The iron form of methane mono-oxygenase and its mode of action

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All bacteria that grow at the expense of methane as their sole carbon and energy source elaborate a complex enzyme system for conversion of the hydrocarbon substrate into methanol in the presence of NADH and dioxygen [1]:

\[
\text{CH}_4 + \text{NADH} + \text{H}^+ + \text{O}_2 \rightarrow \text{CH}_3\text{OH} + \text{NAD}^+ + \text{H}_2\text{O}
\]

Which type of methane mono-oxygenase (MMO) system is formed depends on the concentration of copper ions in the environment [2]. At high Cu/biomass ratios a membrane-associated enzyme (pMMO) predominates which appears to involve copper at its active site. At low Cu/biomass ratios a soluble enzyme (sMMO) is formed which has been well characterized, contains non-haem iron as its active-site metal and is the subject of this paper. The best-characterized enzymes are from *Methylococcus capsulatus* strain Bath [2–5] and *Methylosinus trichosporium* OB3b [6,7]. Although fundamentally similar with respect to their physical features, there are subtle mechanistic differences between them which still need to be resolved. The sMMO complex comprises three proteins: a 38.6 kDa reductase (Fe$_2$S$_2$; FAD-containing) which interacts with NADH to relay electrons to the 250 kDa hydroxylase, which contains a p-0-bridged

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di-iron centre and provides the binding site to bring both methane and dioxygen together to form methanol; the third protein (protein B) contains no prosthetic groups and has been reported as having a molecular mass varying between 15 and 30 kDa. It is an important regulatory protein for the whole complex and has been implicated in regulating the rate of the reaction, the redox potential of the hydroxylase, regioselectivity of substrate hydroxylation, coupling of NADH consumption to substrate oxidation and various spectroscopic features of the hydroxylase [8].

Earlier studies on the interaction of the three protein components had revealed that the hydroxylase component contained a binuclear iron centre that could exist in three oxidation states (Fe$^{II}$Fe$^{III}$[H$_{ox}$]; Fe$^{II}$Fe$^{II}$[H$_{red}$] and Fe$^{II}$Fe$^{III}$[H$_{red}$]) [9–12]. The H$_{ox}$ species was EPR-silent as the result of antiferromagnetic coupling between the two Fe(III) atoms, which were bridged by a μ-hydroxo species rather than the μ-oxo-bridge, as seen in haemerythrin and ribonucleotide reductase. The mixed-valent state (H$_{red}$) produced by one electron reduction was now EPR-active giving a rhombic EPR signal with g values less than 2.0. Complete reduction to the Fe(II) Fe(II) state gave rise to an EPR signal at g ≈ 14 arising from a ferromagnetically coupled spin system that appeared to have lost the Fe–Fe backscattering (as seen in EXAFS), suggesting that the oxygen bridge between the iron atoms may have broken or that the hydroxo bridge has been converted into a water bridge.

