Pulsed EPR Studies of Particulate Methane Monooxygenase from *Methylococcus Capsulatus* (Bath): Evidence for Histidine Ligation

Sean J. Elliott,[†] David W. Randall,[‡,§] R. David Britt,[§] and Sunney I. Chan*[,†]

A.A. Noyes Laboratory of Chemical Physics
127-72 California Institute of Technology
Pasadena, California 91125
Department of Chemistry, University of California Davis
Davis, California 95616-0935

Received August 25, 1997

Particulate methane monooxygenase (pMMO), a membrane-bound metalloenzyme found in all obligate methanotrophs, catalyzes the 2-electron, dioxygen-dependent oxidation of methane to methanol.[1,2] We have previously proposed that the catalytic site of pMMO is a copper cluster on the basis of the observed dependence of cellular growth and catalysis with copper ion concentration.[3,4] The number of copper ions strongly bound by pMMO is large, up to 15 copper ions per pMMO unit, and we have suggested that they are primarily arranged in multinuclear clusters that are unlike other multinuclear copper centers found in metallobiochemistry.[2,5] We recently presented a model that differentiated the pMMO copper content into subsets of copper ions that are associated with catalysis (C-clusters), on the basis of their susceptibility to oxidation by air from the Cu(I) to the Cu(II) state, and those copper ions that can only be oxidized by ferricyanide[5] (E-clusters).

Electron spin−echo modulation spectroscopy (ESEEM) has been a useful tool for the identification and characterization of nitrogenous ligands in copper(II)-containing metalloproteins.[7−12] In this paper, we present our initial characterization of the copper(II) ions of oxidized pMMO from *Methylococcus capsulatus* (Bath) by ESEEM spectroscopy.

Dithionite-reduced pMMO-containing membranes were oxidized by exposure to air at 4 °C for 20 min or by treatment with ferricyanide (as a 10 mM solution). All ESEEM and ENDOR experiments were performed at liquid helium temperature, on a laboratory-built instrument,[13] using a three-pulse ($\pi/2 - \tau - \pi/2 - T - \pi/2$) stimulated echo pulse sequence and a Davies pulse sequence[14] for the ESEEM and ESE-ENDOR experiments, respectively. Figure 1 presents representative Fourier transforms[15] of the time domain ESEEM patterns obtained from pMMO samples oxidized by either air or ferricyanide and collected at a frequency of 10.280 GHz with applied magnetic field values that correspond to fields below, above, and directly at the maximal ESE-EPR intensity for the observed signal at $g = 2.06$ (see the Figure 1 caption for details).

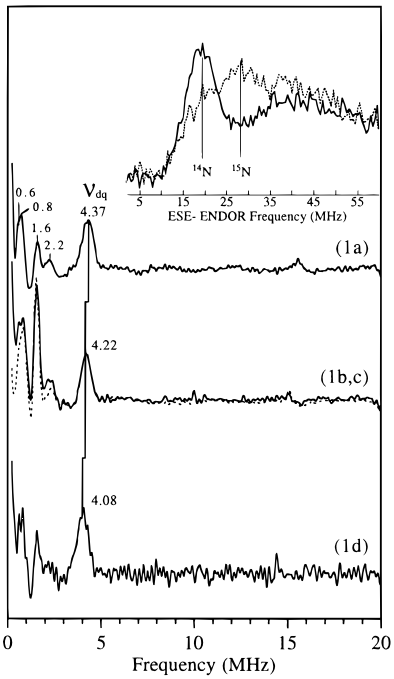

Figure 1. Three-pulse ESEEM spectra of ferricyanide- (solid line) and air-oxidized (dashed line) pMMO-containing membrane samples. The applied magnetic field varies from 3600 G (1a), 3560 G (1b,c) and 3400 G (1d) with the other conditions being $\tau = 198$ ns (1a, 1d), $\tau = 132$ ns (1b,c); microwave frequency, 10.280 GHz; pulse width, 20 ns; pulse sequence repetition rate, 3 ms; temperature, 4.2 K. Inset: Davies ESE-ENDOR spectra of air-oxidized natural isotopic abundance (solid line) and globally labeled ^{15}N (dashed line) pMMO-containing membrane samples. Experimental parameters: microwave frequency = 10.203 GHz; magnetic field = 3543 G; $\tau = 200$ ns; $T = 5$ μs; RF power = 100 W; temperature = 4.2 K.

