X-ray Absorption and EPR Studies on the Copper Ions Associated with the Particulate Methane Monooxygenase from *Methylococcus capsulatus* (Bath). Cu(I) Ions and Their Implications

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Received May 28, 1996[⊗]

Abstract: Parallel X-ray absorption edge and EPR studies of the particulate methane monooxygenase *in situ* reveal that the enzyme contains unusually high levels of copper ions with a significant portion of the copper ions existing as Cu(I) in the "as-isolated" form (70−80%). The observation of high levels of reduced copper in a monooxygenase is surprising considering that the natural cosubstrate of the enzyme is dioxygen. Toward clarifying the roles of the various copper ions in the enzyme, we have successfully prepared different states of the protein in the membrane-bound form at various levels of reduction using dithionite, dioxygen, and ferricyanide. EPR intensity analysis of the fully-oxidized preparations indicates that the bulk of copper ions are arranged in cluster units. The fully-reduced protein obtained by reduction by dithionite has been used to initiate the single turnover of the enzyme in the presence of dioxygen. Differential reactivity toward dioxygen was observed upon analyzing the copper reduction levels in these synchronized preparations. The enzyme is capable of supporting turnover in the absence of external electron donors in the highly reduced states. These results suggest the presence of at least two classes of copper ions in the particulate methane monooxygenase. As a working hypothesis, we have referred to these classes of copper ions as (1) the catalytic (C) clusters, which function principally as the catalytic core of the enzyme, and (2) the electron-transfer (E) clusters, which are presumed to be the source of endogenous reducing equivalents and therefore function in an electron-transfer capacity.

Introduction

The enzyme methane monooxygenase found in methanotrophic bacteria catalyzes the conversion of methane to methanol using dioxygen as a cosubstrate at ambient temperatures and pressures.[1] This system has attracted considerable attention since it provides an ideal natural model to study methane activation and functionalization, a subject of significant current interest.[2] Two distinct forms of methane monooxygenase (MMO) are known to exist at different cellular locations, a cytoplasmic ("soluble") MMO and a membrane-bound ("particulate") MMO.[1,3] The soluble MMO (sMMO) is a complex three-component system consisting of a hydroxylase, a reductase, and a small regulatory protein.[3,4] The sMMO has been investigated extensively by several research groups.[5−20] Recently, the X-ray crystal structure of the sMMO hydroxylase isolated from *Methylococcus capsulatus* (Bath) has been solved.[20] The hydroxylase active site contains a non-heme binuclear iron cluster. In contrast, the particulate methane monooxygenase

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[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1996.

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S0002-7863(96)01778-7 CCC: $12.00

(pMMO) is a copper protein.[1b,21] This enzyme is much less well-characterized despite its technological importance and natural preponderance. The main obstacle in studying the pMMO is the unusual instability of the enzyme activity. Activity is frequently lost upon cell lysis, detergent solubilization, and freeze−thaw cycles.[21−23] Enzymatic activity is also very sensitive to exogenous ligands as well as the choice of buffer. As a result, a highly active and purified preparation of the enzyme has been slow in forthcoming.

Despite the enzyme lability *in vitro*, the pMMO appears to be expressed in all methanotrophs. So far the sMMO has been detected in only the following strains and species: *Methylococcus capsulatus, Methylosinus trichosporium, Methylosinus sporium, Methylocystis sp. M, Methylomonas methanica 68-1*, and *Methylobacterium* CRL-26.[24−28] Yet, in strains capable of expressing either the sMMO or pMMO, the sMMO is expressed under copper-limiting conditions (no copper added to the growth medium and/or high cell density).[29−33] Otherwise, the pMMO is expressed.[22,29−33] Copper ions not only regulate the expression of the pMMO but have been found to be crucial for pMMO activity.[21,29] The expression of the pMMO is accompanied by the formation of an extensive network of intracytoplasmic membranes, where the membrane-bound pMMO resides.[22,29−33] In addition, an increase in carbon to biomass conversion efficiency is also observed.[32]

Results published to date indicate that the sMMO and pMMO have distinct properties, most notably their different range of sensitivity to substrates.[3,22] While the sMMO is capable of oxidizing a vast array of hydrocarbons (more than 250 compounds known todate), the pMMO has a very narrow substrate specificity, and is only capable of oxidizing C5 hydrocarbons or smaller.[3,22,34] The pMMO is also more sensitive to inhibition by oxygen analogs and metal chelators.[1b] Recently, stereochemical analysis of chiral ethanol products derived from pMMO-catalyzed hydroxylation of chiral ethane points to a concerted reaction mechanism proceeding with complete retention of substrate configuration.[35] In contrast, significant inversion of configuration was observed in the case of the reaction catalyzed by the sMMO, suggesting the formation of a sequestered substrate-based radical intermediate, albeit very short-lived.[36] These results clearly indicate that the two enzymes operate with fundamentally different mechanisms. Thus, the oxygen-containing intermediates employed by these two enzymes as hydroxylating agents must be significantly different species.

In a previous report, we have summarized our preliminary findings on several intriguing aspects of this seemingly intractable system.[37] First, we have found that in *M. capsulatus* (Bath) the presence of copper ions in the growth medium is important for the assembly of the pMMO, that these copper ions are eventually incorporated into the membrane fractions of the organism, and that the enzyme activity is proportional to the copper/protein ratio in the membranes.[21,37] The enzymatic activity also seems to correlate with the level of membrane-bound EPR-active copper ions. Second, the pMMO-associated polypeptides (45 kDa, and the 26 kDa acetylene-binding polypeptide) were found to be the most abundant polypeptides in the membranes, suggesting that the pMMO is overexpressed and is the major protein in these membranes.[37] Third, EPR and magnetic susceptibility measurements suggest the presence of spin-coupled trinuclear copper clusters in the highly-oxidized membrane preparations. On the basis of the turnover chemistry, we proposed that these copper ions constitute the active site(s) of the pMMO.[21]

In the course of our preliminary EPR and magnetic susceptibility characterization of the pMMO membranes, we have also observed that the "as-isolated" membranes typically contain significant amounts of diamagnetic copper ions.[21] Varying, yet similar, levels of diamagnetic copper ions can be inferred from magnetic susceptibility measurements. These observations point to an unusual heterogeneity associated with these membrane-bound copper ions. The nature and location of the EPR-silent copper ions and their participation in dioxygen and methane activation remain intriguing and important pieces of the pMMO puzzle. In this paper, we present our characterization of the copper ions in the pMMO system by parallel X-ray absorption edge spectroscopy, EPR, and magnetic susceptibility measurements. Conclusive evidence for Cu(I) ions as the source of the aforementioned diamagnetic copper ions as well as the level of copper reduction in the pMMO system will be presented. Finally, we will summarize our attempts to prepare samples with more well-defined redox states with respect to the as-isolated preparations and our efforts to characterize these preparations.

Experimental Methods

Growth of Methanotrophs. The organisms used in the studies were maintained on Petri plates containing nitrate mineral salts (NMS) medium[38] with added $CuSO_4$ (20 mM) and solidified with 1.7% agar. Cultures were maintained under an atmosphere of 20% methane in air, and streaked onto fresh plates every 4−6 weeks. Large-scale cultures were grown in 300 mL batches in 2 L Erlenmeyer flasks using the above medium, a 20% methane-in-air atmosphere, and continual shaking or in a fermentor containing 8 L of the above described medium with added 30 mM $CuSO_4$ and 20 mM CuEDTA. Each batch was inoculated with a culture grown to late-log phase. *M. capsulatus* (Bath) was grown at 42 °C. Cells were harvested in late-log phase (typically 48−52 h

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after inoculation) by centrifugation at 15 000 rpm for 15 min, washed once with 20 mM Pipes (pH 7.2), and resuspended in the Pipes buffer.

