.03 s.

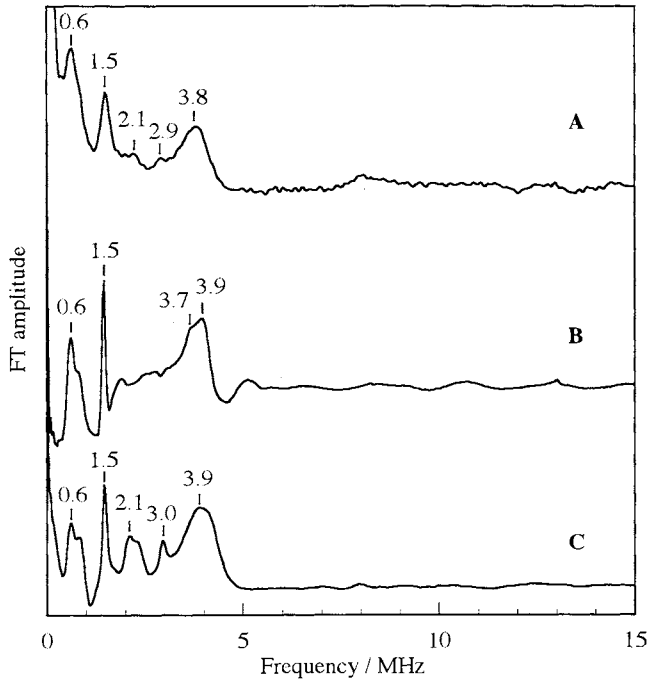

Figure 2. Three-pulse ESEEM spectra of the lyophilized EDTA-treated membranes (A), Cu-diethylenetriamine-imidazole complex (B), and Cu-tetraimidazole complex (C). Experimental conditions were as follows: microwave frequency, 8.805 GHz; magnetic field strength, 305.3 mT; τ value, 310 ns; sample temperature, 15 K.

were carried out as reported previously[10,11] except that an Air Product Heli-Tran cryostat was used to obtain lower sample temperatures. The three-pulse sequence ($\pi/2$- $\pi/2$-T- $\pi/2$-τ-echo) was applied with a pulsed widths of $t_{\pi/2}$ = 25 ns. Prior to the Fourier transformation, the Linear Prediction Singular Value Decomposition method was used to reconstruct the data lost to the instrumental dead time.[6,7]

Stimulated echo ESEEM spectra for the EDTA-treated membranes and copper complexes are shown in Figure 2. The copper complexes are copper (II) - diethylenetriamine-imidazole and copper (II) - tetraimidazole, both measured in a frozen glass. The ESEEM signals are attributed to magnetically coupled remote ^{14}N of equatorially bound imidazole.[8] The low frequency components at 0.6 and 1.5 MHz and the shoulder at ~0.9 MHz correspond to nuclear quadrupole frequencies from one manifold where the Zeeman and hyperfine energies cancel (A $\approx$ 1.6 MHz).[8] The other manifold exhibit the double-quantum transition at 3.9 MHz. For copper (II) - tetraimidazole complex that contains more than a single imidazole ligand, multinuclear combination peaks at 2.1, 2.3 and 3.0 MHz are evident. Such multinuclear combination peaks appear because of the presence of more than one equivalent nuclear spin interacting to the same electron spin, and their amplitudes increase with the increase of the number of the equivalent nuclear spins.[12] Hence the appearance of the combination peaks indicates that there are at least two imidazole coordinated to the type II copper site in the enzyme.

