

COMMENTARY

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Mechanism of methane monooxygenase – a structural and quantum chemical perspective

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Abstract The diiron site of methane monooxygenase (MMO) has the unique ability to activate methane. Structural studies of the MMO diiron site have revealed a limited number of coordination sites for dioxygen and dioxygen derived species. Using quantum mechanical studies of the MMO reaction, several possible reaction paths have been investigated. Energetically feasible geometries have been obtained for the different reaction steps, where the substrate activation is best described by an almost pure hydrogen abstraction step, followed by the formation of a metal-carbon bond.

Key-words Methane monooxygenase · C-H activation · Diiron site · Quantum mechanical calculations · Protein structure

The last couple of years have seen great advances in our understanding of the catalytic diiron core of the soluble methane monooxygenase (MMO) and its unique ability to hydroxylate methane [1–3]:



Crystallographic studies have provided a structural framework for discussing the mechanism of this reaction [4–6]. The active site of the enzyme is buried in the core of the protein, where four neighbouring α -helices provide two histidines and four glutamic acids as iron

ligands. On one face of the diiron site a hydrophobic pocket is found, well suited for harbouring methane and other substrates.

From the structural studies both on MMO, and on the structurally related R2 subunit of ribonucleotide reductase (RNR R2), observations have been made which may be of some generality for this type of metal site [7]. One is that several of the glutamates bound to the iron ions have great structural flexibility and can readily change their coordination mode. Also the number of water/hydroxo/oxo ligands coordinated to the site can change between different forms. The site can therefore accommodate significant changes in the iron-iron distance as well as allow the generation of new open coordination sites on the diiron centre where dioxygen and substrates can interact. Even so, the likely coordination sites for oxygen species or substrates/products are limited and localized on the face of the iron site exposed to the hydrophobic pocket (Fig. 1) or in between the two iron ions.

Crystallographic studies on different forms of the MMO type diiron sites has allowed the assignment of plausible protonation states of groups coordinating the diiron site, based on coordination and hydrogen bonding distances. This has led us to suggest that the net charges of all the structurally determined sites so far are zero. The main reason for this is of course the low dielectric constant of the protein core, but it may also be related to the high structural flexibility of the coordination sphere of the diiron site which allows the site to access many different coordination modes, some of which avoid the energetic costs of charge build-up. Based on this “net charge rule” we have suggested that the charge of the site is unlikely to change during the reaction cycle and that electron transfer to the site will be at least thermodynamically coupled with proton transfer. When this rule is applied, several mechanistic suggestions in the literature fail and are therefore, in our opinion, less likely to describe the true situation.

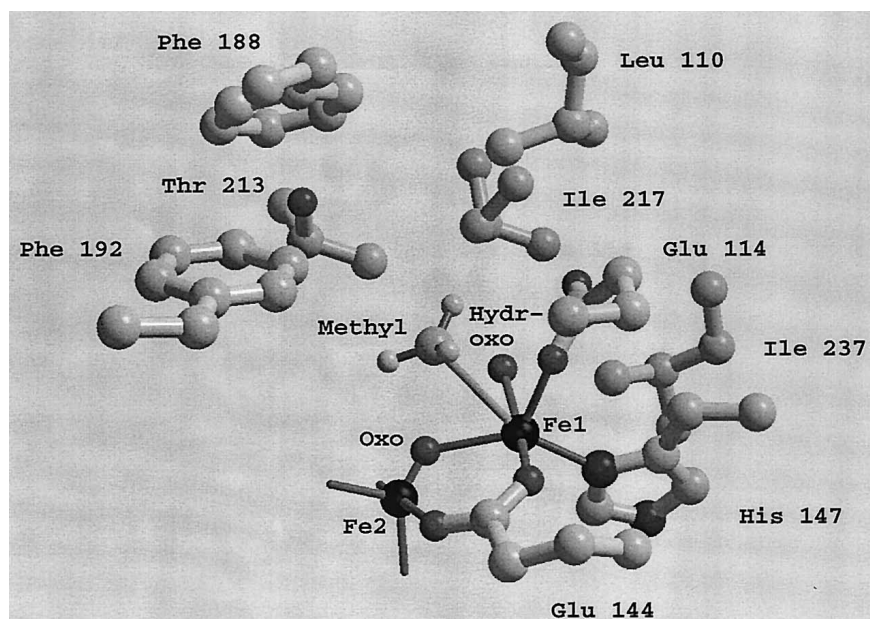
Kinetic and time resolved spectroscopic studies have provided important information on the sequence and

