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The metal centres of particulate methane mono-oxygenase

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Abstract

pMMO (particulate methane mono-oxygenase) is an integral membrane metalloenzyme that catalyses the oxidation of methane to methanol. The pMMO metal active site has not been identified, precluding detailed investigation of the reaction mechanism. Models for the metal centres proposed by various research groups have evolved as crystallographic and spectroscopic data have become available. The present review traces the evolution of these active-site models before and after the 2005 *Methylococcus capsulatus* (Bath) pMMO crystal structure determination.

Keywords

copper; crystal structure; iron; metalloenzyme; methane mono-oxygenase; methane oxidation

Introduction

pMMO (particulate methane mono-oxygenase) is an integral membrane metalloenzyme that oxidizes methane to methanol in methanotrophic bacteria [1]. pMMO is a trimer, with three copies each of three subunits: pmoB or α (~47 kDa), pmoA or β (~24 kDa) and pmoC or γ (~22 kDa) [2]. The trimeric polypeptide arrangement is observed in both X-ray crystallographic [3] and cryoelectron microscopic [4] structures. All methanotrophs utilize pMMO; a few strains can also express a sMMO (soluble methane monooxygenase) under conditions of copper starvation [5]. The active site of sMMO is a carboxylate-bridged di-iron centre and the catalytic cycle has been studied extensively [6]. In contrast, the active site of pMMO remains unidentified, with significant controversy pervading the field over the last decade [7,8]. In the present review, the evolution of current models for the pMMO metal centres is traced in the context of crystallographic and spectroscopic data.

The pMMO metal centres in 2004 (pre-crystal structure)

Before the crystal structure determination of *Methylococcus capsulatus* (Bath) pMMO, several laboratories proposed models for the metal centres on the basis of metal analysis and spectroscopic data [9]. For purified *M. capsulatus* (Bath) pMMO, copper contents per 100 kDa $\alpha\beta\gamma$ protomer of 15–20 (Chan laboratory) [10,11], eight to ten (DiSpirito laboratory) [12], two to three (Rosenzweig laboratory) [13] and two (Dalton laboratory) [14] were reported. Most, but not all [11], preparations also contained 0.75 to two iron ions per protomer. Chan and co-workers proposed that the large number of copper ions were arranged in five to seven trinuclear copper centres. These clusters were divided into two

functional groups, the C and E clusters, of which the C clusters represent the catalytic site and the E clusters provide reducing equivalents. The primary evidence for these clusters was the interpretation of a broad isotropic EPR signal at $g \sim 2.1$ [15–18]. Although DiSpirito and co-workers also reported a high copper stoichiometry [12,19], much of the copper in their preparation was associated with the copper chelator methanobactin [20,21]. Their active-site model thus included copper, iron and methanobactin [19].

In contrast with the Chan laboratory's EPR report, purified *M. capsulatus* (Bath) pMMO samples from the other groups as well as whole-cell or membrane-bound preparations from different organisms gave EPR spectra with a type 2 Cu(II) signal [12–14,19,22–25]. None of these exhibited the signal attributed to the trinuclear copper centre, and Antholine and co-workers have suggested alternative interpretations of that spectrum [23,26]. The type 2 signal corresponds to only part of the total copper [13,14], consistent with our XANES (X-ray absorption near-edge spectrum) data of *M. capsulatus* (Bath) pMMO showing a mixture of Cu(I) and Cu(II) [13,27]. According to our EXAFS data, *M. capsulatus* (Bath) pMMO contains a copper cluster with a short Cu–Cu distance of 2.51 Å (1 Å = 0.1 nm) that increases to 2.65 Å upon chemical reduction with dithionite [13,27]. This finding was the first direct evidence for a copper-containing cluster in pMMO and influenced interpretation of the crystal structure (see below). Combining the EPR and XAS (X-ray absorption spectroscopy) data, we proposed that pMMO contained a mononuclear type 2 Cu(II) centre and some type of copper cluster [9,13]. Because our spectroscopic data suggested the presence of contaminating haem [13,27], we did not include iron in the model.

