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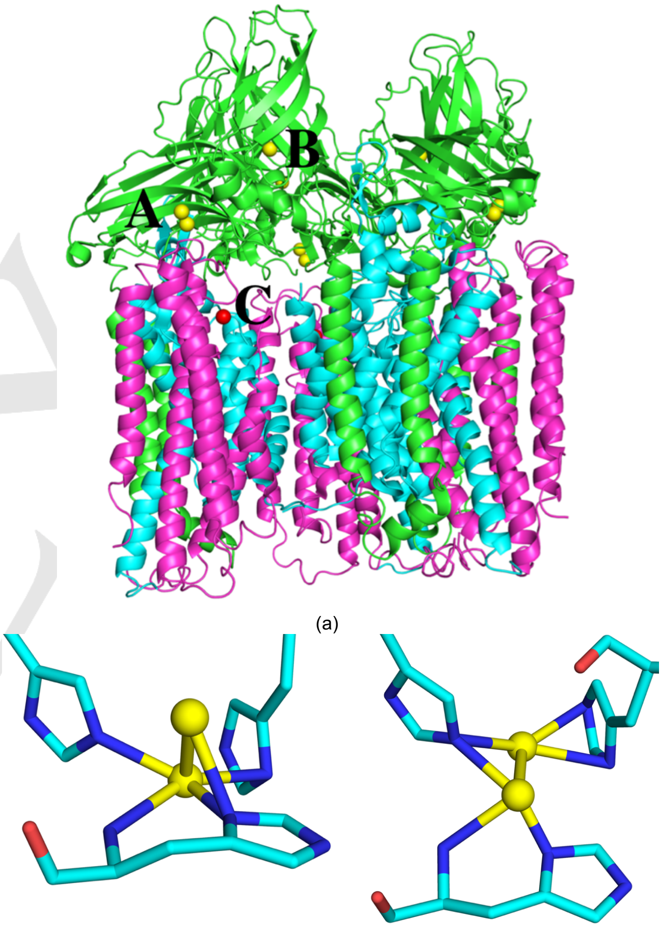
# Quantum refinement does not support dinuclear copper sites in crystal structures of particulate methane monooxygenase

*Lili Cao,[a] Octav Caldararu,[a] Amy C. Rosenzweig[b] and Ulf Ryde*[a]*

***Abstract:*** *Particulate methane monooxygenase (pMMO) is one of the few enzymes that can activate methane. The metal content of this enzyme has been highly controversial with suggestions of a dinuclear Fe site or mono-, di- or trinuclear Cu sites. Crystal structures have shown a mono- or dinuclear Cu site, but the resolution is low and the geometry of the dinuclear sites is unusual. We have employed quantum refinement (i.e. crystallographic refinement enhanced with quantum-mechanical calculations) to improve the structure of the active site. We have compared a number of different mono- and dinuclear geometries, in some cases enhanced with more protein ligands or one or two water molecules to determine which structure fits two sets of crystallographic raw data best. In all cases, the best results are obtained with mononuclear Cu sites, occasionally with an extra water molecule. Thus, we conclude that there is no crystallographic support for a dinuclear Cu site in pMMO.*

Methane is one of the most difficult organic substrates to activate, with a C–H bond dissociation energy of 435 kJ mol$^{-1}$.[1] In biology, there are only two groups of enzymes that can perform this reaction. The first is the soluble methane monooxygenases,[2] which contain a dinuclear Fe active site, similar to that of the ribonucleotide reductases. This is a well-characterised group of enzymes, the reaction mechanism of which is understood in some detail.[2] The second group is the particulate (i.e. membrane-bound) methane monooxygenases (pMMO), which is the predominant enzyme in methanotrophic bacteria, except at copper-limiting conditions.[1,3] The pMMOs have been more difficult to characterise, and the metal content has been highly controversial, involving suggestions of dinuclear Fe centres, as well as mono-, di- and trinuclear Cu sites.[1,4–6]