Membrane Isolation. Cytosolic and membrane fractions were separated by passing a cell suspension (~0.5 g wet weight of cells per mL) three times through a French pressure cell at 20 000 psi. Unlysed cells and cell debris were removed by centrifugation at 15 000 rpm for 40 min. The supernatant was then ultracentrifuged at 60 000 rpm for 90 min to pellet the membrane fraction. The clear supernatant obtained after ultracentrifugation was used as the cytosolic fraction. The membrane pellet was washed by suspending it in 0.25 N NaCl in 20 mM Pipes (pH 7.2) using a Dounce homogenizer. The membrane fraction was repelleted by ultracentrifugation and resuspended in a volume of 20 mM Pipes buffer (pH 7.2) equivalent to the volume of the original cell suspension. This process was repeated one or two more times until the supernatant was virtually free of heme-containing soluble proteins and/or soluble protein as determined by absorption at 280 and 412 nm.

MMO Activity Assay. The MMO activity of samples was measured by the propene epoxidation assay. For whole cell assays, sodium formate was used as reductant; for membrane fractions the reductant was NADH. In both cases the reductant was added to the cell or membrane suspensions to give a final concentration of 5 mM in a total volume of 1.0 mL. The assay was performed at 45 °C, and at ~5−7 min intervals a 1 mL aliquot of the solution was removed and injected directly onto a gas chromatograph (Carbograph 60/80 mesh, AllTech) at 165 °C for chemical analysis. The propene oxide produced from the propene epoxidation reaction catalyzed by the pMMO was detected by flame ionization. The activity of the pMMO was determined from the limiting initial slope of a propene oxide concentration vs time plot. Specific activity was then obtained by dividing the activity by the total amount of protein in the sample determined by the Lowry method.[39] A small volume of 100 mM $CuSO_4$ was added to a number of samples in order to assay the activity of the pMMO in the presence of excess copper.

Metal Ion Analysis. Metal ion analysis (copper, iron, zinc, cobalt, manganese, and nickel) was performed by induced-coupled plasma-mass spectroscopy (ICP-MS). The copper concentration of the samples was determined relative to standard solutions of $Cu(NO_3)_2$ ranging in concentration from 7.3 to 155 mM in 0.1 N HNO_3. A solution of 0.1 N HNO_3 in distilled water was used as a copper-free control. Samples of membrane fractions were burned in acid-washed crucibles. The ash was then dissolved with distilled water containing 1 N HNO_3 and then diluted using 0.1 N HNO_3 solution prior to analysis. The values reported are the averages of three separate determinations. The same samples were used for iron analysis, but the standards were prepared by diluting an iron atomic absorption standard purchased from Sigma Chemical (St. Louis, MO) with 0.1 N HNO_3.

Sample Preparations and Redox Poising. Fully-reduced samples were prepared by treating the membrane suspensions with dithionite or a dye (thionine) under anaerobic conditions. The as-isolated pMMO membranes were purged repeatedly with dioxygen-free argon, mixed anaerobically with deoxygenated dithionite solution (20 mM dithionite in 20 mM Pipes, pH 7.2) and repelleted. Highly- or fully-oxidized preparations were obtained by incubating the isolated membranes with potassium ferricyanide. After the membranes were suspended in Pipes buffer (20 mM, pH 7.2) containing potassium ferricyanide (20 mM) and the mixture was allowed to stand on ice for 30 min, the membranes were repelleted and washed repeatedly with oxidant-free buffer until no trace of the oxidant was observed in the supernatant ($\lambda_{max,ferricyanide}$ = 420 nm). Synchronized-turnover samples were prepared by treating deoxygenated membranes with dithionite (5 mM) anaerobically, followed by reexposing the suspension to air with vigorous shaking for 10 min or less (in the absence of hydrocarbon cosubstrate). The reduction and reoxidation processes were monitored by EPR spectroscopy. The reduced, highly-oxidized, as-isolated and reoxidized membranes were pelleted immediately upon their preparations and resuspended in deoxygenated glycerol and/or buffer for EPR and X-ray absorption measurements.

EPR Spectroscopy. EPR spectra were recorded on a Varian E-line Century X-band spectrometer. In the EPR experiments, sample temperature was maintained at 77 K with a liquid nitrogen dewar or at 4.2 K with an ESR-900 Oxford Instruments (Oxford, England) liquid helium cryostat. The EPR samples were prepared by sealing 200 mL of membrane suspensions under an atmosphere of Ar in quartz EPR tubes at a total protein concentration of ~50 mg/mL in 20 mM Pipes (pH 7.2). Following preparation of the samples, the membrane fractions were rapidly frozen in liquid nitrogen. Quartz EPR tubes equipped with a septum port were used in anaerobic reductive experiments. Solutions of freshly-prepared sodium dithionite were purged with argon and added anaerobically to the sample with a gas-tight Hamilton syringe. The concentration of the dithionite solution was determined by spectrophotometric titration with ferricyanide ($\epsilon_{420\ nm}$ = 1010 M^{-1} cm^{-1}).

X-ray Absorption Edge Spectroscopy. X-ray absorption spectroscopy data were collected at Stanford Synchrotron Radiation Laboratory, SSRL (beam line 7-3, 8-pole wiggler, unfocused, Si(220) double crystal monochromator, 50% detuned to minimize higher harmonics, 3 GeV, 40−90 mA, internal energy calibration).[40] All samples were loaded into lucite EXAFS cells (23 mm × 2 mm × 3 mm; ~140 mL) with 62 mm Mylar windows. During the measurements the samples were kept at 10 K maintained by using an Oxford Instruments continous-flow liquid helium CF1208 cryostat. Fluorescence data were measured using an Ar-filled ionization chamber detector[41] equipped with a Ni filter and Soller slits. Data represent an average of 10−24 scans. Data reduction included energy calibration assigning the first inflection point of the Cu foil to 8980.3 eV, pre-edge subtraction using a polynomial function, spline removal, and normalization. Multiple samples (three reduced, five as-isolated, two air-oxidized, two ferricyanide-treated, one reoxidized turnover) were recorded to ascertain reproducibility.

Measurement of EPR Intensity and Spin Concentration Quantitation. Copper nitrate standard solutions (7550, 3895, 779, 156, and 77.9 μM) in ultrapure water containing 10 mM EDTA were prepared. The EPR spectra of these copper standards were recorded at 4.2 K with 0.01 and 0.05 mW of microwave power. Upon performing baseline correction, the spectra were double-integrated from 2500 to 3500 G and a standard curve correlating the EPR intensity with copper concentration was then generated. The EPR spectra of different preparations described above were recorded with microwave powers of 0.01 and 0.05 mW, and double-integrated from 2500 to 3500 G. The copper spin concentration measured against copper nitrate standards was then inferred on the basis of the EPR signal intensity and the standard curve. The same samples used in EPR spin quantitation experiments were subsequently analyzed for metal content by ICP-MS as descibed earlier. The level of EPR-detectable copper ions for each preparation was calculated as the ratio of the copper concentration determined by EPR-spin count and the value obtained from metal content analysis by ICP-MS.

Magnetic Susceptibility Measurements. Magnetic susceptibility data were collected using a SQUID magnetometer (model MPMS by Quantum Design, San Diego, CA). Membrane fractions were isolated as described previously prior to the experiments. The pelleted membrane preparations were frozen in liquid nitrogen, and immediately lyophilized at ambient temperature for 12 h at a pressure of 3 μm of Hg just prior to magnetic susceptibility measurements. Lyophilized membranes as obtained were quickly packed into a gelatin capsule free of any paramagnetic impurities. The capsule containing lyophilized membranes was then purged repeatedly with helium gas to remove residual moisture and, particularly, oxygen before susceptibility measurements. Magnetization saturation data were obtained at 1.8 K by varying the magnetic field from 0 to 5.5 T. Temperature-dependent magnetization data were collected over the temperature range of 1.8−300 K and at a field strength of 1 T. The paramagnetic component of the magnetization was deduced by subtracting the diamagnetic contribution from the observed magnetization.

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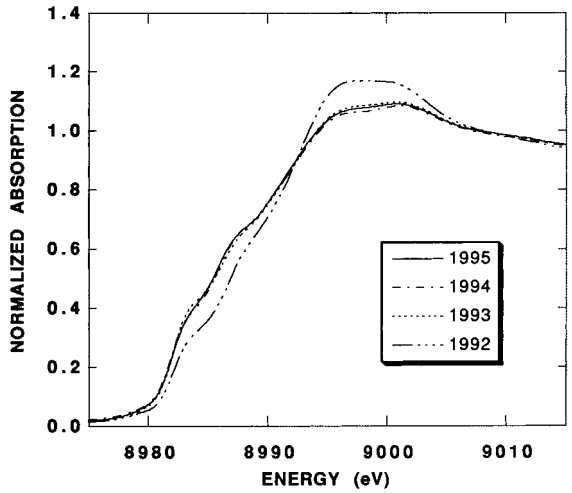

Figure 1. Cu K-edge X-ray absorption spectra of successive as-isolated pMMO samples. The legend indicates which year the respective samples were prepared and studied.