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Fig. 1 The active site of the *Methylococcus capsulatus* methane monooxygenase with the methyl-radical complex **6** in Scheme 1 modelled into the iron core. The pocket residues lining the likely substrate binding site are Phe192, Phe188, Leu110, Ile237, Ile217 and Thr213. Only the hydrogens of the methyl group are shown. The residues coordinating Fe2 are left out for clarity



redox states of reaction intermediates in the MMO catalysed hydroxylation. The proposed reaction intermediates involve an initial hypothetical dioxygen iron complex (compound O) followed by a directly observable peroxide intermediate (compound P). Full reduction of dioxygen can occur in a substrate independent manner and leads to the formation of a formally Fe(IV)-Fe(IV) centre (compound Q). In subsequent steps this species can activate the substrate for the oxygen insertion reaction.

Several different structures have been suggested for the above reaction intermediates and their possible roles in the reaction have been discussed. Although the structures of these intermediates are likely to describe relevant oxidation states, it is not clear to what extent they are actual structures on the true reaction path. The real challenge is now to elucidate the true reaction path of MMO, i.e. to derive the detailed structures of the intermediates in this path, as well as understanding the energetic barriers separating them and to what extent the reaction steps are concerted. Quantum chemical methods have the potential to provide such information. These methods have only very recently achieved the power and reliability necessary for meaningful applications to bioinorganic problems of the size of MMO. We believe that, when used critically, these methods will allow one to distinguish between different mechanistic suggestions for the MMO catalysed chemistry. We have just begun the work to examine the MMO mechanism and instead of discussing several possible mechanisms in this commentary, we will focus on results and features of the best mechanism obtained so far and its relationship to available experimental data.

Only the catalytic iron core of the enzyme can be treated in the quantum chemical calculations and it is not yet possible to treat the full ligand sphere. A quan-

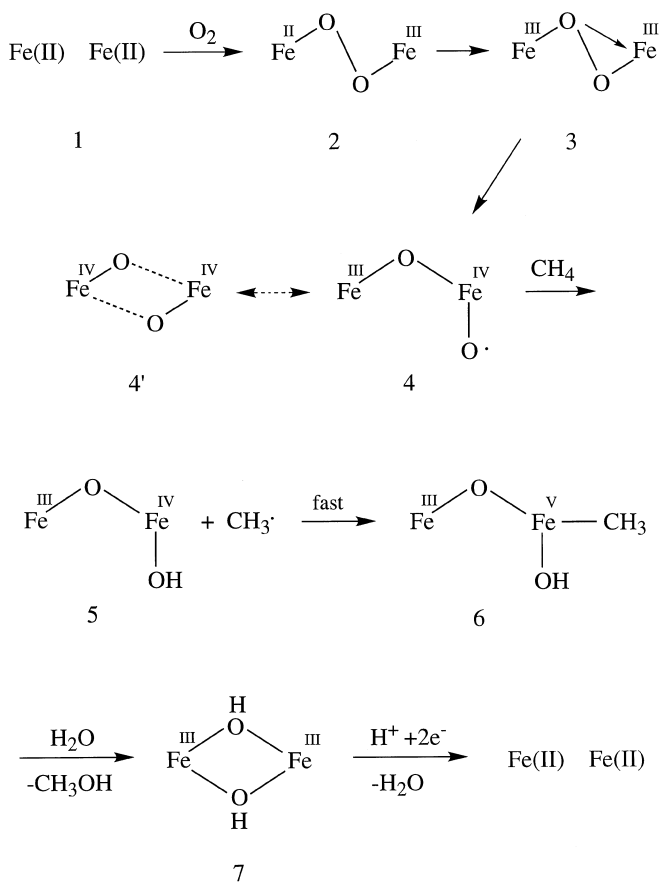
tum chemical model system must initially be chosen in which amino acid ligands present in the enzyme are replaced by simpler groups. The active site of MMO, being buried in the low dielectric medium of the protein core, is probably a particularly well suited system for a quantum chemical calculation in vacuo. Also, the structure of the active site of MMO does not directly suggest any significant involvement of other protein components than the diiron core in catalysing the chemical steps.