In 2005, the first crystal structure of a pMMO was published (2.8 Å resolution), showing a heterotrimeric $\alpha_3\beta_3\gamma_3$ structure with a dinuclear and a mononuclear Cu site in the soluble periplasmic parts of the PmoB subunit, and a monomeric Zn site in the PmoC subunit, within the membrane (these three metal sites will be called A, B and C in the following; cf. Figure 1).[7] Site A was suggested to be the active site and involves three histidine residues, which are conserved in all pMMO sequences, except those from one phylum of bacteria.[1] All three residues interact with the metals with their sidechain imidazole group, but one of them is the first residue of PmoB and the amino-terminal N atom also coordinates to one of the metals. This gives a binding site similar to the histidine brace in the lytic polysaccharide monooxygenase enzymes,[8] although that site involves only two His ligands and binds only one Cu. In 2011, a crystal structure was reported at a slightly improved resolution, 2.68 Å.[9] It also showed Cu ions in site A, but it was modelled as mononuclear in two of the protomers and dinuclear in one protomer. In addition, it showed a Zn ion in site C (the Zn ions in these structures derive from the crystallization buffer). Three additional crystal structures of pMMO have been published, showing only a single Cu ion in site A.[10]



(a)

(b)

(c)

**Figure 1.** (a) Trimeric structure of the pMMO (3RGB)[9] with metal sites A–C indicated (Cu ions in yellow, Zn ions in red; PmoA, PmoB and PmoC subunits in magenta, green and cyan, respectively). Geometry of Cu site A in protomers 1 (b) and 2 (c), involving three histidine residues and the amino terminal group. Cu ions are yellow balls, whereas C, N and O are shown in cyan, blue and red sticks, respectively.

Unfortunately, the crystal structures did not settle the location and composition of the pMMO active site. Computational studies have supported site A and have indicated that both mono- and dinuclear Cu sites are reactive enough to oxidise CH$_4$.[3,11] On the other hand, Chan and coworkers have argued that the active site instead consists of three Cu ions, not observed in the crystal structure, but coordinated by residues from PmoA and PmoC.[5] Other groups have instead suggested

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that site C, with its conserved ligands, is a dinuclear Fe active site, similar to that in the soluble methane monooxygenases.[6]

A prime problem with the mechanistic studies of pMMO is the poor resolution of the crystal structures, giving metal sites with unclear composition and unusual geometries (Figures 1b and c). These issues may be addressed by using computational methods. Quantum refinement is an approach to supplement crystallographic refinement with quantum mechanical (QM) calculations for a small but interesting part of the protein.[12] It is standard crystallographic refinement, in which the empirical potential, used to ensure chemically reasonable bond lengths and angles in the structure, is replaced by more accurate QM calculations. Quantum refinement has been shown to locally improve crystal structures and to be able to determine protonation and oxidation states of metal sites.[13,14] Here we apply quantum refinement to two crystal structures of pMMO[9] with the aim of improving the structure of the Cu site A and determining the number of copper ions observed crystallographically.

We started by performing quantum refinement of site A in the first protomer of the 3RGB structure[9] using a dinuclear Cu site. This gave a topology similar to that in the starting crystal structure (Figure 1b), but with a more reasonable geometry. The two Cu ions are on top of each other, with all three imidazole groups directed towards the Cu ions. One of the Cu ions is coordinated by all four N atoms, whereas the other binds to only one or two N atoms (Figure 2). The distance to the amino-terminal N atom is typically longer than that to the imidazole groups. The detailed distances depend on the oxidation states of the two Cu ions, as is shown in Table S1 and discussed in the supplementary material.

The electron-density maps in Figure 2 show that the dinuclear site fits the crystallographic raw data[9] rather poorly. In particular, there is negative difference density around the upper Cu ion, indicating that it is not correctly modelled or that it does not have full occupancy. This is also quantified by the real-space difference density Z-score (RSZD)[15] which is 1.9–2.1 (C2W0 in Table 1). Still, the structures are appreciably better than the starting structure, for which RSZD was 4.7.