Results

Redox States of Copper Ions in the Native Preparations. Evidence for Cu(I) ions. Cu K-edge absorption spectra of the as-isolated pMMO membranes collected over the years are shown in Figure 1. The intensities of the observed fluorescence signals were unusually strong relative to those of typical concentrated copper-containing proteins in solution, lending further credence to the assertion that the pMMO membranes are highly enriched in copper ions. The observed fluorescence intensities of pMMO-enriched membranes correspond to ~3−6 mM levels of copper ions at least, in contrast to submillimolar to a few millimolar levels as commonly obtained for other copper-containing protein solutions specifically prepared for X-ray absorption measurements. This high level of copper ions in the membranes is clearly a unique feature of the pMMO system. Preparations used for X-ray absorption studies reported here contain ~150−170 nmol or higher of copper/mg of membrane proteins. After considering several possibilities, we concluded previously that the bulk of these membrane-bound copper ions is associated with the pMMO.[21]

X-ray absorption spectroscopy has proved to be the ideal method to ascertain the oxidation states of copper ions since it provides a direct spectroscopic probe of Cu(I) ions.[42] Analysis of X-ray absorption spectra of the as-isolated preparations reveals that a significant portion of the membrane-bound copper ions exist in the cuprous form. The absorption feature at ~8984 eV in the Cu K-edge X-ray absorption spectra has been shown to be associated quantitatively with reduced copper.[42] This has been attributed to the 1s → 4p electric dipole-allowed transition in Cu(I) ions of various coordination environments according to ligand field analysis.[42] This diagnostic signature of Cu(I) ions has been observed in all of the Cu K-edge X-ray absorption spectra of the as-isolated pMMO membranes obtained to date, albeit with varying degrees of intensity, hence indicative of different levels of reduction.

Metal Ions in Membranes Containing Overexpressed pMMO. The presence of other metal ions in addition to copper in the membranes has been reported.[21] Besides an anomalous amount of copper, these membranes also contain some iron. The level of iron in our membrane preparations varies, but a Cu/Fe ratio of 7−20/1 is typical. The iron detected in the membranes mostly is from heme-containing proteins which constitute >30−50% of the detected iron, iron−sulfur proteins, and nonspecific iron. Occasionally, zinc and nickel were also detected. However, no stoichiometric amount of iron has been detected in the *purified* pMMO.[43] The presence of iron in the pMMO suggested by a recent report[44] is clearly a result of unaccounted-for heme-containing contaminants in the preparations as well as the experimental protocols employed in the study, particularly when the organisms were cultivated under unusually high iron concentrations. High levels of iron during growth (60 μM in contrast to a typical 1−2 μM) would lead to higher amounts of nonspecific iron as well as sMMO expression, resulting in sMMO-containing membranes if care was not exercised during membrane-washing. Furthermore, in the aforementioned report,[44] the copper ions were thought to be associated with a copper-binding cofactor which has a MW of ~1200. This species, which roughly corresponds to a hexa- to dodecapeptide, was considered capable of accommodating with specificity six Cu(II) ions, i.e., one Cu ion per one or two amino acid residues. Thus, the result reported does not make sound chemical sense. As such, this preparation[44] was devoid of any metallic cofactors and low activity if any should be expected.

Enzyme Stability and Activity. As we became increasingly more successful in improving our "pMMO" preparations with time from 1991 to the present, these preparations contain progressively more Cu(I) ions as judged by X-ray edge spectroscopy. The improvements on pMMO preparations can be assessed by two criteria: stability of the activity and specific activity. The pMMO-enriched membranes used in this study do exhibit higher stability as the level of reduction increases. Except for the 1992 preparation, for which the activity was more labile, other preparations since 1993 have been exceptionally stable. The activity was stable with respect to several freeze−thaw cycles, and prolonged storage at −80 °C. X-ray absorption samples stored since 1993 are still active now. These preparations exhibited no activity reduction after several days of storage at 4 °C and often showed increasing activity as they "aged". The enhanced stability is indeed remarkable for these preparations, considering what has been known about the enzyme lability in the past. The maximal specific activity possible, however, varies from preparation to preparation. In addition, for a given preparation, its specific activity can be enhanced upon aging under aerobic conditions, or upon dioxygen treatment *(vide infra)*. Therefore, many indicators have been used to evaluate a certain preparation. The pMMO preparations used in the present study exhibit either no reduction or enhanced activity with prolonged storage, they are stable with respect to freeze−thaw cycles, they exhibit a specific activity of at least 5 and as high as 60 nmol/(min·mg), and sustain turnover for at least 30 min to more than 1 h during activity assays, indicating high catalytic productivity numbers per enzyme. The details of the procedure to obtain these preparations will be presented in our upcoming pMMO purification paper.[43]

Redox-Poising. Fully-Reduced and Fully-Oxidized Preparations. Since large variations in the Cu(II)/Cu(I) ratio of as-isolated pMMO preparations indicate heterogeneity in the redox state of the enzyme molecules, we have developed methods to prepare pMMO in a more well-defined, poised, redox state than the as-isolated form. As shown in Figure 1, the as-isolated

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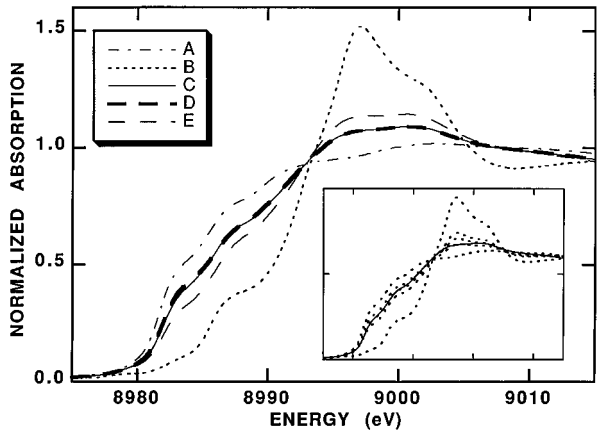

Figure 2. Cu K-edge X-ray absorption spectra of (A) dithionite-reduced, (B) ferricyanide-oxidized, (C) as-isolated, (D) reoxidized turnover, and (E) dioxygen-treated pMMO samples. The inset shows, from left to right, fully reduced; 80/20, 70/30, and 60/40 fully reduced/oxidized; and fully-oxidized samples, with the as-isolated data superimposed as the solid line.

pMMO membranes obtained since 1992 contained varying levels of reduced copper ions. As indicated by the intensity of the 8984 eV feature as well as by the shift in the edge energy, the level of reduction increased progressively as improvements in pMMO preparations were made. This observation prompted us to attempt to fully reduce the membrane-bound copper ions. Among various reductants tested (thionine ($E^{\circ\prime} = 0.064$ V), NADH ($E^{\circ\prime} = -0.320$ V), ascorbate ($E^{\circ\prime} = 0.058$ V), various quinol analogs (1,4-benzoquinol, tetramethyl-1,4-benzoquinol, ubiquinol Q-10), and dithionite ($E^{\circ\prime} = -0.527$ V)), dithionite yielded the best result, capable of reducing most if not all of the membrane-bound copper ions. The extent of copper reduction can be ascertained by examining the intensity of the absorption at ~8984 eV in the Cu K-edge spectra. The pronounced shoulder at 8984 eV observed in the absorption spectra of dithionite-reduced samples provides a clear indication of Cu(I) ions. On the basis of the energy position and absorption intensity of the ~8984 eV feature, we infer that the majority of membrane-bound copper ions are reduced in the dithionite-treated samples (Figure 2A). EPR measurements concur with this result. As shown in Figure 3A, the dithionite-reduced sample does not exhibit any EPR-detectable species, implying that most if not all of the membrane-bound copper exists as Cu(I) in this sample.