The spectra shown in Figure 1, as well as similar spectra obtained with other values of τ, all indicate a consistent set of features at 0.6, 0.8, 1.6, and 2.2 MHz, with a fifth transition of greater intensity and width at approximately 4.25 MHz. Such spectral features are characteristic in shape, frequency, and intensity of weakly hyperfine-coupled ^{14}N nuclei in close proximity to an EPR active species, such as Cu(II), near the "exact cancellation" limit. At this limit, in one of the $M_S = \pm^1/_2$ submanifolds, the ^{14}N nuclear Zeeman and hyperfine interactions cancel,[16] giving three intense low-frequency transitions at 0.6, 0.8, and 1.6 MHz. These transition frequencies are controlled by the ^{14}N nuclear quadrupole interaction (nqi) and are invariant with applied magnetic field. Moreover, the sum of the frequencies of the first two transitions should equal the frequency of the third. A fourth transition, often referred to as the double quantum transition, ν_{dq}, arises from the $\Delta M_I = 2$ transition of the other $M_S = \mp^1/_2$ submanifold.[16] It is expected to exhibit a broad line shape and to shift in frequency linearly with the applied magnetic field. As clearly shown in Figure 1, only the transition at ~4.25

[†] California Institute of Technology.
[‡] Current address: Department of Chemistry, Stanford University, Stanford, California 94305.
[§] University of California, Davis.

(1) Bédard, C.; Knowles, R. *Microbiol. Rev.* **1989**, *53*, 68−84.
(2) Nguyen, H.-H. T.; Shiemke, A. K.; Jacobs, S. J.; Hales, B. J.; Lidstrom, M. E.; Chan, S. I. *J. Biol. Chem.* **1994**, *269*, 14995−15005.
(3) Prior, S. D.; Dalton, H. *J. Gen. Microbiol.* **1985**, *131*, 155−163.
(4) Semrau, J. D.; Zolandz, D.; Lidstrom, M. E.; Chan, S. I. *J. Inorg. Biochem.* **1995**, *58*, 235−244.
(5) Nguyen, H.-H. T.; Nakagawa, K. H.; Hedman, B.; Elliott, S. J.; Lidstrom, M. E.; Hodgson, K. O.; Chan, S. I. *J. Am. Chem. Soc.* **1996**, *118*, 12766−12776.
(6) Semrau, J. D.; Chistoserdov, A.; Lebron, J.; Costello, A.; Davagnino, J.; Kenna, E.; Holmes, A. J.; Finch, R.; Murrell, J. C.; Lidstrom, M. E. *J. Bacteriol.* **1995**, *177*, 3071−3079.
(7) Mims, W. B.; Pesiach, J. *Biochemistry* **1976**, *15*, 3863−3869.
(8) Mondovi, B.; Graziani, M. T.; Mims, W. B.; Oltzik, R.; Peisach, J. *Biochemistry* **1977**, *16*, 4198−4202.
(9) Kosman, D. J.; Peisach, J.; Mims, W. B. *Biochemistry* **1980**, *19*, 1304−1308.
(10) Lu, J.; Bender, C. J.; McCracken, J.; Peisach, J.; Severns, J. C.; McMillin, D. R. *Biochemistry* **1992**, *31*, 6265−6272.
(11) Peisach, J. *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklàr, Z., Eds.; Chapman & Hall: New York, 1993; pp 21−33 and references therein.
(12) Magliozzo, R. S.; Bubacco, L.; McCracken, J.; Jiang, F.; Beltramini, M.; Salvato, B.; Peisach, J. *Biochemistry* **1995**, *34*, 1513−1523.
(13) Sturgeon, B. E.; Britt, R. D. *Rev. Sci. Instrum.* **1992**, *63*, 2187−2192.
(14) Davies, E. R. *Phys. Lett.* **1974**, *47A*, 1−2.
(15) Mims, W. B. *J. Magn. Reson.* **1984**, *59*, 291−306.
(16) The correct assignment of $M_S = +^1/_2$ or $M_S = -^1/_2$ cannot be achieved with knowing the sign of the hyperfine tensor *A*, which is not known currently.