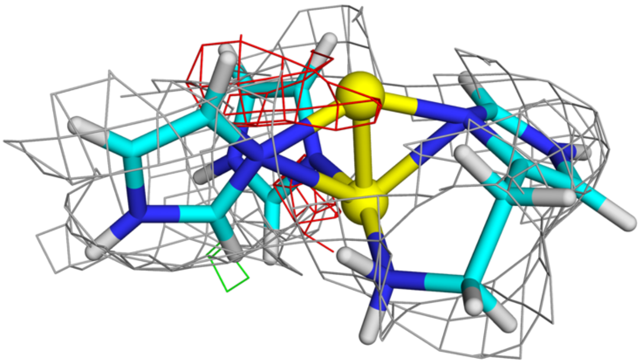


**Figure 2.** QM-refined structure of site A in protomer 1 of the 3RGB structure, modelled by two Cu ions in the fully reduced state, including electron-density maps[9] ($2mF_o - DF_c$ map, contoured at 1.0 $\sigma$, grey, and the $mF_o - DF_c$ maps, contoured at +3.0 $\sigma$, green, and –3.0 $\sigma$, red). Atom colours are the same as in Figure 1 (and H as white sticks).

**Table 1.** RSZD and strain energies ($\Delta E_{str}$, kJ mol$^{-1}$) for the various QM-refined structures in the three protomers (1–3).

| PDB[a] | Structure[b] | State[c] | RSZD | | | $\Delta E_{str}$ | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 1 | 2 | 3 |
| 3RGB | C2W0 | Red | 2.1 | 2.8 | 2.0 | 179 | 195 | 176 |
| | | MV | 1.9 | 2.9 | 2.6 | 224 | 178 | 160 |
| | | Ox | 2.1 | 2.9 | 2.8 | 300 | 247 | 224 |
| | Y2W2 | Red | 3.2 | 2.2 | 3.0 | 153 | 144 | 160 |
| | | MV | 1.5 | 1.9 | 3.2 | 149 | 110 | 104 |
| | | Ox | 1.5 | 2.1 | 2.9 | 158 | 126 | 104 |
| | Y2W0 | Red | 2.7 | 2.5 | 1.8 | 220 | 226 | 227 |
| | | MV | **1.3** | 2.7 | 2.1 | 170 | 136 | 124 |
| | | Ox | **1.3** | 2.8 | 2.2 | 214 | 179 | 167 |
| | C1W0 | Red | 1.7 | **1.1** | **1.5** | 85 | 62 | 76 |
| | | Ox | 1.6 | 1.7 | 1.6 | 75 | **47** | **37** |
| | C1W1 | Red | 1.7 | | | 90 | | |
| | | Ox | 1.5 | 2.4 | 2.0 | **64** | 53 | 52 |
| 3RFR | C2W0 | Red | 2.7 | | | 311 | | |
| | | MV | 1.6 | | | 292 | | |
| | | Ox | 1.9 | | | 357 | | |
| | C1W0 | Red | 1.4 | **0.4** | **0.9** | 88 | 101 | 89 |
| | | Ox | **1.3** | **0.4** | **0.9** | **32** | **47** | **37** |
| | C1W1 | Red | 2.6 | | | 82 | | |
| | | Ox | 2.3 | 1.3 | 1.0 | 42 | 68 | 58 |

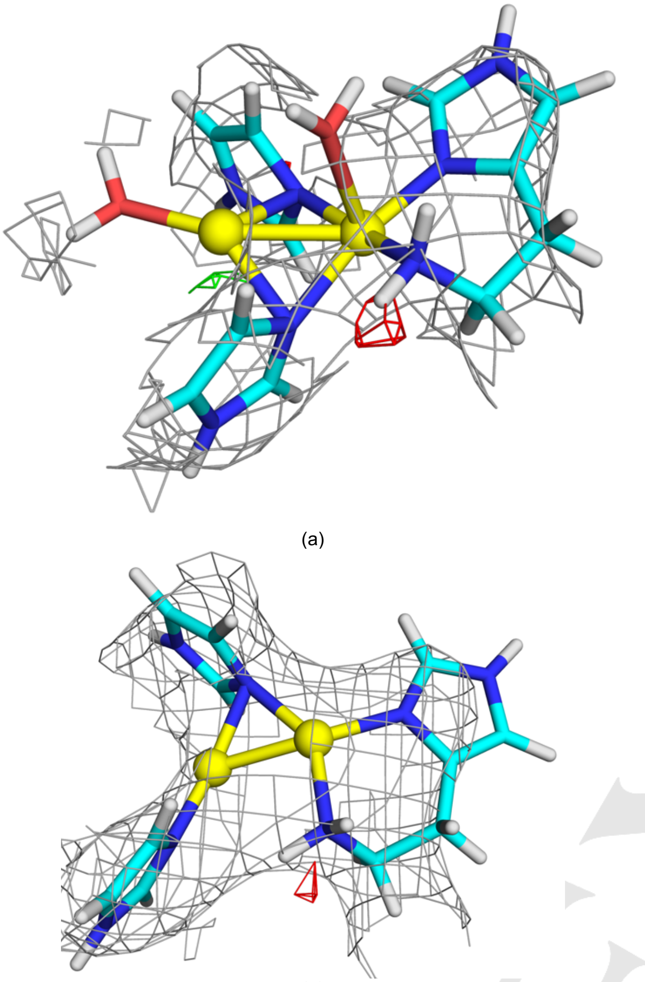
[a] PDB code. [b] Number of Cu ions (C2 or C1; Y2 represent planar structures, cf. Figure 3) and number of water molecules, W0–W2. [c] Oxidation state of the Cu ion(s), fully reduced (Red), oxidised (Ox) or mixed valence (MV; $Cu^I$+$Cu^{II}$).

