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Structure of the key species in the enzymatic oxidation of methane to methanol

Rahul Banerjee[1,2], **Yegor Proshlyakov**[3], **John D. Lipscomb**[1,2], and **Denis A. Proshlyakov**[3]

[1]Department of Biochemistry, Molecular Biology & Biophysics, University of Minnesota, Minneapolis, Minnesota 55455, USA.

[2]Center for Metals in Biocatalysis, University of Minnesota, Minneapolis, Minnesota 55455, USA.

[3]Department of Chemistry, Michigan State University, East Lansing, Michigan 48824, USA.

Abstract

Methane monooxygenase (MMO) catalyses the O_2-dependent conversion of methane to methanol in methanotrophic bacteria, thereby preventing the atmospheric egress of approximately one billion tons of this potent greenhouse gas annually. The key reaction cycle intermediate of the soluble form of MMO (sMMO) is termed compound Q (Q). Q contains a unique dinuclear Fe^{IV} cluster that reacts with methane to break an exceptionally strong 105 kcal mol^{-1} C-H bond and insert one oxygen atom[1,2]. No other biological oxidant, except that found in the particulate form of MMO, is capable of such catalysis. The structure of Q remains controversial despite numerous spectroscopic, computational and synthetic model studies[2–7]. A definitive structural assignment can be made from resonance Raman vibrational spectroscopy but, despite efforts over the past two decades, no vibrational spectrum of Q has yet been obtained. Here we report the core structures of Q and the following product complex, compound T, using time-resolved resonance Raman spectroscopy (TR^3). TR^3 permits fingerprinting of intermediates by their unique vibrational signatures through extended signal averaging for short-lived species. We report unambiguous evidence that Q possesses a bis-μ-oxo diamond core structure and show that both bridging oxygens originate from O_2. This observation strongly supports a homolytic mechanism for O-O bond cleavage. We also show that T retains a single oxygen atom from O_2 as a bridging ligand, while the other oxygen atom is incorporated into the product[8]. Capture of the extreme oxidizing potential of Q is of great contemporary interest for bioremediation and the development of synthetic approaches to methane-based alternative fuels and chemical industry feedstocks. Insight into the formation and reactivity of Q from the structure reported here is an important step towards harnessing this potential.

Correspondence and requests for materials should be addressed to D.A.P. (dapro@chemistry.msu.edu) or J.D.L. (lipsc001@umn.edu)..

Author Contributions R.B., Y.P. and D.A.P. developed the enhanced instrument and performed the experiments, D.A.P. analysed the data, and R.B., D.A.P. and J.D.L. designed the experiments and wrote the manuscript.

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Online Content

Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Transient kinetic studies of sMMO have revealed eight reaction cycle intermediates, thereby providing the most comprehensive description of enzymatic O_2 activation and C-H bond oxidation currently available for any di-iron oxygenase[1,9–11] (Fig. 1a). It is broadly accepted that the linear increase in the decay rate constant for **Q** with concentration of methane indicates reaction between **Q** and substrates with concomitant formation of the product complex **T**. **Q** is formed in a single turnover system by mixing O_2-containing buffer solution with the heterocomplex of diferrous sMMO hydroxylase ($MMOH^{red}$) and regulatory B component (MMOB). In the absence of methane, the yellow **Q** has a lifetime of several seconds, accumulates in high yield, and can be trapped by rapid freeze-quench (RFQ) techniques. Several spectroscopic studies of trapped **Q** have been successful[2,3,10], but not RFQ-resonance Raman, perhaps owing to weak resonance Raman enhancement and/or photosensitivity of **Q**. To minimize photolysis, we acquired the resonance Raman spectrum of **Q** in a continuously flowing reactant stream, while at the same time extending spectral accumulation to many hours[12].

Previous studies have shown that **Q** maximizes at $\Sigma t \approx 3$ s after initiation of the reaction between $MMOH^{red}$/MMOB and O_2 at pH 7.0, 4 °C in the absence of substrate[1] (Extended Data Fig. 1a). Accordingly, we observe the electronic absorption spectrum of **Q** at this time in the TR[3] flow cell (Fig. 1b).