S0002-7863(97)02968-5 CCC: $15.00
Published on Web 03/20/1998

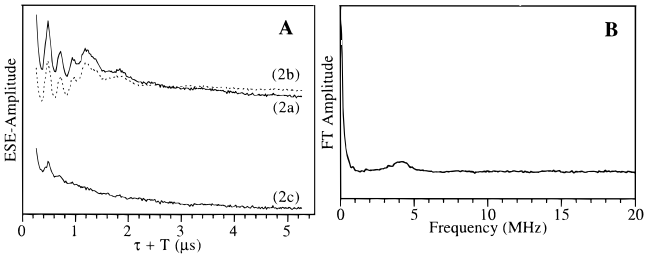

Figure 2. Time domain (A) and frequency domain (B) three-pulse ESEEM spectra for ferricyanide- and air-oxidized (2b) pMMO-containing membranes and their difference spectrum (2c, B). Experimental parameters: magnetic field = 3560 G; τ = 198 ns; pulse width, 15 ns; repetition rate, 5 ns; temperature, 4.2 K.

MHz shifts with varying field, indicating that it is indeed υ_{dq}. The remaining nqi feature observed in Figure 1, the weak 2.2 MHz feature, can be assigned to υ_C, a combination of the nqi components observed at 0.6 and 1.6 MHz. The presence of such a feature indicates *multiple* magnetically equivalent ^{14}N nuclei at the Cu(II) center.[10]

Whereas the ESEEM experiments can detect coupling between the Cu(II) center and weakly hyperfine-coupled nitrogens, ESE-ENDOR can identify nitrogen nuclei more strongly coupled to a paramagnetic center. The inset of Figure 1 shows Davies ESE-ENDOR spectra for air-oxidized samples of pMMO that have been cultured from either natural abundance nitrogen isotopes (^{14}N, solid line) or globally labeled ^{15}N nitrogen (dashed line). Spectra were collected using a short microwave pulse length (15 ns) to minimize the contribution from strongly coupled protons and, in the natural abundance sample, the data show a blue-copper-like hyperfine coupling that shifts appropriately to higher frequency[17] upon incorporation of the ^{15}N isotope, indicating the presence of a *strongly* hyperfine-coupled nitrogenous ligand in addition to the weakly coupled ^{14}N nuclei observed in the ESEEM experiments. Such signals typically demonstrate multiple features, which are not resolved in the present study due to poor angle selection at the magnetic field where the experiments were performed. However, the expected Larmor (and quadrupole, for the ^{14}N case) splitting is well within the band width of the observed transitions.

In assigning the chemical identity of the ^{14}N nuclei detected in the FT spectra shown in Figure 1, we note that the ferricyanide-oxdized sample contains oxidized C- and E-clusters, whereas the air-oxidized sample only contains C-clusters that do not contain the $S = {}^3/_2$ ground-state observed for E-clusters.[5] Considering the time-domain 3-pulse ESEEM experiments for ferricyanide versus air-oxidized preparations (Figure 2), we note that the overall echo intensity for the ferricyanide-oxidized sample is larger, though not as great as would be intuited by the oxidation of the three E-clusters thought to be present per protein unit. This is due to the anomalously low signal intensity for the ferricyanide-oxidized sample, resulting from observing only the $|\pm{}^1/_2\rangle$ levels of the $S = {}^3/_2$ manifold at the *g*-value used.[5] Moreover, a great portion of the decay is expected to occur during the deadtime of the experiment because of the expected shorter T_2 for fully oxidized E- and C-clusters. The contribution from the C-clusters should remain about the same (a 20% increase is expected) between the two preparations. In any case, the difference spectrum (and corresponding Fourier transform) should reveal the presence of additional modulation components that are inherent to the ferricyanide-oxidized sample regardless of the difference in overall echo intensity. Yet, as shown in Figure 2, such a difference spectrum does not yield the set of modulations that corresponds to ^{14}N nuclei. We take this result as evidence that the copper ions in pMMO that are solely oxidized by ferricyanide (i.e., E-clusters) are not magnetically coupled to remote nitrogen nuclei. Thus, the C-clusters of pMMO, which are the apparent active site(s) of pMMO, contain the only multiple weakly coupled ^{14}N nuclei, in addition to at least one strongly coupled ^{14}N. This is the first spectroscopic finding that directly distinguishes the ligand environment (and therefore the chemical properties) of the two distinct sets of pMMO copper-sites.