The strain energy (i.e. the difference in QM energy of the QM region when optimised in vacuum or in the crystal),[12] is a measure of how well the metal site fits into the crystal structure. It is high for all three structures, 179–300 kJ mol$^{-1}$ (Table 1). Thus, QM refinement can improve the structure of site A in pMMO, giving reasonable geometries of the ligands, but the results indicate that the site is not well modelled by two Cu ions. In particular, the ligands are too few for the higher oxidation states.

Therefore, we tried to enhance the site with two water molecules. After many attempts, we were able to obtain some reasonable structures and the best are shown in Table 1 (Y2W2). The RSZD scores are improved for the higher oxidation states (1.5) and the strain energies are also appreciably better, 149–159 kJ mol$^{-1}$. However, the maps in Figure 3a show that there is no electron density around the water molecules. Consequently, we also optimised the corresponding structures without the water molecules (Y2W0 in Table 1 and Figure 3b, i.e. Cu ions in the same plane and not on top of each other as in Figure 2). They gave slightly better RSZD scores (down to 1.3), but somewhat higher strain energies (170–220 kJ mol$^{-1}$).

We have also tried to enhance the QM system with nearby potentially coordinating groups, as suggested by some QM studies.[16] However, as detailed in the supplementary material only the carbonyl group of His-137 was found to coordinate and such structures reproduced the crystallographic raw data quite poorly.

Finally, we instead tried to model site A with a single Cu ion (C1W0 in Table 1). This gave chemically more reasonable structures and the electron density maps in Figure 4a show that this state fits the crystallographic raw data[9] almost as well as the best dinuclear structures with RSZD = 1.6–1.7. Moreover, the strain energies were much lower, 75–85 kJ mol$^{-1}$, reflecting a minimal distortion by the protein.



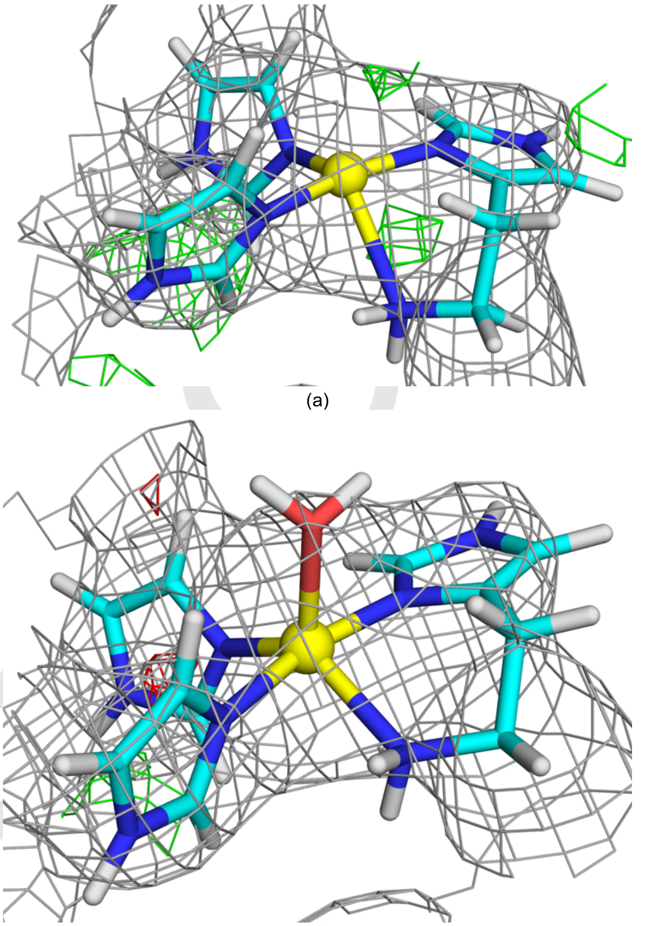
(a)