The absolute resonance Raman spectra of the reaction mixture at $\Delta t \approx 3$ s ($\lambda_{ex} = 351$ nm) are dominated by non-resonant vibrations of bulk solution due to the relatively low attainable enzyme concentration (0.25 mM) (Extended Data Fig. 2). Repetitive switching between $^{16}O_2$-and $^{18}O_2$-saturated oxygen streams, while maintaining the same diferrous $MMOH^{red}$/MMOB stream, eliminates minute variability between sample preparations. The O_2 isotope difference spectra reveal weak vibrations that involve dioxygen-derived atoms, thus identifying sMMO intermediates, while all other vibrations cancel out (Fig. 1c, trace (i), and Extended Data Fig. 2).

Two distinct oxygen vibrations were detected at $\Delta t \approx 3$ s in the absence of substrate (Fig. 1c, trace (i)): a major mode at 690 cm^{-1} (identified herein by the ^{16}O isotopomer and the $^{16}O/^{18}O$ downshift, $\Delta^{18}O = 36$ cm^{-1}) and a weaker mode at 556 cm^{-1} ($\Delta^{18}O = 23$ cm^{-1}). No noticeable laser power dependence was observed, thus excluding photochemistry under these conditions (Extended Data Fig. 3). Neither of the vibrations was observed at a long delay time ($\Delta t \approx 30$ s).

The catalytic relevance of the observed intermediate(s) was probed by mixing substrates into the reactant stream, which is expected to fully quench **Q**[1]. Accordingly, the 690 cm^{-1} mode disappeared upon addition of methane or furan (Fig. 1c, traces (ii) and (iii)). A concomitant fourfold increase in the intensity of the 556 cm^{-1} mode shows that this vibration arises from a different intermediate that evolves from **Q** as it reacts with substrate. The rate constants for **Q** decay with methane or furan predict a predominant accumulation of **T** at $\Delta t \approx 3$ s (ref. 1; Extended Data Fig. 1b, c). A broad near-ultraviolet electronic absorption band for **T** allows its resonance enhancement ($\lambda_{ex} = 351$ nm) (Extended Data Fig. 4). Based on substrate-induced build-up and its transient nature, the 556 cm^{-1} vibration mode is assigned to **T**. The observation of the resonance Raman spectrum of **T** in the absence of substrate suggests that

the spontaneous decay of **Q** involves similar intermediates as found in the substrate mediated process.

The exceptionally large deuterium kinetic isotope effect in the reaction of **Q** with methane[8] reveals further correlation between **Q** and the 690 cm^{-1} mode. This step is slowed by a factor of 50 when using CD_4 rather than CH_4 due to the large tunnelling component in the reaction coordinate[13] (Extended Data Fig. 1d). Indeed, the loss of intensity of the 690 cm^{-1} vibration and the increase of the 556 cm^{-1} vibration were much smaller when using CD_4 as substrate (Fig. 1c, trace (iv)).

An initial assignment of the structure for the di-iron cluster of **Q** can be made by comparing its resonance Raman spectrum to those of model complexes (Table 1, Extended Data Table 1). The observed frequency of 690 cm^{-1} for **Q** is much less than the $\nu_{Fe=O}$ of terminal ferryl complexes[12,14]. Moreover, the $\Delta^{18}O$ shift of 36 cm^{-1} for **Q** is greater than the expected value for an Fe^{IV}=O diatomic oscillator at this frequency. Therefore, the possibility of an isolated, terminal Fe^{IV}=O moiety in **Q** can be excluded (Fig. 3, panel (v)), as can be a peroxy complex, which would exhibit the ν_{O-O} at even higher frequency[15]. In contrast, the ν_{Fe-O} of end-on Fe^{III}-OH, Fe^{IV}OH and Fe^{II}-O_2 complexes appear at lower frequencies than observed here[16–18]. Protonated ligands typically exhibit a pronounced downshift upon bulk water deuteration, which we did not observe for **Q** (Fig. 1d). Indeed, the vibration frequency and $\Delta^{18}O$ isotopic shift for **Q** agree with values observed in only one class of synthetic di-iron models, namely bis-μ-oxo 'diamond core' complexes[19].