The catalytic cycle in *Mc. capsulatus* was shown to involve all three components and it was suggested that the di-iron centre of the hydroxylase underwent a series of redox changes to facilitate binding and activation of dioxygen to the high-valent ferryl species ultimately responsible for the electrophilic abstraction of a hydrogen atom from methane to produce a methyl radical. The resultant radical would then interact with an iron-bound hydroxyl species to generate methanol (Figure 1) [13,14]. In *Ms. trichosporium* a similar cycle has been proposed, although the presence of the regulatory protein B was not considered essential, since chemically reduced hydroxylase was able to undergo a single turnover on addition of methane and O$_2$ [6]. Addition of protein B would, however, accelerate the initial velocity of the reaction by up to 150-fold [15,16]. In the cycle as shown in Figure 1 the generation of the metal-bound oxene (ferryl) occurs through
the addition of two reducing equivalents and O<sub>2</sub> to the oxidized hydroxylase. In the case of cytochrome P-450, in which a similar intermediate has been proposed, it is also known that the two reducing equivalents and O<sub>2</sub> can be replaced by H<sub>2</sub>O<sub>2</sub> in the well-known 'peroxide shunt' pathway [17]. The same is true for MMO in which the NADH<sub>2</sub>, O<sub>2</sub>, protein B and reductase can be substituted by H<sub>2</sub>O<sub>2</sub> [18,19]. The rates of hydrocarbon oxidation by the peroxide-driven system are only about 10% of those observed for the complete system, and the product distribution from substrates in which primary, secondary and tertiary carbons are available for attack differed in the two systems. Attack at primary carbon atoms was significantly reduced in the peroxide-driven system, suggesting that a weaker electrophilic species may be involved when the peroxide shunt is used. Certainly the distribution of products from the oxidation of propane or 2-methylbutane are reminiscent of attack by the relatively weaker 'OH species which would be formed by homolytic O−O bond cleavage. However, 2-methylpropane oxidation [20] by the two systems gave identical product distributions, so it is possible that both homolytic and heterolytic O−O bond cleavage could occur. Indeed we have proposed that multiple pathways are involved in substrate oxidation [20]. The type of mechanism used depends on the nature of the substrate and oxidizing species. In the case of trans-2-butene oxidation by the complete sMMO system, 68% alcohol and 32% epoxide were given. With the peroxide-driven hydroxylase only 3% of the alcohol was formed and 97% of the epoxide was formed. Thus it is possible that the weaker 'OH would have greater difficulty in abstracting H from RCH<sub>2</sub>-H (435 kJ/mol (R = H); 377 kJ/mol (R = alkene)) than breaking the −CH=CH− bond in 2-butene (π bond = 293 kJ/mol) and producing an epoxide by direct oxygen insertion. We have also observed that the nature of the hydrocarbon substrate can also affect the mechanism. The oxidation of carbon monoxide and pyridine by the complete sMMO system gives carbon dioxide and pyridine N-oxide respectively as products, and yet these were the only two substrates from the dozen tested for which we were unable to detect any carbon-centred radical intermediates [14]. We have suggested that these compounds are oxidized via direct oxygen insertion and not via hydrogen atom abstraction [20]. It is possible that, for alkenes at least, this may be the preferred route since they are extremely good MMO substrates but give very weak EPR signals in the radical-trapping experiments.

In contrast with the results obtained with the complete sMMO system, which required protein B for maximum activity [5], the peroxide shunt system was progressively inhibited by increasing amounts of protein B until complete inhibition was observed at a ratio of protein B/hydroxylase of 2:1 [19]. These effects for *M. capsulatus* were also observed in the *Ms. trichosporium* system, which showed a shift in product distribution ratio as the amount of protein B was increased [18]. In the absence of protein B, there was a lower preference for primary carbon hydroxylation than in its presence. Since one would expect the same high-valent iron oxene species to be present irrespective of the oxygen donor (and this is essentially true whatever H<sub>2</sub>O<sub>2</sub> or O<sub>2</sub> is used, as judged by the qualitative nature of the products), then the only reason for differing product distributions must reside in the interaction with protein B. Complex explanations for these data with H<sub>2</sub>O<sub>2</sub>, the complete system and the single-turnover system have been advanced [8,21] suggesting that protein B binds to the hydroxylase to alter the structure of the active site and thereby alter the way substrates approach the active oxygen species. Such explanations, however, need substantiating by experimental fact and currently there are too few data (either structural or dynamic) on the interaction between the hydroxylase and protein B to give a meaningful analysis.