(b)

**Figure 3.** QM-refined structures of site A in protomer 1 of the 3RGB structure with two (a) or no (b) extra water molecules (MV state). Atom colours and electron-density maps[9] are the same as in Figure 2.

There remained some positive difference density on top of the Cu ion (green mesh in Figure 4a), which corresponds to the additional Cu ion in the original structure. Yet, it is too close to the Cu ion and with a too low density to be another metal. Therefore, we tried to model it as a water molecule instead (C1W1 in Table 1), giving reasonable structures for the oxidised state. The electron density maps in Figure 4b show that this state fits the crystallographic raw data[9] even better than without the water molecule, with a RSZD score of 1.5 and a strain energy of only 64 kJ mol$^{-1}$.

We have also studied site A in the other two protomers of the 3RGB structure, which have somewhat different structures of site A in the original structure, as can be seen in Figure 1c. The quantum refinement also gave a more planar structure, shown in Figure 5a (details in the supplementary material). However, the same types of structures could be found also in these two protomers (Figures 5b and 5c) and the results in Table 1 show that a mononuclear site still gives the best RSZD scores and strain energies in both protomers.



(a)

(b)

**Figure 4.** QM-refined structures of site A in protomer 1 of the 3RGB structure, modelled with a single Cu ion without (a) or with (b) an extra water molecule (oxidised state). Atom colours and electron-density maps[9] are the same as in Figure 2.

Finally, we considered also the 3RFR crystal structure,[9] which contains a dinuclear site A only in protomer 1. Again, the results in Table 1 show that the best results were obtained with a mononuclear site in all three protomers (details in the supplementary material).

Thus, quantum refinement of two crystal structures of pMMO gives quite conclusive results for the nature of the Cu site A: The mononuclear models always yield lower strain energies than the dinuclear models (37–85 kJ mol$^{-1}$, compared to 104–300 kJ mol$^{-1}$). Thus, the dinuclear structures are not chemically reasonable even after quantum refinement.

Likewise, the RSZD score is also lowest for the mononuclear sites in the 3RFR structure and protomers 2 and 3 of 3RGB (by 0.3–0.8). However, for protomer 1 in 3RGB, the lowest score was actually obtained for the structure in Figure 3b, but only marginally (1.3, compared to 1.5 for the best mononuclear site; however, as discussed in the supplementary material, this depends on how the score is calculated).