The fully-oxidized form of the enzyme, it turns out, is much more difficult to prepare. For all oxidants tested to date (1,4-benzoquinone, tetramethyl-1,4-benzoquinone, ubiquinone Q-10, *p*-amino-*N*,*N*-dimethylaniline ($E^{\circ\prime} = 0.38$ V), *p*-aminophenol ($E^{\circ\prime} = 0.314$ V), $Co(phen)_3^{3+}$ ($E^{\circ\prime} = 0.370$ V), ferricyanide ($E^{\circ\prime} = 0.338$ V)), ferricyanide yields the best result, capable of oxidizing most, if not all, of the membrane-bound copper ions with a single incubation. Occasionally, residual Cu(I) ions are still observed, however. The level of oxidation achieved appears to be a function of both oxidant tension and incubation time.

The most dramatic changes in the X-ray absorption edge and EPR spectra are observed in the case of ferricyanide-treated membranes. The bulk of the pMMO-associated copper ions are seen to be oxidized in this preparation as indicated by a complete loss of the absorption edge feature at ~8984 eV, and the shift in the edge features to higher energy (Figure 2B). This observation allows us to ascertain that the major fraction of the membrane-bound copper ions are present as Cu(II) in this sample. The oxidation level of the copper ions in the ferricyanide-treated membranes was also independently verified on the basis of the metal content and the saturated magnetic moment of the sample in conjunction with the X-ray absorption measurements. Concomitantly, the EPR spectrum of this preparation shows an increase in intensity. A dramatic change in the EPR signal line shape is also noted (Figure 3B). While a four-line hyperfine pattern EPR spectrum typical of Cu(II) ions in pseudo-square-planar coordination is often observed for the as-isolated sample, the EPR spectrum of the highly-oxidized preparation exhibits dramatically different characteristics. The spectrum now clearly shows only the broad and quasi-isotropic features of the previously proposed spin-coupled copper clusters. The origin of this EPR absorption has been attributed to the $|-1/2\rangle \rightarrow |+1/2\rangle$ transition in the ground quartet manifold of a spin-coupled trinuclear copper cluster. Since there is no intense feature toward high g values, particularly near $g \sim 4$, the EPR spectrum is consistent with a quartet with a very small axial zero-field splitting parameter, D. The overwhelming presence of the isotropic signal suggests that the bulk of the membrane-bound copper ions in this sample are organized into cluster units of undetermined homogeneity and defined magnetic/chemical properties. Thus, the EPR signature of the copper cluster can be directly observed simply upon fully oxidizing the copper ions in the membranes, and it is not necessary to employ high microwave powers to saturate the "type 2" copper EPR in order to discern this signal as we have done in the past.

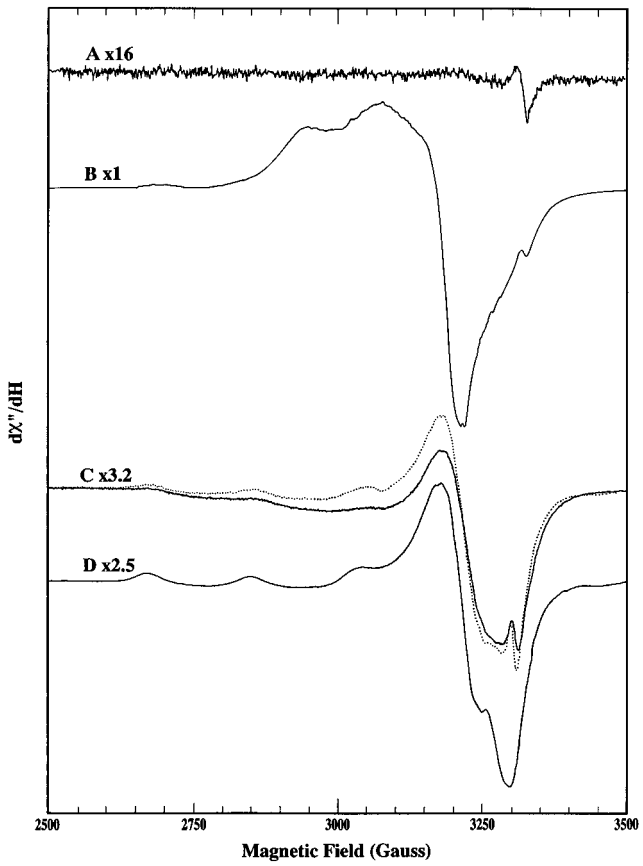

Figure 3. EPR spectra of (A) dithionite-reduced, (B) ferricyanide-oxidized, (C) (dotted line) as-isolated prior to reduction, and (solid line) reoxidized turnover, and (D) dioxygen-treated pMMO samples. Spectra were recorded at the X-band frequency at 4.5 K with a microwave power of 0.05 mW, modulation frequency of 100 kHz, and modulation amplitude of 10 G.

Dioxygen Chemistry. Synchronized-Turnover Experiments. Since a fully-reduced enzyme can be produced by dithionite, we have attempted to use this species to synchronize the generation of other homogenous states. In our earlier work, we have reported preliminary evidence that the copper clusters

Table 1. Percentage of Cu(II) Ions in Different Preparations As Determined from X-ray Absorption Edge and EPR Spin Quantitation

preparation	percentage of Cu(II) ions deduced from X-ray absorption edge data	percentage of Cu(II) ions based on Cu EPR intensity	percentage of Cu(II) ions predicted by the two turnover models
dithionite-reduced	0	0	0
as-isolated	30 ± 10	23 ± 5	29[a]/27[b]
synchronized turnover with air for <10 min	30 ± 10	$19 + 4$	29[a]/27[b]
turnover with pure O_2 for 2 h	50 ± 10	57 ± 7	57[a]/53[b]
fully-oxidized with $[Fe(CN)_6]^{3-}$	100	41 ± 7 ($\sim$103[c])	100

[a] Model: four E clusters and three C clusters. [b] Model: three E clusters and two C clusters. [c] Value obtained after correction for the intensity anomaly expected from the intensity of the $|-1/2\rangle \rightarrow |+1/2\rangle$ transition within the $S = 3/2$ quartet manifold.

participate in dioxygen activation.[21] However, if each of the putative trinuclear Cu(I) clusters are involved in hydroxylation chemistry and under synchronized turnover, then ca. one-third of the copper ions should remain reduced after reaction with dioxygen. This is so because the monooxygenase reaction coupled with substrate oxidation only consumes two electrons. The third electron must stay in the system. A consequence of the O−O bond cleavage paradigm as applied to the pMMO system is that one of the copper ions in the cluster merely provides the third electron to facilitate heterolytic cleavage of the O−O bond and should remain reduced after turnover. However, if the reaction is carried out in the absence of hydrocarbon substrates, the hot "oxygen intermediate" is not consummated and it ultimately must also be reduced to water, because of its high reduction potential. This "catabolic" step requires an additional two electrons, which must come either from the remaining Cu(I) ions in the enzyme, including the "odd" electron remaining within the cluster, and/or from oxidizable amino acid residues in the protein matrix.

Upon exposing a completely-reduced preparation to air, a significant fraction of the copper ions is rapidly oxidized as indicated by the fast recovery of the sample EPR intensity to the level prior to reduction within <10 min (Figure 3C). XAS edge analysis of this preparation *(vide infra)* revealed, however, that ~70% of the total copper ions in this synchronized sample still exist as Cu(I) ions (Figure 2C,D). The remaining population of Cu(I) ions is higher than expected, hence demonstrating that all of the pMMO-associated copper ions do not exhibit the same reactivity toward dioxygen. As mentioned above, if these membrane-bound copper ions are the pMMO site(s) of dioxygen activation, prolonged exposure of the sample to dioxygen, or increasing the dioxygen tension, should yield a more oxidized sample. Treating as-isolated, reoxidized, or fully-reduced pMMO membranes with pure dioxygen for 1−2 h does yield a more oxidized preparation as indicated by the Cu K-edge absorption spectrum (Figure 2E). However, edge analysis *(vide infra)* reveals that ca. 40−50% of the total membrane-bound copper ions still exist as Cu(I) ions. The remaining Cu(I) ions appear to resist further oxidation by dioxygen as demonstrated by the failure to obtain a highly or fully-oxidized preparation upon prolonged O_2 exposure. In parallel EPR measurements, the dioxygen-saturated sample exhibits intense features typical of magnetically-isolated or weakly-coupled copper ions (Figure 3D). Thus, the level of reduced copper ions in these synchronized preparations is inconsistent with our expectation regarding the function of these copper clusters, namely, that they *all* participate in dioxygen activation and hydrocarbon hydroxylation. This finding represents the most significant insight to emerge from the present study.