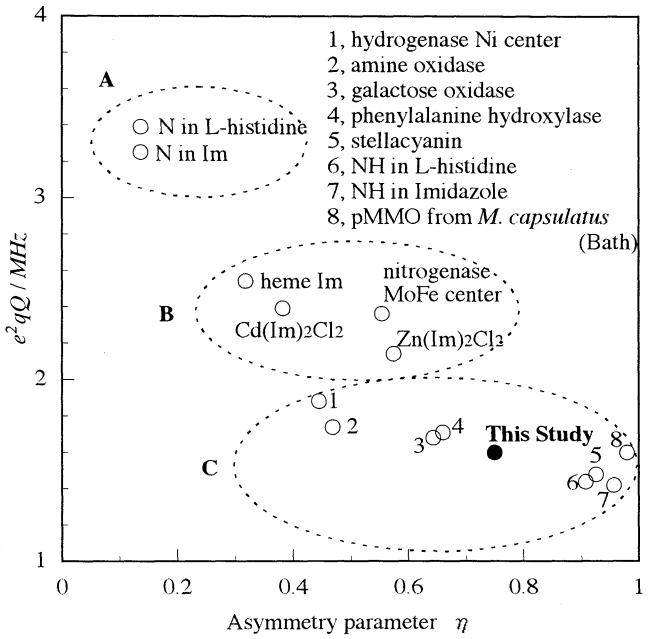

Figure 3. ^{14}N quadrupolar parameters for imidazole, metal imidazole complexes and metalloproteins. Three regions in the figure are set off. Region A is for the deprotonated ^{14}N in histidine and imidazole (Im). Region B contains the parameters for metal imidazole models in which the observed ^{14}N is directly coordinated by a metal ion, and for similar metalloprotein centers. Region C contains the quadrupolar parameters for protonated ^{14}N of imidazole as in L-histidine and in copper-proteins.

The quadrupole coupling constant, e^2qQ and asymmetry parameter, η can be computed from the nuclear quadrupole frequencies. Figure 3 summarizes the quadrupole coupling parameters for metal imidazole complexes and metalloproteins,[12] as well as present results of e^2qQ = 1.60 MHz and η = 0.75 for pMMO from *M. trichosporium* OB3b. The parameters for pMMO from *M. trichosporium* OB3b were found to be similar to pMMO from *M. capsulatus* (Bath) and other copper-proteins.[9,13]

The above results, therefore, establishes the existence of at least two imidazole coordinated to the type II copper site in the enzyme.

This present work is partly defrayed by the Grant-in-Aid for Scientific Research on Priory-Areas-Research from the Ministry of Education, Science, Sports and Culture of Japan (10145211). *M. trichosporium* OB3b was kindly provided by Professor J. D. Lipscomb of University of Minnesota.

References and Notes

1 J. A. Zahn and A. A. DiSpirito, *J. Bacteriol.*, **178**, 1018 (1996).
2 M. Takeguchi, K. Miyakawa, and I. Okura, *J. Mol. Catal.*, **132**,145 (1998).
3 H.-H. T. Nguyen, A. K. Shiemke, S. J. Jacobs, B. J. Hales, M. E. Lidstrom, and S. I. Chan, *J. Biol. Chem.*, **269**, 14995 (1994).
4 H.-H. T. Nguyen, K. H. Nakagawa, B. Hedman, S. J. Elliott, M. E. Lidstrom, K. O. Hodgson, and S. I. Chan, *J. Am. Chem. Soc.*, **118**, 12766 (1996).
5 M. Takeguchi, K. Miyakawa, and I. Okura, *J. Mol. Catal., in press.*
6 H. Barkhuijsen, R. De Beer, W. M. M. J. Bovee, and D. Van Ormondt, *J. Magn. Reson.*, **61**, 465 (1985).
7 D. S. Stephenson, *Prog. NMR Spectrosc.*, **20**, 515 (1988).
8 W. B. Mims and J. Peisach, *J. Chem. Phys.*, **69**, 4921 (1978).
9 S. J. Elliott, D. W. Randall, R. D. Britt, and S. I. Chan, *J. Am. Chem. Soc.*, **120**, 3247 (1998).
10 K. Fukui, H. Ohya-Nishiguchi, and H. Kamada, *Inorg. Chem.*, **36**, 5518 (1997).
11 K. Fukui, H. Ohya-Nishiguchi, M. Iwaizumi, and Y. Xu, *Bull. Chem. Soc. Jpn.*, **71**, 2787 (1998).
12 J. Lu, C. J. Bender, J. McCracken, J. Peisach, J. C. Severns, and D. R. McMillin, *Biochem.*, **31**, 6265 (1992).
13 W. B. Mims and J. Peisach, in "Advanced EPR; Application in Biology and Biochemistry," ed by A. J. Hoff, Elsevier, Amsterdam (1989), p. 1.