The defining feature of a diamond core structure is the presence of two vibrationally coupled μ-oxo bridges resulting in tetratomic vibrations (Table 1). This coupled motion yields a unique signature that allows discrimination of the diamond core from other structures by using the mixed isotopomer of oxygen ($^{16}O^{18}O$) (ref 19). If the diamond core comprises both oxygen atoms from O_2, then mixed oxygen substitution in **Q** (**Q**-$^{16}O^{18}O$) will result in a new vibration appearing symmetrically between those of the **Q**-$^{16}O_2$ and **Q**-$^{18}O_2$ (Fig. 2b, c, green). However, the absence of a diamond core or the incorporation of only one of two oxygen atoms from O_2 (Fig. 3, panel (iv)) will yield the same vibrational frequencies as observed for **Q**-$^{16}O_2$ and **Q**-$^{18}O_2$ with smaller $\Delta^{18}O$ (Fig. 2a–c, blue, red). Subtraction of ν(**Q**-$^{16}O^{18}O$) from the average of ν(**Q**-$^{16}O_2$) and ν(**Q**-$^{18}O_2$) (Fig. 2c, trace (iii)) reveals a new frequency at ~673 cm^{-1} in the resonance Raman spectrum of the asymmetrically labelled derivative, showing definitively that it arises from a diamond core di-iron cluster of **Q**. Notably, the new vibration also indicates that **Q** retains both oxygen atoms from O_2. The absence of **Q**-$^{16}O^{18}O$ vibration in the $^{16}O_2$-$^{18}O_2$ difference (Fig. 2c, trace (i)) shows that no exchange occurs between O_2 atoms and bulk water (Extended Data Fig. 5).

Subtraction of ν(**T**-$^{16}O^{18}O$) from the average of ν(**T**-$^{16}O_2$) and ν(**T**-$^{18}O_2$) results in cancellation of all oxygen vibrations (Fig. 2c, trace (iii)), demonstrating that **T** inherits only one of two oxygen atoms from O_2. This vibration is unchanged for samples prepared in D_2O (Fig. 1d), strongly suggesting that **T** does not possess a protonated bridging oxygen or terminal Fe-OH moiety, which are the only other species with similar resonance Raman vibrational fingerprints (Table 1). Thus, we assign **T** as a mono-μ-oxo-bridged structure derived from **Q** by transfer of one oxygen atom to product, as observed experimentally[8]

(Fig. 3, panel (vi)). We found no indication that labelled product is directly bound to the di-iron cluster in **T**. Together with the **Q** data, these observations allow each oxygen atom from O_2 to be tracked throughout the reaction cycle.

The observation that both bridging atoms in **Q** are derived from the same O_2 molecule has important implications for the mechanism of O_2 activation in sMMO. The dinuclear iron cluster in the **P** intermediate (Fig. 3, panel (i)) that precedes **Q** is predicted to possess a symmetrical *cis*-μ-1,2 peroxo bridge[20]. Proton migration in **P** facilitates O-O bond cleavage to form **Q**, which could occur either heterolytically (as in cytochrome P450)[21] or homolytically (as in some dinuclear copper-O_2 complexes and mimics of NO reductase)[22,23]. A heterolytic cleavage mechanism would result in formation of a water molecule from an O_2-derived atom (Fig. 3, panel (iv), and Extended Data Fig. 5c). Our results show that neither of the O_2-derived atoms is lost from the cluster, nor do we see vibrations of O_2-derived water bound to the iron atoms. While it is possible to formulate a mechanism for symmetrical diamond core formation by heterolytic O-O cleavage (Extended Data Fig. 5b), such a scenario would involve large asymmetric changes in the oxidation states of the cluster irons and would probably show solvent oxygen incorporation into the diamond core of **Q**, which we do not observe. Consequently, O_2 activation in sMMO is likely to proceed via homolytic cleavage (Fig. 3, panels (ii) and (iii), and Extended Data Fig. 5a), highlighting different mechanistic strategies of haem and non-haem enzymes for generation of potent oxygenating species.