Level of Copper Reduction and Spin Quantitation. The level of copper reduction can be estimated by examining the Cu K-edge spectrum of these preparations. The intensity of the ~8984 eV feature, however, reflects both the amount of reduced copper and the nature of the coordination environment. As the samples contain multiple coppers, it is not possible to isolate the individual contributions. Using the assumption that the dithionite-reduced sample contains 100% Cu(I) and the ferricyanide-oxidized sample contains 100% Cu(II) and that there was no significant coordination difference among the various copper sites in their respective oxidation states, a series of percent-weighted numerical additions of the two spectra was performed and the best match to the edge structure/energy position was assessed. Edge analysis then suggests that ~70% ± 10% of the total copper ions exist as Cu(I) ions in the as-isolated preparations employed in these experiments, in which high and unusually stable activity was also observed (Figure 2C). Errors given indicate the level at which the agreement between calculation and the data deviates beyond acceptability within the context of this model. Consistent with this level of copper reduction, EPR spin quantitation on these as-isolated samples also indicates that ~23% ± 5% of the total membrane-bound copper ions are EPR-visible (Table 1).

The level of agreement between X-ray absorption edge data and EPR spin quantitation allows us to conclude that the EPR-silent copper ions in the as-isolated preparations must be reduced. The uncertainty for this result is no greater than 10% of the total membrane-bound copper ions, the same as the error associated with the model and experimental protocols. This spin-count result thus eliminates one of the possible sources for the diamagnetic copper ions observed from magnetic susceptibility measurements on the as-isolated samples noted earlier. Magnetic susceptibility measurements indicate a significant level of diamagnetic copper ions in the as-isolated samples. Antiferromagnetically-coupled copper ions such as type 3 copper ions could give rise to the aforementioned diamagnetism. However, considering the level of agreement between the X-ray absorption results and the EPR spin quantitation data, the presence of a significant level of antiferromagnetic Cu(II) ions is very unlikely. At most, no more than 10% of the total membrane-bound copper ions can be postulated to exist in this form in the as-isolated preparations.[21] Hence, both X-ray data and spin-count results indicate that, in typical as-isolated preparations, approximately 70% of the total membrane-bound copper exists as Cu(I). This result resolves any mystery from previous magnetic susceptibility data.

In contrast, spin quantitation results on ferricyanide-oxidized preparation indicate a very different story. Even though X-ray absorption data indicate that all of the membrane-bound copper ions are oxidized, spin quantitation at nonsaturating microwave power conditions (0.01 and 0.05 mW) reveals that only ~35−40% of the membrane-bound copper ions are EPR-detectable (Table 1). This result, however, is further evidence in support of the presence of a spin-coupled trinuclear copper cluster in the fully-oxidized preparations. Ferromagnetic-coupling of three copper ions ($S = 1/2$) would give rise to a $S = 3/2$ quartet ground spin state in addition to two $S = 1/2$ doublet spin states (see the next section). As EPR absorption mainly arises from the allowed $|-1/2\rangle \rightarrow |+1/2\rangle$ transition within the quartet

manifold at low temperature ($T < 10$ K), an intensity anomaly is thus expected. Under these conditions where the quartet spin state is expected to dominate the EPR spectra, EPR spin quantitation results obtained from the fully-oxidized pMMO preparations can be analyzed using the energy levels derived for $S = 3/2$. Assuming that the sample geometry, the filling factor (η), and the loaded quality factor (Q) are identical, the total number of spins in the fully-oxidized samples can be inferred from copper sulfate/EDTA standards ($S = 1/2$) through the expression

$$N_{\text{samp}} = N_{\text{ref}} \frac{I_{\text{samp}}}{C_{\text{samp}}|V_{ij}|^2_{\text{samp}}} \frac{C_{\text{ref}}|V_{ij}|^2_{\text{ref}}}{I_{\text{ref}}}$$

where N is the number of spins, I is the measured EPR signal intensity obtained by double integration, C is the normalization constant, and $|V_{ij}|^2(P_{ij})$ is the transition probability matrix element for the transition $i \rightarrow j$. The transition probabilities P_{ij} are given by

$$P_{ij} = |V_{ij}|^2 = |\langle\phi_i|\mu\cdot\mathbf{H}_1|\phi_j\rangle|^2$$

where μ is the magnetic moment; $\mathbf{H}_1$ is the microwave field; and ϕ_i and ϕ_j are the eigenfunctions of the spin Hamiltonian. Short of any significant changes in g values, about 40% of the expected intensity based on the total membrane-bound copper ions (assuming magnetically-isolated Cu(II) ions) should be detected by EPR. The level of EPR intensity observed in the ferricyanide-treated preparations, when compared with Cu(II) ions standards ($S = 1/2$), is consistent with this analysis.

EPR Spectra of the Fully-Oxidized Preparations. Even though it is almost certain that the copper clusters in the pMMO do not exist as symmetric triads, the quantum mechanics that describe the EPR spectrum of the ferromagnetically-coupled fully-oxidized trinuclear copper cluster in its $S = 3/2$ quartet ground state remain unchanged. When the exchange coupling parameters among the three Cu(II) ions are nonidentical, the degeneracy of the excited doublet states is lifted, however. Considering only isotropic coupling interactions, it is relatively straightforward to deduce the spin energy levels of the asymmetric trimer from the Hamiltonian

$$\hat{\mathbf{H}} = \sum_{i<j} J_{ij}\hat{\mathbf{S}}_i\cdot\hat{\mathbf{S}}_j$$

where J_{ij} denotes the exchange interaction between the ith and jth copper ions. The expressions for the energy levels are complicated and given as

$$E(3/2) = (1/4)(J_{12} + J_{23} + J_{13})$$

$$E_1(1/2) = -(1/4)(J_{12} + J_{23} + J_{13}) + (1/2)(J_{12}^2 + J_{23}^2 + J_{13}^2 - J_{12}J_{23} - J_{12}J_{13} - J_{13}J_{23})^{1/2}$$

$$E_2(1/2) = -(1/4)(J_{12} + J_{23} + J_{13}) - (1/2)(J_{12}^2 + J_{23}^2 + J_{13}^2 - J_{12}J_{23} - J_{12}J_{13} - J_{13}J_{23})^{1/2}$$

where $E(3/2)$ denotes the energy level of the quartet spin state and $E_1(1/2)$ and $E_2(1/2)$ denote the energy levels of the two doublet spin states. The above expressions can be used to calculate the energy separations between the ground state and the excited states of the copper cluster with any symmetry.