The metal centres in the *M. capsulatus* (Bath) pMMO structure

We determined the 2.8 Å resolution crystal structure of *M. capsulatus* (Bath) pMMO in 2005 [3,28]. Two copper centres were modelled in the structure, both in the soluble regions of the pmoB subunit (Figure 1). The first site is mononuclear with the copper ion co-ordinated by His⁴⁸ and His⁷², and a glutamine residue, Gln⁴⁰⁴, positioned nearby. The second site was assigned as dinuclear based on the electron density and the EXAFS data (see above). Three strictly conserved histidine residues, His³³, His¹³⁷ and His¹³⁹ are within co-ordinating distance of two nitrogens, including the N-terminal group of His³³, apparently ligated to each copper ion. The detection of two to four oxygen/nitrogen ligands by EXAFS [27] suggests that solvent ligands may be present at the copper sites, but were not detectable at 2.8 Å resolution. A third metal centre identified in the structure was occupied by zinc from the crystallization buffer. This 'zinc' site is within the membrane and is co-ordinated by conserved residues Asp¹⁵⁶, His¹⁶⁰ and His¹⁷³ from pmoC as well as Glu¹⁹⁵ from pmoA (Figure 1A). Since zinc is not found in purified pMMO, this site is probably occupied by another metal ion *in vivo*. We also noted the possibility that the histidine and carboxy residues in this location could accommodate a di-iron centre akin to that in sMMO. Given that the multiple trinuclear copper centres proposed by Chan and co-workers [15–18] were not observed, the possibility of depleted metal centres was also considered, and we identified a conserved patch of hydrophilic residues within the pmoA and pmoC subunits as a possible metal-binding site. This region houses many potential ligands, including His³⁸, Met⁴², Asp⁴⁷, Asp⁴⁹ and Glu¹⁰⁰ from pmoA and Glu¹⁵⁴ from pmoC (Figure 1B). Because no recombinant expression system has been developed for pMMO, the functional relevance of the metal sites in the crystal structure has not been investigated by mutagenesis.

The pMMO metal centres in 2008 (post-crystal structure)

Since the publication of the *M. capsulatus* (Bath) crystal structure, additional spectroscopic and crystallographic studies have been conducted. The interpretation of these data has been shaped by the crystal structure, and has led to models of the metal centres that differ from

those put forth in 2004. The Chan laboratory reported new EPR redox potentiometric data in 2007 [29]. In this study, the type 2 Cu(II) signal and the signal attributed to the tricopper centre were resolved with the type 2 signal disappearing at a potential of -121.3 mV, leaving a signal at $g = 2.05$ assigned to a Cu(II)Cu(II)Cu(II) cluster. This type of cluster was then modelled into the conserved hydrophilic patch noted in the crystal structure [3] (Figure 1B) with Glu¹⁵⁴ and His³⁸ as ligands to the first Cu(II) ion, Met⁴² and Asp⁴⁷ co-ordinated to the second Cu(II) ion, and Asp⁴⁹ and Glu¹⁰⁰ co-ordinated to the third Cu(II) ion. In the model, an oxygen atom bridges the three copper ions (Figure 2A). If this modelled cluster represents the active site, the remaining nine to twelve copper ions in the Chan preparation still need to be accounted for. In another 2007 paper, the Chan group reported binding of ten Cu(I) ions to the C-terminal cupredoxin-like domain of pmoB expressed in *Escherichia coli* [30] (Figure 2B). The Cu(I) ions bind co-operatively with micromolar affinity and are needed for proper folding according to CD data. It should be noted, however, that this domain is well folded in the crystal structure and binds no metal ions [3] (Figure 1C). These Cu(I) ions are proposed to constitute the E clusters, but their nuclearity and whether sufficient ligands are present were not elaborated [30].