A previous combined Mössbauer and Extended X-ray Absorption Fine Structure study of **Q** revealed an exceptionally short Fe–Fe distance[3]. The model complexes available at the time suggested that the short Fe–Fe distance could be accommodated by a bis-μ-oxo ‘diamond-core’ structure[3]. In recent years, other structures of **Q** have been proposed based on new synthetic model complexes[20,24]. It was found that formation of an Fe^{IV}=O moiety in an open-core low-spin Fe^{IV}/Fe^{IV} or high-spin Fe^{III}/Fe^{IV} complex resulted in major increases in reactivity compared with the low-spin closed diamond core Fe^{IV}/Fe^{IV} or Fe^{III}/Fe^{IV} models with the same ligand structure[24]. Contrary to several open-core proposals for **Q**[20] that developed from this finding (Fig. 3, panel (v)), we do not detect an Fe^{IV}=O moiety indicative of an open core in **Q**. A coupled, polyatomic vibration in **Q** shows that it does have a diamond-core structure. Consequentially, the key to the reactivity of nature's most potent oxidant is likely to come from the spin state of the cluster, putting emphasis on further synthetic work towards mimicking the high-spin nature of **Q**, while retaining the high-valent diamond core.

METHODS

Chemicals

All chemicals used were of the highest grade available and were purchased from Sigma-Aldrich. $^{18}O_2$ was obtained from ICON. The asymmetrically labelled oxygen isotope ($^{16}O^{18}O$) was synthesized by E. H. Appelman from Argonne National Laboratory[12]. D_2O was obtained from Cambridge Isotope Laboratories.

Biological materials

MMOH was purified from *Methylosinus trichosporium* OB3b and MMOB from a heterologous expression system in *Escherichia coli* according to purification protocols described previously[11,30]. Low molar extinction of transient intermediates compounded by the nature of the TR^3 experiment required a large amount of the MMOH protein, estimated to be about 30 g for the experimental results presented here, with more than half used in the instrumentation development and optimization phases.

Sample preparation

Anaerobic solutions of MMOH in 50 mM MOPS, pH 7.0, 5% glycerol and MMOB in 50 mM MOPS, pH 7.0 were prepared in sealed vials by exchanging the headspace gas with argon while stirring on ice for 1 h. The chemical reduction of MMOH was conducted by anaerobic addition of methyl viologen (200 μM) and sodium hydrosulphite (4 mM) while stirring for 15 min at room temperature. MMOH was subsequently separated from the small molecule reducing agents by passage through a Sephadex G-25 PD-10 desalting column equilibrated in 75 mM MOPS buffer at pH 7.0 containing 5% glycerol, 0.1% Triton X-100, 200 μM $Fe(NH_4)_2SO_4$ and 2.0 mM L-cysteine (buffer A). The glycerol stabilizes the diferrous enzyme while Triton X-100 prevents protein aggregation in the flow cuvette that leads to disruption of the laser beam. The exogenous iron included in Buffer A in the form of the Fe^{II}-cysteine complex is used to maintain MMOH activity and does not reconstitute the protein active site[31]. Extensive controls have been carried out to ensure that it does not contribute to the observed vibrational spectra. Control stopped-flow experiments showed that neither glycerol nor Triton X-100 affected the decay rate constant for **Q**. Anaerobic MMOB was added to $MMOH^{red}$ to obtain a 1:1 MMOH active sites to MMOB ratio (480 μM). The protein was transferred to the secondary glove bag (Plas-Labs) where it was installed on the syringe infusion pump. The running enzyme syringe was continuously chilled to 4 °C using a Peltier cooling block mounted atop the syringe since $MMOH^{red}$ slowly precipitates at room temperature during the time required for a measurement (typically 1–5 h). For the experiments with deuterated solvents, the MMOH sample was exchanged by passage through the PD-10 desalting column equilibrated in buffer A prepared in D_2O (pH reading was adjusted to correct for D^+ vs. H^+ concentration). The isotopic enrichment of deuterated samples is estimated at 90%. The TR^3 experiment was conducted at 5–6 °C to avoid freezing of the D_2O buffer.