The EPR spectrum of the highly oxidized preparations at low temperatures ($T < 10$ K) have been interpreted in terms of a $S = 3/2$ quartet species with a small axial zero-field splitting. The EPR spectrum of ferricyanide-treated preparations at liquid helium temperature, where the copper ions of the clusters are fully-oxidized as confirmed by X-ray absorption spectroscopy, is consistent with this interpretation. For a $S = 3/2$ spin system, the EPR spectrum can be deduced from the spin Hamiltonian

$$\hat{\mathbf{H}} = \beta g\vec{\mathrm{H}}\cdot\hat{\mathbf{S}} + D[\hat{\mathbf{S}}_z^2 - (1/3)S(S+1)] + E[\hat{\mathbf{S}}_x^2 - \hat{\mathbf{S}}_y^2]$$

where D and E are the axial and rhombic zero-field splitting parameters, respectively, g is the average isotropic g value of the trimer, and $\vec{\mathrm{H}}$ is the applied magnetic field. For a system possessing a near trigonal axis of symmetry, E is expected to be small. An isotropic g is assumed in accordance with observations from EPR experiments even though a small anisotropy is likely. The anisotropy of the spectrum then arises mostly from the ZFS terms (D and E). In the case of small D and E, the spin Hamiltonian predicts three EPR transitions ($+3/2 \leftrightarrow +1/2$, $+1/2 \leftrightarrow -1/2$, and $-1/2 \leftrightarrow -3/2$) of relative intensities 3:4:3. However, the $\pm 3/2 \leftrightarrow \pm 1/2$ transitions are very anisotropic, and are outside the range of the typical X-band EPR experiments, even in the case of relatively small zero-field splittings ($|D| \sim 0.05$ cm $^{-1}$). Also, they are expected to be highly broadened as a result of the large distribution in ZFS parameters (D-strains) in a protein environment as well as variations in D among the copper clusters. As such, these transitions are either not observable at X-band or too smeared out to be observable. Only the central $|-1/2\rangle \leftrightarrow |+1/2\rangle$ transition is sufficiently isochronous to be discerned in the powder spectrum. For small axial zero-field splittings, i.e., $|D| \ll g\beta\mathbf{H}$, the resonance field for this transition is only weakly orientation dependent, concentrating in the spectral region around $g = 2$. In the case of large D, i.e., $|D| \gg g\beta\mathbf{H}$, this resonance field becomes more orientation-dependent. The derivative powder EPR spectrum is dominated by features near $g \sim 4$ arising from those clusters with the applied magnetic field $\mathbf{H}$ along the x and y principal axes of the zero-field splitting tensor. These axes lie within the triangular planes of the various trinuclear clusters. When the applied field is aligned along the z principal axes, namely, parallel to the normals to the triangular planes, the resonance field occurs at $g = 2$. The EPR spectra observed for the fully-oxidized samples (ferricyanide-treated) are consistent with the central $|-1/2\rangle \rightarrow |+1/2\rangle$ transition of a quartet species with small D. Since only this central transition is observed, an intensity anomaly is expected on the basis of the concentration of Cu(II) ions present. Only 40% of the expected EPR intensity is anticipated, as observed.

Finally, we have found that this EPR signal is very difficult to saturate. The basis of this power saturation behavior, of course, is the thermal accessibility of the two low-lying excited states (the $S = 1/2$ doublets), which provide efficient relaxation pathways for the electron spins via the Orbach process.[45] Exchange-coupled trinuclear Cu(II) clusters are rare; however, a few examples are known.[46] These compounds often exhibit an EPR powder spectrum with a quasi-isotropic resonance at $g \approx 2$ devoid of any half-field transition ($\Delta M_s = 2$) or fine structure. The copper ions in the pMMO apparently are arranged in some triangular form, and adjacent ions are in

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sufficient close juxtaposition that the motions of the spins are correlated when the ions are in the Cu(II) state. These copper ions are not expected to be equivalent as Jahn−Teller distortion will lift the site symmetry from C_{3h} or D_{3h} to some lower symmetry. The observed ferromagnetic-coupling is most likely the consequence of spin frustration.

Levels of Oxidized Copper Ions and Specific Activity. As shown above, the as-isolated membranes contain significant levels of reduced copper ions. Cupric ions are often found to constitute only ∼30% or less of the total membrane-bound copper ions in as-isolated preparations. Upon treating the membranes with dioxygen, the membrane-bound reduced copper ions can be oxidized further, albeit rather slowly. Up to ∼50−60% of the total membrane-bound copper ions can be prepared in the divalent state via this route. This increase in the level of cupric ions is also accompanied by a substantial increase in the specific activity and the total turnover numbers (higher final oxidation product (propylene oxide) concentration). In a typical preparation, the as-isolated membrane preparation exhibits a specific activity of ∼5.7 nmol of propylene oxide produced/(min·mg of protein). The same preparation upon dioxygen treatment exhibits a specific activity of ∼20.2 nmol of propylene oxide produced/(min·mg of protein). Thus, a >300% increase of activity was observed. This result strongly suggests that either (i) a significant portion of the Cu(I) ions become capable of directly participating in hydroxylation chemistry upon O_2 activation or (ii) there is conformational hysteresis associated with this system, which is relieved by allowing the enzyme to react with O_2. This kind of conformational hysteresis is commonly observed for membrane proteins, particularly those involved in signal and energy transduction. Higher levels of oxidized copper ions can also be obtained by further prolonged storage at 4 °C under O_2-saturated ambient atmosphere ("aerobic aging"), hence allowing more reacting time for dioxygen. However, enzymatic activity decreases dramatically (eventually becoming totally inactive) upon further copper oxidation. This latter observation strongly suggests that the remaining Cu(I) ions are also crucial for pMMO activity.

Discussion

The overexpressed pMMO intracytoplasmic membranes as isolated contain an abnormally high level of copper ions. This level of copper in pMMO membrane preparations has resulted in unusually intense Cu X-ray fluorescence signals, not commonly observed for other copper-containing proteins. The presence of a copper storage protein has yet to be conclusively ruled out, but in light of (a) the pMMO-activity-stimulation effect of membrane-bound copper ions and (b) the redox-active nature of the copper ions, the bulk of the membrane-bound copper ions appear to be associated with pMMO. This conclusion is supported by our pMMO isolation effort. In these studies, we found that pMMO is in fact the major protein in these membranes, ca. 70−80% of the total membrane proteins, and the membrane-bound copper ions are found to be mostly associated with the pMMO.[43] Since pMMO constitutes the bulk of the membrane proteins, and the molecular mass of pMMO is on the order of 94 kDa, based on a subunit composition of three polypeptides of molecular masses 45, 26, and 23 kDa,[43] the level of copper observed in the pMMO-containing membranes used in the present study (ca. 150−170 nmol of Cu/mg of total protein) translates into 20 ± 3 copper ions per pMMO molecule. This copper content is close to the stoichiometry (ca. 15) determined for the purified protein.[43] Furthermore, since the overexpressed pMMO membranes were subjected to a large number of salt washes, the amount of adventitious copper associated with the membranes is minimal, certainly not large enough to grossly distort the conclusions reached here, particularly given that the pMMO is overexpressed in the membranes, the high copper content associated with the pMMO, and the fact that most of the coppers in the purified membranes as well as the enzyme are reduced as isolated. On the basis of our best estimate, the amount of adventitious copper associated with the membranes cannot amount to more than 1−2 copper ions per protein molecule, or ∼10% of the total copper content.

However, it remains whether the ∼70% (or 40% after longer dioxygen treatment) of the Cu(I) ions that do not react with dioxygen might not be actually associated with the pMMO. We address this issue specifically here. The answer to this question is provided by the intensity and nature of the EPR signal after all the copper ions have been oxidized by ferricyanide. Only the cluster EPR signal is observed, after all the copper ions have been oxidized according to the X-ray absorption edge. In particular, no type 1 or type 2 copper EPR is discerned in the spectrum which would otherwise be characteristic of isolated type 1 or type 2 Cu(II) ions normally found in copper proteins. Type 3 coppers are EPR invisible, but contribute to the copper X-ray edge as Cu(II). We find that the intensity of the cluster EPR signal accounts for all the coppers in the sample. Thus, it is very unlikely that the Cu(I) ions that do not react with dioxygen could be accounted for by other copper-containing species in the membrane preparation. The uncertainty in our estimate of the level of pMMO-associated copper ions in the membranes is again at worse 10%. Thus, despite the inevitable presence of other copper proteins in the membranes, it is clear that pMMO is by far the most abundant copper protein in the preparations and the analysis of the spectroscopic results is not compromised by the relatively insignificant level of these "contaminants". On the basis of Cu/protein ratio data, it is evident that the pMMO contains multiple copper sites. It also seems certain that all of these copper ions are not in identical environments. Moreover, the differential reactivity of these copper ions toward dioxygen suggests that the copper ions are sequestered into distinct cluster sites, most likely with different functions, reminiscent of the P and M clusters of nitrogenase.[47] The new observations that we present here clearly add new dimensions to the complexity of the problem we are now facing in our attempt to analyze the accumulated spectroscopic information.