Several of the proposed intermediates in the sMMO catalytic cycle have now been characterized using rapid-scan stopped-flow rapid-quench Mössbauer spectroscopy and Raman spectroscopy techniques [8,22]. The diferrous oxygen adduct (Fe<sup>II</sup>Fe<sup>III</sup>O<sub>2</sub>) has not been detected and its rate of formation (>10<sup>7</sup> M<sup>−1</sup>s<sup>−1</sup>) is probably diffusion controlled. The next intermediate in the cycle (peroxo, P) (Figure 2), which is the result of electron transfer from Fe<sup>III</sup> to O<sub>2</sub>, has been identified in the *M. capsulatus* enzyme. The physical characteristics of the peroxy complex are shown in Table 1. The Mössbauer spectrum exhibits a single symmetric doublet, and an isotope-sensitive (O<sup>16</sup>−O<sup>18</sup>) Raman band at 905 cm<sup>−1</sup> suggests that the peroxy ion is bound to iron (one or both) through both oxygen atoms. Possible structures for the peroxy complex are shown in Figure 3. The high-valent iron oxo intermediate (Q) is formed after heterolytic cleavage of the O−O bond in 2-butene.
This species has been detected in both the *Mc. capsulatus* and *Ms. trichosporium* enzyme systems, and its UV–vis and Mössbauer spectral characteristics are shown in Table 1. Two Mössbauer doublets are observed for the *Mc. capsulatus* intermediate Q, indicating inequivalent environments for the two iron atoms. In contrast, a single doublet is seen in the *Ms. trichosporium* Mössbauer, indicating equivalent strongly coupled iron atoms and a symmetrically bound activated oxygen species. Likely structures for intermediate Q are presented in Figure 3. The substrate radicals resulting from hydrogen atom abstraction by the iron–oxo intermediate have been identified in spin-trapping experiments, in which the individual radical adducts were identified by their characteristic EPR spectra [14]. An enzyme product complex (T) was seen in the *Ms. trichosporium*, but not the *Mc. capsulatus*, enzyme system using p-nitrobenzene as substrate [23]. The rates of formation and decay of several of the intermediates are included in Table 1 and some of these have been shown to be substrate-dependent. The rate-determining step in the catalytic cycle is almost certainly release of product. In all of these experiments, observation of intermediates P, Q and T required the presence of two equivalents of protein B. Protein B has also been implicated in the redox reactions of the hydroxylase. Here again there have been different values recorded for the two bacterial systems. The $E'_{1/2}$ values for the two redox couples (versus normal hydrogen electrode) have relied upon EPR and Mössbauer spectrosopies to determine the relative amounts of the mixed-valent and fully reduced hydroxylase species in the presence of redox-active mediators. The values for the couples:

**Figure 2**
Rate constants for intermediates in MMO cycle during a single turnover at 4°C

Adapted from refs. [8,22–24].

**Table 1**
Characterization of peroxo and high-valent iron–oxo intermediates

The data for *Ms. trichosporium* were taken from refs. [8] and [23] and those for *Mc. capsulatus* from refs. [22] and [24].

<table>
<thead>
<tr>
<th></th>
<th><em>Ms. trichosporium</em></th>
<th><em>Mc. capsulatus</em> (Bath)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(OB3b)</td>
<td>H-peroxo (P)</td>
</tr>
<tr>
<td></td>
<td>Fe$_{ox}^{IV}$ (Q)</td>
<td>Fe$_{ox}^{IV}$ (Q)</td>
</tr>
<tr>
<td>UV–visible</td>
<td>$A_{1320} \sim 7500$</td>
<td>$A_{1320} \sim 7500$</td>
</tr>
<tr>
<td></td>
<td>$A_{420} \sim 7500$</td>
<td>$A_{420} \sim 7500$</td>
</tr>
<tr>
<td>$A_{420} \sim 1500$</td>
<td>$A_{420} \sim 1500$</td>
<td></td>
</tr>
<tr>
<td>$A_{320} \sim 3600$</td>
<td>$A_{320} \sim 3600$</td>
<td></td>
</tr>
<tr>
<td>$A_{320} \sim 7200$</td>
<td>$A_{320} \sim 7200$</td>
<td></td>
</tr>
<tr>
<td>$A_{320} \sim 1400$</td>
<td>$A_{320} \sim 1400$</td>
<td></td>
</tr>
<tr>
<td>Mössbauer</td>
<td>$\delta_1$ (mm·s$^{-1}$)</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>$\Delta E_Q$ (mm·s$^{-1}$)</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>$\delta$ (mm·s$^{-1}$)</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td>$\Delta E_Q$ (mm·s$^{-1}$)</td>
<td>0.55</td>
</tr>
<tr>
<td>Raman (cm$^{-1}$)</td>
<td></td>
<td>905(O$^{16,18}$ stretch)</td>
</tr>
<tr>
<td>Rate of formation</td>
<td>$4^\circ C + 2B$ (s$^{-1}$)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>$4^\circ C + 2B$ (s$^{-1}$)</td>
<td>25</td>
</tr>
<tr>
<td>Rate of decay</td>
<td>$4^\circ C + 2B$ (s$^{-1}$)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>$4^\circ C + 2B$ (s$^{-1}$)</td>
<td>0.45</td>
</tr>
</tbody>
</table>
Fe(III)Fe(III) $\rightarrow$ Fe(II)Fe(III) $\rightarrow$ Fe(II)Fe(II) $\rightarrow$ Fe(II)Fe(II)