TR^3 measurements

While the core design of the instrument is similar to the cryogenic TR^3 study described previously[12], a brief overview of the instrument and experimental design are provided to describe the extensive revisions that were required for the current study. The TR^3 experiment relies on active mixing of the continuous streams of $MMOH^{red}$/MMOB and oxygen-saturated buffer at a volumetric ratio of 1:1. The maximal concentration of **Q** in the absence of substrate was estimated to be 95 μM at the probe point, which arises from the 40% active fraction of MMOH[11].

Sample is delivered to a fused silica flow cuvette (25 mm long, internal cross section 0.15 × 1.5 mm, Starna Cells) where it is probed approximately 5 mm from the mixer outlet (dead

volume ~3 μl). The time resolution is achieved by varying the flow rates of the mixed stream driven by computer controlled syringe infusion pumps (high-pressure OEM modules, Harvard Apparatus). Flow rates varying between 100 μl per min and 2.5 μl per min per line result in ageing times of 1 – 36 s. The mixer with the attached flow cuvette are mounted on a translation stage driven by an actuator drive (model ZST25, Thorlabs, Inc.) via a motion control board (KFLOP, DynoMotion). This actuator maintains a constant reciprocating translation of the cuvette along the sample flow direction over a distance of ~1 mm at a speed of 0.02 mm s^{-1}. This limits the residence time of the slow-moving protein solution along the walls of the flow cuvette in the laser beam. Scattering from the near-wall areas of the cell was rejected optically. The temperature of gas is maintained at 4 °C by re-heating the nitrogen stream from a dry-ice/ethanol bath with a two-channel temperature controller (Model 34, Cryocon). In addition, the temperature of the core mixing chamber is further controlled by attached miniature Peltier cells. All wetted surfaces in the TR^3 setup are comprised of protein-compatible PEEK, PTFE (in anaerobic atmosphere), fused silica and glass.

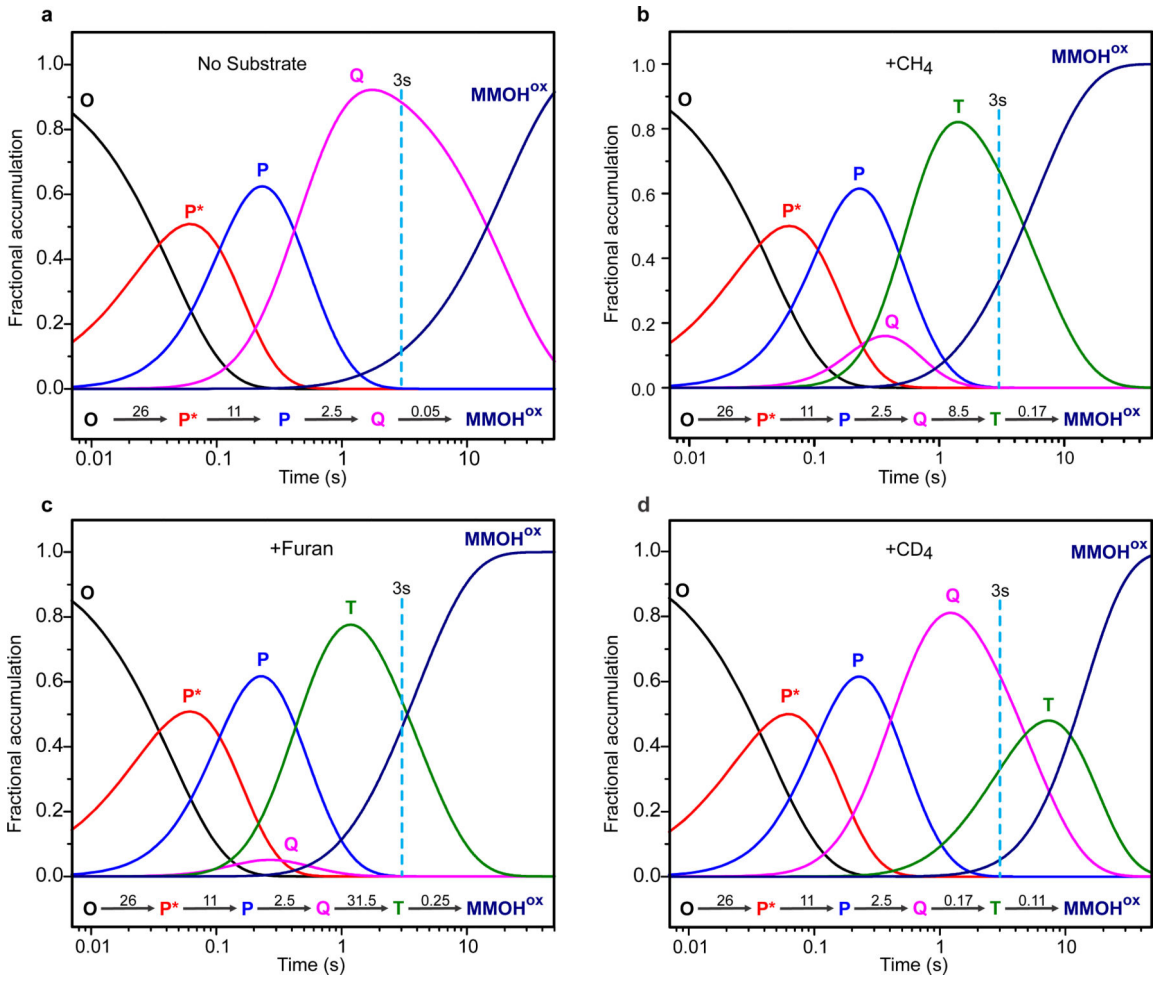
The oxygen-saturated buffer was prepared by addition of 1 ml of O_2 gas to 6 ml of argon-sparged anaerobic buffer at room temperature in a 10 ml gastight syringe. During operation of the TR^3 experiment, the oxygen-saturated buffers were automatically and repeatedly purged and switched from one isotope to the other using computer-controlled actuated HPLC valves (Upchurch Scientific & Rheodyne, IDEX Health & Science). Resonance Raman spectra were collected in 4–12 pairs of oxygen isotope runs in the course of a single TR^3 experiment to randomize the non-isotopic variations in the sample composition. Results of three to eight separate experiments performed over the course of several months using different enzyme preparations were averaged together, taking into account normalization of resonance Raman intensity.