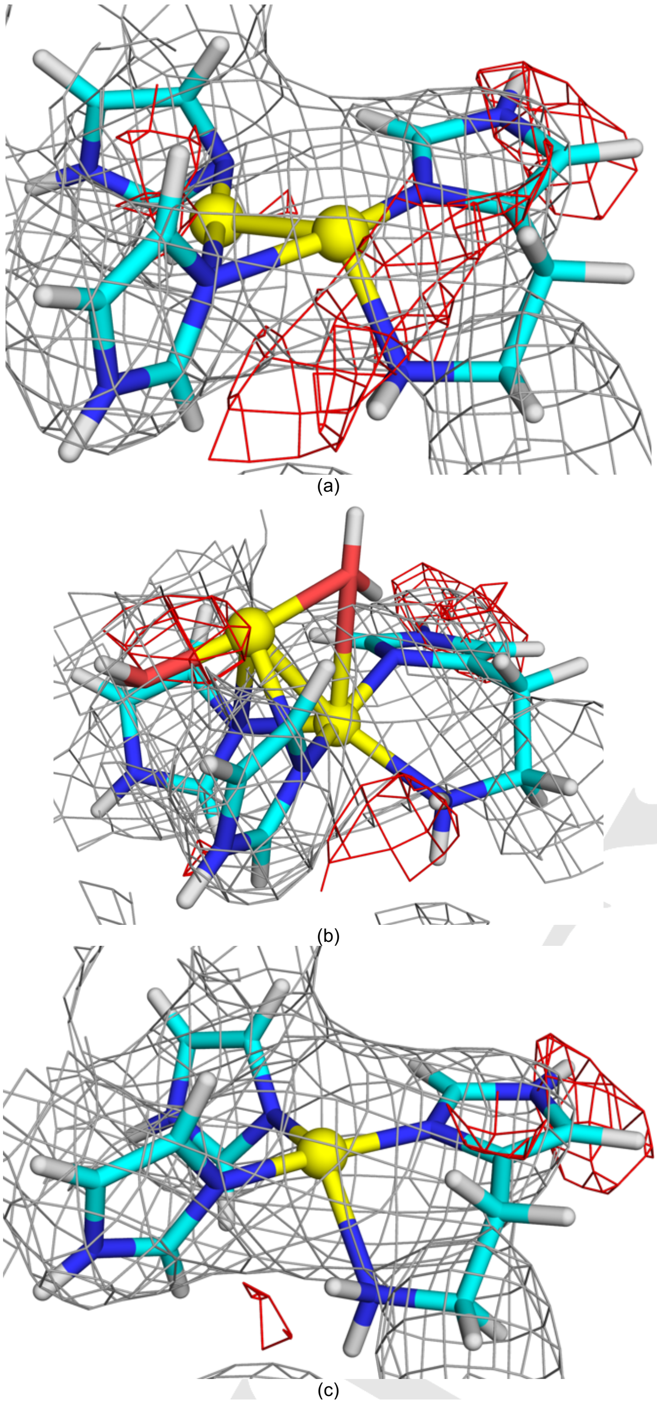


**Figure 5.** QM-refined structures of site A in protomer 2 of the 3RGB structure with (a) two Cu ions, (b) two Cu ions and two waters or (c) one Cu ion in the reduced states. Atom colours and electron-density maps[9] are the same as in Figure 2.

To further investigate the catalytic relevance of a mononuclear copper site, we have studied a putative reaction mechanism of pMMO using the mononuclear site in Figure 4. Following the studies by Yoshizawa and Shiota,[11] who studied both dinuclear and mononuclear Cu sites, but used site B (Figure 1) as the mononuclear site (see supplementary material for additional discussion), we started from a Cu(III)–oxo species. This complex can bind methane and extract a hydrogen atom with a barrier of only 22 kJ mol$^{-1}$, giving an intermediate with a methyl radical and a OH group (Figure 6). Finally, the latter rebounds to the radical, forming methanol with a barrier of 53 kJ mol$^{-1}$. The net reaction is strongly exothermic. The first half takes place on the triplet spin surface, but at the intermediate, it shifts over to singlet spin, as was also observed in the previous study.[11] These results show that a mononuclear site can oxidise methane and that the activation barriers actually are lower than those obtained in the previous study (79 and 107 kJ mol$^{-1}$ for the mono- and dinuclear sites, respectively).[11]

Consequently, we find no conclusive evidence that the crystal structures of pMMO involve a dinuclear Cu site A. Instead, a mononuclear site fits the crystallographic raw data at least as well. Moreover, it gives chemically much more reasonable structures, which are similar in both crystals and all protomers. This finding, if applicable to functional pMMO in the cell, has strong implications for its possible reaction mechanisms.

## Methods

Structures were obtained with quantum refinement[12,13] using the 3RGB and 3RFR crystal structures at 2.8 and 2.68 Å resolution, respectively.[9] QM calculations were performed with the TPSS[17] method and the def2-SV(P)[18] basis set. QM regions are shown in Figures 2–5.