In the quantum chemical studies of the MMO mechanism, the hybrid density functional (DFT) method B3LYP [8–11] as implemented in the GAUSSIAN94 program [12] was used. Medium size basis sets (LANL2DZ) were used for the geometry, while very large basis sets [6–311 + G(2d,2p)] were used for the final energies [13]. The energies were corrected for zero-point vibration. In the initial quantum chemical studies, simple ligand models were used. The bridging formate carboxylate group of the actual structure was included, but all other ligands were replaced by hydroxyl and water ligands. This choice of ligands was obviously made partly for economic reasons for this initial study where general trends are the main goal, but the ligands are also chosen based on a vast experience of quantum chemical modelling. The adequacy of the models is mainly demonstrated by the results obtained. The present model is thus able to reproduce the suggested core Fe-(μ -O)₂-Fe structure of compound Q [14] (see below) and is also able to activate methane with a reasonable energy, apart from the obvious fact that the oxidation states are the desired ones.

More recently, better models have been used with formates and imidazoles replacing the actual glutamates and histidines (P. Siegbahn, unpublished work). Several possible reaction intermediates have been energy-optimized and either rejected as being too unstable

to lie on the reaction pathway or retained as having a plausible energy. For key steps, we have tried to locate the transition state structure and so obtain the activation energy for the cycle as a whole.

Scheme 1 shows the series of possible intermediates that emerges from the quantum chemical study and which constitutes likely steps of the mechanism. On this scheme, the $\text{Fe}_2(\text{II},\text{II})$ starting cluster **1** binds and activates O_2 . Although not entirely settled at this moment, the dissociation probably takes place as follows. O_2 first binds in a superoxo $\text{Fe}(\text{III})-(\mu\text{-O}_2)\text{-Fe}(\text{II})$ species **2**. This species has iron spins of 3.97 and 3.53 and is ferromagnetically coupled to a ${}^9\text{A}$ electronic state. Our preliminary investigations indicate that the O-O dissociation (i.e. breakage of the oxygen-oxygen bond) takes place on this spin surface. As the O-O bond dissociates, one of the oxygens is bridge bonded (**3**) but the other oxygen is bound only to the active iron and has a large spin with the opposite direction to the iron spin. The calculation of the barrier for O_2 dissociation is not finished at present but appears to be about 15 kcal/mol using the present model. Once the O-O bond is broken, a surface crossing is proposed to give a key $\text{Fe}(\text{III})\text{-O-Fe}(\text{IV})\text{-O}\cdot$ species **4** with an oxo group having strong



Scheme 1 Schematic representation of the catalytic reaction sequence for the activation of methane by MMO leading to information of methanol, as obtained from quantum chemical calculations

oxy radical character. This oxygen radical species is in equilibrium with an $\text{Fe}_2(\text{IV},\text{IV})$ dioxo species **4'** which we identify as compound Q, the last experimentally observed intermediate before methane oxidation takes place.

EXAFS and Mössbauer data led to a proposal for the structure of MMO compound Q [14]. The cluster model chosen by those workers to interpret the data contains a Jahn-Teller distorted $\text{Fe}(\mu\text{-O})_2\text{Fe}$ with essentially the same metric parameters as given by the quantum chemical studies of structure **4'**. Structure **4'** is also five-coordinate, another feature we did not expect, but which seems to be another consequence of the Jahn-Teller distortion [8].