Functions of the Cu(I) Ions in the pMMO System. X-ray absorption spectroscopy provides a powerful probe for the presence of Cu(I) ions. The presence of these diamagnetic Cu(I) ions in the as-isolated pMMO membranes is established upon analysis of the Cu K-edge X-ray absorption spectra as well as the redox-active nature of these membrane-bound copper ions, including their reactivity toward dioxygen. These copper ions can exist in both Cu(I) and Cu(II) oxidation states in the membranes. The fact that the as-isolated membrane-bound copper ions obtained at different times contain different Cu(II)/Cu(I) ratios indicates that the membrane-bound copper ions are redox-active toward dioxygen, and different copper sites in the membranes exhibit different reactivity. As shown by X-ray absorption data, these copper ions are sensitive to dioxygen tension, and can be oxidized with varying ease in the presence of O_2. The reduced copper ions for any given as-isolated preparations can be oxidized by O_2, concomittant with an increase in *in-vitro* activity. These results strongly suggest that the membrane-bound copper ions participate in dioxygen activation, and constitute the active site(s) of the pMMO. Since the native and highly active preparations contain cuprous ions,

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the functional form of the enzyme must be the fully-reduced or partially reduced form. The presence of significant levels of Cu(I) ions is indeed an interesting feature of the system. This revelation renders the pMMO a unique Cu protein since with most redox-active copper-containing enzymes, the oxidized form is most often isolated. This very unusual feature of the pMMO is likely to have significant ramifications regarding its dioxygen activation and methane hydroxylation chemistry. Furthermore, the requirement of high levels of reduced copper ions may contribute to the unusual instability of the enzyme activity *in vitro*.

In our view, Cu(I) ions in the pMMO system are directly correlated to the dioxygen and methane activation chemistry performed by the enzyme. While this report shows that there are distinctions among the membrane-bound copper ions, the physical nature of the catalytic site is not resolved. A postulated turnover cycle which incorporates general features of our current understanding of dioxygen activation,[48−53] involving a trinuclear copper cluster site, can be proposed. The fully-reduced copper cluster would bind dioxygen, followed by two-electron reduction and possibly proton transfer to form a copper−peroxy intermediate. Assuming that the pMMO follows a pathway similar to many other monooxygenases, O−O bond cleavage proceeds subsequently, generating a copper−oxygen intermediate capable of alkane hydroxylation. The exact nature of this species, the pMMO hydroxylating agent, has yet to be elucidated. In the presence of alkane substrate(s), hydroxylation will rapidly occur and the product is formed. In such a mechanism involving a trinuclear copper cluster, two electrons are consumed for each turnover in agreement with the overall reaction scheme. However, in general and most often found to be the case, a third electron is needed to facilitate irreversible heterolytic O−O bond breakage after two-electron reduction of dioxygen and to generate the hydroxylating agent.[48,49] Unlike iron, copper ions do not have ready access to higher oxidation states such as Cu(III), and the formation of the hydroxylating oxygen intermediate requires at least one more electron to lower the kinetic barrier for heterolytic O−O cleavage. The third electron can be supplied by the remaining copper ion in the cluster and retained after each turnover. Thus, the copper cluster is in effect one-electron-reduced after each turnover. If one assumes that all copper ions exhibit similar reactivity toward dioxygen and all clusters react with O_2 at the same time, 1/3 of the total membrane-bound copper should remain reduced in the synchronized turnover samples. The level of reduction observed in synchronized turnover experiments by exposing fully-reduced preparations to dioxygen is not in agreement with this expectation. On the basis of the X-ray absorption edge data, we must conclude that ca. ∼50% of the copper clusters are not directly participating in dioxygen chemistry. While the formation of an "activated-oxygen" species is presumed to be a necessity, the structural requirements to generate such a species are not well understood. Recent reports have shown that dioxygen cleavage and aliphatic hydroxylation can proceed in binuclear-copper model systems with varying rates and efficiencies depending on the conditions of solvent, temperature, and copper ligand identity.[54] In such model systems, O−O bond homolytic cleavage is thought to be the case, implying the generation of oxygen- and substrate-based radicals in the process of hydroxylation. Recently, pMMO-mediated hydroxylation of chiral ethane was shown to proceed with 100% retention of substrate configuration; hence, a radical mechanism is unlikely.[35] This result implies that model chemistry is insufficient to explain pMMO-mediated hydroxylation. While it is unlikely, it may prove neccessary to invoke exotic species such as high-valent Cu(III) ions to account for the unusual stereospecificity of the pMMO.

The existence of a significant level of cuprous ions in the pMMO indicates that the enzyme may buffer itself with a reservoir of endogenous reducing equivalents. This possibility is intriguing. On the basis of the reduction levels in several as-isolated preparations, the pMMO system is indeed very rich in electrons. Since Cu(I) ions can readily form dioxygen adducts with O_2, their function as site(s) of dioxygen activation is certain. However, with an *excess* of Cu(I) ions as observed, these copper ions can function as an additional source of reducing equivalents, particularly under scenarios when the hydrocarbon cosubstrate is not available, or when the electron pressure is low. Effectively, these additional copper ions serve in the capacity of electron transfer. These Cu(I) ions can channel electrons to dioxygen-reduction sites and reduce the "activated" oxygen further to water, as is evident in the O_2-turnover experiments. This "undesirable" oxidase function occurs in the absence of hydrocarbon substrates. The built-in oxidase function could be a means of detoxifying the enzyme from highly reactive activated oxygen species. The reduction of dioxygen to water (a four-electron process) requires additional electrons which are provided by these "buffer" Cu(I) ions. These copper ions, which also appear to be arranged in cluster units, constitute another class of copper ions present in the pMMO, apart from the copper ions which directly participate in the dioxygen reduction. The picture that emerges from this analysis is that there are at least two classes of copper ions in the pMMO system. One class of copper clusters (C clusters) functions primarily as the catalytic centers of the enzyme. The other class of copper ions (E clusters) can function in an electron-transfer capacity. These two functions performed by the two classes of copper ions in the pMMO are, however, not necessarily mutually exclusive.

C and E Copper Clusters. A Hypothesis. In a separate report, we will describe isolation and purification of the pMMO as well as the progress we have made to-date toward characterizing the protein.[43] Experiments are also in progress toward clarifying the function of the different possible classes of copper clusters within the pMMO. The level of copper ions in the present X-ray absorption samples is in the range of 150−170 nmol of Cu/mg of protein, which corresponds to 20 ± 3 copper ions per pMMO. The copper content is close to the stoichiometry of ca. 15 coppers that has been determined for the purified protein. The data, taken together, seem most consistent with the following model, which we presently take as a working hypothesis: the pMMO has a stoichiometry of ∼15−20 copper ions, sequestered into 5−7 clusters of 3, which may be further grouped into 2−3 catalytic (C) clusters and 3−4 electron-transfer (E) clusters according to their function (Figure 4). Under normal synchronous turnover in the presence of O_2, this model would predict 27−29% reoxidation of the copper ions to Cu(II). Under prolonged incubation of the as-isolated or fully-reduced preparations with O_2 in the absence of substrate, we expect

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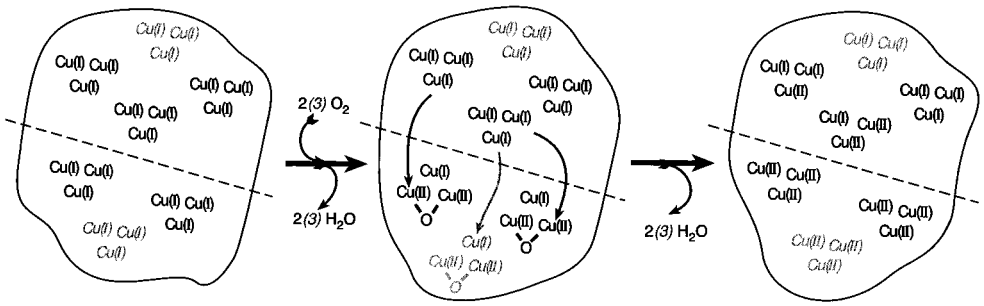

Figure 4. Schematic presentation of the C and E clusters in the pMMO and single turnover in the absence of hydrocarbon substrate, assuming a 15 or 21 Cu stoichiometry per enzyme.

further reduction of the dioxygen intermediate to water with concomitant consumption of two additional reducing equivalents per dioxygen molecule. Such a scenario would result in complete reoxidation of the two (or three) C clusters as well the withdrawal of two (or three) additional reducing equivalents from the E clusters, depending upon the exact copper stoichiometry. Approximately 53−57% of the pMMO copper ions will be reoxidized at this juncture, a prediction that is in accordance with the X-ray absorption edge data as well as the EPR. A final prediction of this model is that the isotropic EPR signal characteristic of the fully-oxidized trinuclear copper cluster begins to manifest itself only at this stage, also in accord with experiment. At the moment, we are still trying to ascertain whether the level of copper found in the purified pMMO preparations obtained to date has been compromised despite the mild purification conditions employed. The copper stoichiometry determined for the purified protein is 25% lower than the copper content of the purified membranes employed in the present study. The outcome of these studies, however, will not affect the C cluster/E cluster hypothesis proposed here; only the number of E clusters and C clusters need ultimately to be revised when the copper content is obtained with greater accuracy.