$^{H_{0x}}$  $^{H_{302}}$  $^{H_{red}}$

are given in summary form in Table 2.

Criticisms of these data [25] include the fact that some mediators bind to the hydroxylase to change their spectroscopic properties or that they have been studied at temperatures vastly different from those at which the redox reactions were undertaken [27]. To overcome these objections we have undertaken direct electrochemistry using gold electrodes that have been modified with hexapeptides containing cysteine and lysine residues [27]. Such peptides provide a positively charged surface to which the hydroxylase may bind and thus serves as a conduit for electrons. Differential pulse voltammetry produced two waves for the first and second electrons being transferred to the hydroxylase centre at +248 mV ($E^0_2$) and −142 mV ($E^0_3$). Addition of protein B resulted in a shift of both potentials to more negative values. Such an observation was in accord with observations using mediator-assisted electron transfer (see Table 2). This shift to negative values caused by the protein B is consistent with the view that protein B serves to induce a conformational change in the hydroxylase, which is transmitted to the active site. The lowering of the redox potential by protein B would also facilitate binding of dioxygen to the metal centre of the hydroxylase and could therefore serve to regulate the activity of the enzyme system; in the absence of protein B there would be no methane oxidation since $O_2$ would not bind to the hydroxylase. One could therefore postulate that activity of the complex could be regulated through modulation of protein B. We have noted that protein B can exist in two forms: a full-length fully active form (B) and a truncated form that is inactive and has lost 12 amino acid residues from the N-terminus (B’). Separation of these two forms by conventional chromatographic procedures has proved difficult, but, if pure B is left at room temperature, it rapidly degrades to B’ by an autocatalytic mechanism (A. Bhambra and H. Dalton, unpublished work). Since B’ had no effect on altering the redox potential values of the hydroxylase and since it is completely inactive in all enzyme assays (it also binds three times less strongly to the hydroxylase than B), we believe that the cleavage of 12 amino acids from B to form the inactive form is crucial.

The presence of an inactive form of protein B in preparations may demand a reinterpretation.

**Figure 3**

Possible structures for the peroxo complex

Compiled with information from ref. [24].

**Table 2**

Reduction potentials (in mV versus normal hydrogen electrode at pH 7.0) of the hydroxylase from two organisms in the absence and presence of protein B

Data for *M. capsulatus* were taken from refs. [9] and [26] and those for *Ms. trichosporium* from ref. [25].

<table>
<thead>
<tr>
<th></th>
<th>$E^0_0$ (mV)</th>
<th>+ Protein B</th>
<th>$E^0_1$ (mV)</th>
<th>+ Protein B</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. capsulatus</em></td>
<td>+350</td>
<td>−25</td>
<td>+48</td>
<td>−135</td>
</tr>
<tr>
<td></td>
<td>+100</td>
<td>−100</td>
<td>+50</td>
<td>−170</td>
</tr>
<tr>
<td><em>Ms. trichosporium</em></td>
<td>+76</td>
<td>+21</td>
<td>−52</td>
<td>−115</td>
</tr>
</tbody>
</table>
of the kinetic and thermodynamic data in the literature. For example, it has been observed that inhibition of hydroxylase activity occurred when the ratio of protein B/hydroxylase exceeded 2 [15,21]. The inhibitory complex that was assumed to be formed in the kinetic model did not take into account the possible participation of a truncated inactive form of B. An alternative explanation, that protein B was removed by complexation with the reductase, was also offered. Whichever explanation is correct, it is quite clear that protein B has a central role to play in the regulation of methane oxidation through its effect on the O₂-activating hydroxylase.


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