Structure **4'** shows no tendency to react exothermically with methane, but the equilibrium between this species and the $\text{Fe}(\text{III})\text{-O-Fe}(\text{IV})\text{-O}\cdot$ radical described above shows that it is able to arrange to a more reactive structure. The presence of the Jahn-Teller distortion in compound Q explains why this rearrangement is so simple. The calculations of the barriers for the rearrangement are not yet complete but it appears that the barrier is of the same size as the one for the O-O dissociation. Placing a methane in the vicinity of the oxy radical led to exothermic H atom abstraction to form an $\text{Fe}(\text{IV})\text{-OH}$ group and a methyl radical. This radical is formed close to the active site cluster in a cage formed by the protein and so is never truly free. Rather than give a "rebound" mechanism by abstraction of OH, a route we expected on the basis of analogy with P-450 chemistry, the methyl radical instead showed a tendency to recombine with the $\text{Fe}(\text{IV})$ centre to give structure **6**, having an exceptionally weak Fe-C bond (9.2 kcal/mol). This is a key point because the methyl radical is expected to have a very short lifetime, perhaps accounting for the 65% retention of stereochemistry observed in *Methylosinus trichosporium* MMO for a chiral ethane (MeCHDT) as substrate [15].

This alkyl complex, formed by a formal CH addition across the iron oxo, as suggested by Barton and Dollar [16] for related oxidation chemistry, can now lose methanol to form an $\text{Fe}_2(\text{III},\text{III})$ intermediate. The latter must be reduced to regenerate the active $\text{Fe}_2(\text{II},\text{II})$ form, structure **1**, that reacts with O_2 .

The transition state characterised for the C-H abstractions step, **4-5**, illustrates the almost pure H atom abstraction character of the reaction. This result is consistent with the oxy radical character assigned to the terminal oxo group. The observed intramolecular kinetic isotope effect (KIE) of 4.2 observed for *M. trichosporium* MMO is not too different from the calculated KIE of 8 (298 K) for the quantum chemical transition state structure. Recently, higher KIEs of 9 for CH_2D_2 and 19 for CH_4/CD_4 have been seen by Neisheim and Lipscomb [17]. The tunnelling contribution invoked to explain these numbers was not taken into account in the theoretical calculations.

This pathway helps reconcile some of the apparently inconsistent data on MMO. The short radical lifetimes

indicated by the radical clock probes seem inconsistent with the efficient trapping of methyl radical by nitroxides in *Methylococcus capsulatus* MMO [18]. The Fe(V)-Me intermediate proposed here, with its very weak Fe-C bond, helps reconcile the observations, because such an alkyl could be efficiently trapped by nitroxide.

The structures obtained from the quantum mechanical calculations can readily be modelled into the coordination sphere of MMO with few ambiguities. The only important structural information from the MMO active site imposed on the initial quantum mechanical model was the presence of a carboxylate bridge, and therefore the reasonable fit of the models in the MMO diiron coordination geometry further supports their relevance for understanding the MMO mechanism. The bridging formate group in the quantum mechanical model then corresponds to the bridging Glu144 in the structure of MMO. In this geometry the hydroxylation chemistry will take place on the face of the site exposed in the hydrophobic pocket. The reactive oxygen species will however not be in direct contact with any protein residues. This is an important point since it is not sufficient for a biologically selected catalyst simply to promote the chemistry – it must also protect the protein from premature destruction. We find that the asymmetry in the reactive Fe(III)-O-Fe(IV)-O· structure **4** as well as the Fe(III)-O-Fe(V)(O)(CH₃) complex **6** fits well with the assignment of iron 1 as the site coordinating the reactive oxo group as well as activating methane (Fig. 1). Forming a metal-carbon bond as a mechanism for controlling a reactive substrate intermediate is of course an attractive concept, but still controversial. It requires further theoretical and experimental studies and at this point all imaginable reaction paths for the diiron core of MMO have not been tested and the existence of an even more favourable but as yet unexplored pathway cannot be excluded.

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