## Acknowledgements

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**Keywords:** Particulate methane monooxygenase • quantum refinement • density functional theory • copper

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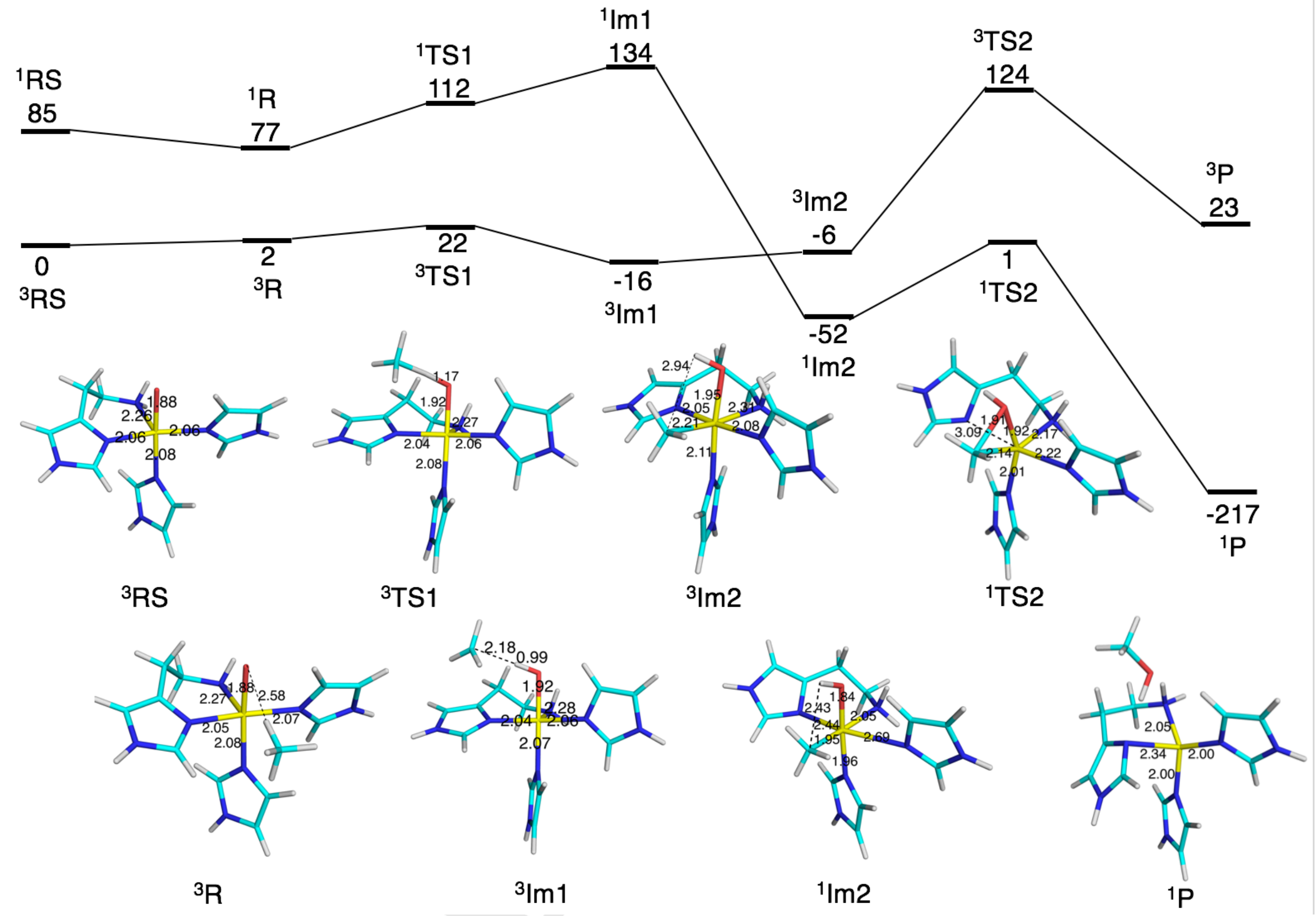

**Figure 6.** Putative mechanism for the reaction of mononuclear site with methane. On the top, reaction energies in kJ mol$^{-1}$ are shown, whereas the lower part shows structures of the various states, with key distances indicated in Å. All states were studied both in the singlet and triplet states, indicated by superscript 1 and 3, respectively.

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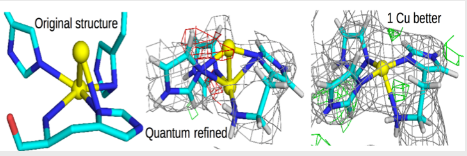

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**Title**

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