Also in 2007, the DiSpirito and Münck laboratories proposed a new model based on Mössbauer spectroscopic data [31]. pMMO purified from cells grown with ⁵⁷Fe exhibits a Mössbauer spectrum with two doublets. The first doublet, also present in whole-cell samples, has $\Delta E_Q(1) = 1.05$ mm/s and $\delta(1) = 0.5$ mm/s, parameters similar to those measured for the di-iron(III) centre in sMMO [32,33]. The second doublet has $\Delta E_Q(2) = 2.65$ mm/s and $\delta(2) = 1.25$ mm/s, parameters characteristic of high-spin Fe(II). The first doublet, attributed to a di-iron(III) centre, corresponds to $\sim 10\%$ of the iron present in purified pMMO. Since the activity of pMMO in whole cells is approx. 10-fold greater than that of the purified sample, DiSpirito and Münck proposed that the diiron centre detected by Mössbauer is the pMMO active site [31]. This di-iron centre was suggested to reside at the crystallographic 'zinc' site (Figures 1A and 3). Multiple controls indicated that the samples were free from sMMO, eliminating contamination as an explanation for the spectrum. However, another possible source of this signal is the di-iron centre in *M. capsulatus* (Bath) haemerythrin, which is up-regulated under pMMO-producing conditions [34,35].

The recent characterization of *Methylosinus trichosporium* OB3b pMMO has added new considerations to these models [36]. This enzyme contains approximately two copper ions per 100 kDa protomer, consistent with previous reports [37], and no detectable iron. The EPR parameters are indicative of type 2 Cu(II) present as two distinct species. Similarly to *M. capsulatus* (Bath) pMMO, the XANES data show a mixture of Cu(I) and Cu(II), and the EXAFS data were best-fitted with a short Cu–Cu interaction at 2.51 Å. The crystal structure of this pMMO was determined to 3.9 Å resolution, and analysis of anomalous data collected at different energies provides several key insights into the metal centres. First, the dinuclear copper centre is present in the same location as in the *M. capsulatus* (Bath) structure. Secondly, the mononuclear copper centre is not observed, which is not surprising since one of its ligands in *M. capsulatus* (Bath) pMMO, His⁴⁸, is not conserved and is replaced with asparagine. Thus it seems likely that the dicopper centre plays an important functional role in pMMO, whereas the mononuclear copper centre is not critical. Thirdly, the *M. capsulatus* (Bath) 'zinc' site is occupied by copper in *M. trichosporium* OB3b pMMO, which was not crystallized in the presence of zinc. Therefore, if a di-iron centre does indeed reside here, it can be displaced by zinc and copper. There is precedent for adventitious zinc occupying di-iron centres [38,39]. Alternatively, the site may bind copper *in vivo*. Finally, no additional metal ions were observed in the structure, including in the C-terminal domain of pmoB and the hydrophilic cluster in pmoA and pmoC.

Conclusions

Despite the efforts of several research groups, definitive identification of the pMMO active site has been elusive. Two metal centres are consistently observed by crystallography: a dicopper centre in the pmoB subunit and a site within the membrane that can be occupied by zinc or copper. The latter site has been proposed to house a di-iron centre on the basis of spectroscopic data. Additional sites both in the membrane and in the C-terminal soluble domain of pmoB have been proposed to house multiple copper ions. None of the observed or proposed sites has been strongly correlated with activity nor has any evidence of substrate or product binding at a specific location been obtained. Such experiments will be critical to resolving the pMMO controversy.

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Abbreviations used

pMMO	particulate methane mono-oxygenase
sMMO	soluble methane mono-oxygenase
XANES	X-ray absorption near-edge spectrum

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Figure 1. Observed and proposed metal-binding sites in the crystal structure of *M. capsulatus* (Bath) pMMO (PDB code 1YEW)

Only one protomer is shown with pmoB in magenta, pmoA in blue, and pmoC in green. Copper ions are shown as cyan spheres, and the zinc ion is shown as a grey sphere. Ligands to the copper centres are labelled. (A) The zinc site and surrounding residues. (B) The hydrophilic patch comprising potential metal-binding residues. (C) The C-terminal cupredoxin-like domain of pmoB.

Figure 2. Proposed models for the pMMO copper centres

(A) Trinuclear copper centre modelled into the hydrophilic patch shown in Figure 1(B).

PDB code 1YEW was used to generate the Figure. (B) Ten copper ions are proposed to bind to the pmoB C-terminal domain shown in Figure 1(C).

Figure 3. Possible location of a di-iron centre in pMMO

The region shown corresponds to Figure 1(A). PDB code 1YEW was used to generate the Figure.