The source of endogenous reducing power must provide certain advantages for the pMMO system and the organism, since, with this high level of cuprous ions, the so-called nonproductive oxidation is more likely to occur, and if not suppressed, would result in autooxidation of the protein, a waste of reducing equivalents, and, ultimately, enzyme inactivation. In many monooxygenase systems known to date, a redox partner, i.e., a reductase, channels electrons to the dioxygen reducing site(s) embedded in the hydroxylase after each turnover. Normally, the reductase would rescue the inactivated hydroxylase simply by reducing it. Since the as-isolated preparations are often found to be mostly reduced, the enzyme in principle can support turnover on its own up to a certain extent, in the case of interrupted electron flow as in starvation conditions *in vivo*, without the need to interact with its reductase. In fact, we have observed that as-isolated membranes in the absence of reductants yield a burst of oxidation products when exposed to substrates with the concomitant increase in Cu(II) ions levels. This turnover phenomena only lasts ca. 4−15 min in contrast to a sustaining turnover in typical assay conditions which could last anywhere from 30 min to 2 h. The E clusters are thus suggested to be an immediate source of reducing equivalents, providing electrons for turnover under limiting electron pressure. The number of turnovers which the enzyme can sustain in the absence of external NADH offers an estimate of the buffer that is serving as the electron storage. To be an effective buffer, the number of E clusters must be larger than the number of C clusters. This feature appears to be an adaptive strategy employed by the organisms. Methanotrophs often go through extended periods without methane, their only source of carbon and energy.[55] As such, the enzyme can use these endogenous electrons for substrate oxidation once they are exposed to methane again. Hence, having the capability of maintaining a robust viability of the pMMO is clearly a survival plus.

Unresolved Issues. On the basis of pMMO characterization results presented here and elsewhere,[21,37,43] and what is known about related systems,[3,49,50] there are several issues yet to be resolved conclusively.

First, we need to fine-tune the mechanism of alkane hydroxylation mediated by the pMMO. Specifically, we need to work out the exact sequence of the steps in the turnover cycle. It is possible that the pMMO needs to be reduced before the hydrocarbon substrate can bind. Presumably dioxygen then binds to the active sites and becomes activated, and hydroxylation of the substrate ensues. The exact order has yet to be elucidated, however. In the absence of substrate, the fully-reduced enzyme still can activate dioxygen. However, this oxidation is unproductive, since it results only in water ultimately. Hence, this route is suppressed by the enzyme. The mechanism for suppression is not clear at the moment, although it must involve control of the electron flow, considering the fact that the system is so rich in reducing equivalents. Enhancing the enzyme substrate-binding affinity or designing a system which activates dioxygen only after hydrocarbon substrate-binding would avert the unproductive oxidation process. The existence of a large number of cuprous ions (up to 80%) as observed here, even under O_2-saturated isolating conditions, suggests that the enzyme may have a preference to bind substrate before dioxygen activation. In other words, there is only limited dioxygen activation without bound hydrocarbon substrate(s), and the enzyme remains mostly reduced. Nevertheless, the reduced enzyme can still react with dioxygen and generate the active oxygen intermediate in the absence of hydrocarbon substrates. This oxygen species is expected to decay rapidly. On the time scale of the synchronized turnover experiments (typical 5 min or more), one often observes an EPR spectrum associated with the resting form(s), namely, the four-line EPR spectrum of magnetically isolated or weakly coupled copper ions in pseudo-square-planar coordination. EPR signals suggestive of transient species have also been detected in these synchronized turnover experiments.

Second, aside from obtaining a more accurate number for the copper stoichiometry, we need to determine the structures of the "so-called" C clusters as well as those of the involved intermediates. In light of recent stereochemical analysis of the chiral ethanol products derived from pMMO-catalyzed hydrox-

(55) (a) In their natural habitat, obligate methanotrophs own their precarious existence to only methane as the source of carbon and energy. However, their ability to form resting bodies (exospores and cysts) enhances their survival in adverse conditions. These resting stages confer resistance to desiccation and heat, and survival in the absence of methane for weeks if not months. (b) Whittenbury, R.; Davies, S. L.; Davey, J. F. *J. Gen. Microbiol.* **1970**, *61*, 205−218.

ylation of chiral ethane, a concerted mechanism with the possible involvement of exotic species such as Cu(III) ions must also be entertained. Also, we surmise that the different copper ions constituting the C clusters may have different functions: two of the copper ions are likely to be responsible for dioxygen binding, while the third carries the additional electron needed to lower the activation barrier for heterolytic O−O bond cleavage and to generate the hydroxylating agent or to stabilize the high oxidation state copper intermediate. With this scenario, we expect structural inequivalence among the copper ions within the C clusters, including different ligand coordinations and structures. This implies that the copper cluster geometry must deviate from the symmetric triad assumed earlier. Finally, the role of the so-called E clusters needs to be confirmed. Experiments must also be designed to ascertain their ligand structures and geometry, as well as spatial distribution *vis-à-vis* the C clusters.

Conclusions

The existence of both Cu(II) and Cu(I) in the native pMMO membranes has provided significant insights into certain intricate features of the pMMO. Different preparations have been found to exhibit different levels of reduction. This heterogeneity at times gave rise to seemingly unpredictable results. In this work, we have devised methods to obtain preparations that are more homogeneous with respect to the redox states of the copper ions. The treatment of the as-isolated membranes with a strong oxidant (potassium ferricyanide) at physiological pH (∼7.2) has resulted in a preparation in which the membrane-bound copper ions can be maintained in a divalent state without dissociating these copper ions from the pMMO complex. This preparation has allowed the direct observation of the copper cluster EPR signal. Using dithionite (as well as certain organic dyes) as reductant, we have also obtained preparations wherein all the copper ions are reduced, as evidenced by X-ray absorption edge spectrsocopy. The fully-reduced protein has been used to synchronize the enzyme turnover in the presence of dioxygen. A detailed X-ray absorption edge study of this turnover has led us to conclude that the copper ions are possibly arranged into catalytic (C) and electron-transfer (E) clusters.

Results obtained so far in the characterization of the pMMO paint an often confusing picture regarding the enzyme, yet it is clear that the present combined X-ray and EPR spectroscopic study has laid certain aspects of the problem in order. On the other hand, it is evident that we have reached that stage of the problem where every experiment is beginning to raise more questions than it is providing concrete answers. With the recent progress toward isolation and purification of the enzyme[43] as well as cloning of the genes for its polypeptides,[43a,56] a more detailed understanding of this important enzyme should be on the horizon.

Acknowledgment. This work was supported by grants from NSF [CHE-9423181 (K.O.H.)] and NIH [GM22432 (S.I.C.) and RR-01209 (K.O.H.)]. The X-ray absorption data were collected at SSRL, which is supported by U.S. Department of Energy, Office of Basic Energy Sciences, Divisions of Chemical and Materials Sciences, with further support provided by the National Institutes of Health, National Center for Research Resources, Biomedical Resource Technology Program, and the Department of Energy, Office of Health and Environmental Research. H.-H T. N. is the recipient of a W. R. Grace fellowship and a National Research Service Predoctoral Award. We also thank Dr. John H.-K. Yip for helpful discussions and Dr. Peter Green for assistance with the ICP-MS analysis.

JA961778G

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