The nqi for the $I = 1$ ^{14}N nucleus is parametrized by the quadrupolar coupling constant, e^2qQ, and the asymmetry parameter, η, both of which can be directly computed from the nqi frequencies and used to indicate the type of ^{14}N nuclei involved in the coupling.[18,19] The parameters for pMMO are quite similar to those observed for other proteins that are known to exhibit copper coordination by histidine(s) (notably other multicopper proteins such as laccase and ascorbate oxidase). Table S1 (Supporting Information) summarizes ^{14}N ESEEM-derived nqi parameters for several copper(II)-containing proteins, as well as the nqi parameters derived here for pMMO, $e^2qQ = 1.60$ and $\eta = 0.98$. Furthermore, the observed ESE-ENDOR features yield a hyperfine coupling of ~38 MHz for the ^{14}N -labeled sample, which agrees with literature values for imidazole nitrogens from histidine residues bound to Cu(II) centers in metalloproteins and model compounds.[20−23] Thus, we conclude the existence of histidine coordination for at least one of the copper ions in the active site of pMMO.

The presence of histidine(s) at the pMMO active site has not been previously observed spectroscopically, and considering the small number of histidine residues present in the protein overall,[6] the above findings further specify the location of the active site of pMMO within the overall structure of the protein. PmoA, a 27 kDa subunit of pMMO which is believed to contain the active site, contains five histidine residues.[6] Kyte-Doolittle hydropathy analysis of this pMMO subunit, and the analogous subunit of the evolutionarily related ammonia monooxygenase[24] (AMO) from *Nitrosomonas europaea*, suggest that both polypeptides are comprised of transmembrane helices primarily (Figure S1, Supporting Information).[25] Early comparison of the gene sequences coding for PmoA and AmoA has shown that three of the five histidines are identical between the two genes (H38, H40, and H168). Focusing on these identical histidines in PmoA, it appears clear that the active site may be localized at the membrane−periplasm interface, assuming a hydropathy analysis of the PmoA sequence as shown in Figure S1, Supporting Information.

Acknowledgment. This work was supported by the National Institutes of Health (GM 22432 to S.I.C. and GM48242 to R.D.B.). D.W.R. acknowledges a fellowship from the University of California, Davis. S.J.E. is a recipient of a National Research Award from the National Institute of General Medical Sciences, NIH.

Supporting Information Available: Table S1 and Figure S2 (2 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA972968+

(17) For $^{14/15}$N ENDOR, the signals are centered at a frequency of one-half the hyperfine coupling, $A(^{14/15}N)/2$, and follow the relation: $|[A(^{15}N)/A(^{14}N)] = 1.403$.

(18) The nuclear quadrupole coupling constant, e^2qQ, and the asymmetry parameter, η, are related to the nqi frequencies by $\nu_\pm = {}^3/_4e^2qQ(1 \pm \eta/3)$ and $\nu_0 = {}^1/_2e^2qQ\eta$.

(19) Mims, W. B.; Peisach, J. *J. Chem. Phys.* **1978**, *69*, 4921−4930.

(20) Roberts, J. E.; Brown, T. G.; Hoffman, B. M.; Peisach, J. *J. Am. Chem. Soc.* **1980**, *102*, 825−829.

(21) Van Camp, H. L.; Sands, R. H.; Fee, J. A. *J. Chem. Phys.* **1981**, *75*, 2098−2107.

(22) Cline, J.; Reinhammar, B.; Jensen, P.; Venters, R.; Hoffman, B. M. *J. Biol. Chem.* **1983**, *258*, 5124−5128.

(23) Scholl, H.-J.; Hüttermann, J. *J. Phys. Chem.* **1992**, *96*, 9684−9691.

(24) Holmes, A. J.; Costello, A.; Lidstrom, M. E.; Murrell, J. C. *FEMS Microbiol. Lett.* **1995**, *132*, 203−208.

(25) Vannelli, T.; Bergmann, D.; Arciero, D. M.; Hooper, A. B. *Microbial Growth on C_1 Compounds*; Lidstrom, M. E., Tabita, F. R., Eds.; Kluwer Acadmeic Publishers: Dordrecht, The Netherlands, 1996; pp 80−87.