Methane saturated buffer (CH_4 or CD_4) was prepared in an identical fashion to the oxygen saturated buffer. The furan substrate was introduced by adding a calculated volume to the reduced protein sample.

Excitation in the near-ultraviolet (351 nm argon laser line, Coherent, model Innova 300) was used to maximize resonance Raman enhancement and eliminate possibility of interference from porphyrins. The laser power was typically 60 mW and was reduced down to 15 mW during power dependence studies. At a flow rate of 10 μl per min and full laser power an estimated 6,700 photons were absorbed per molecule of **Q**.

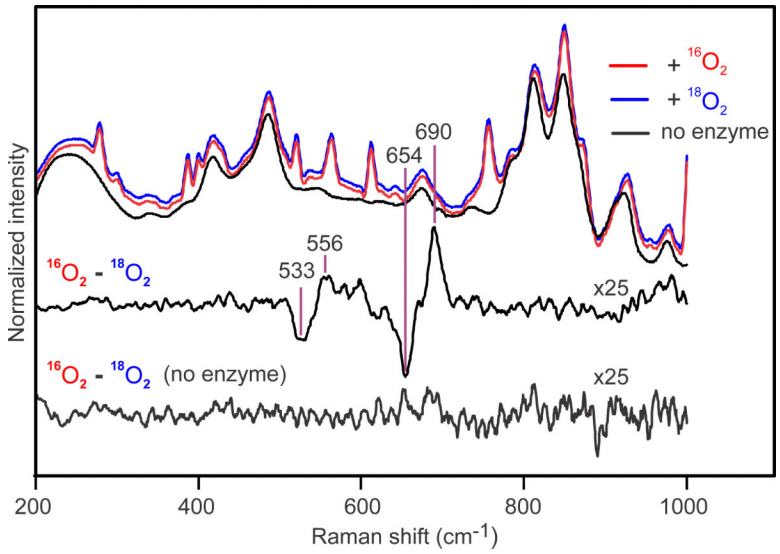
Acetone injected into the flow cell was used as a standard for calibration of the resonance Raman shifts and was subsequently purged with anaerobic buffer. Quantitative analysis of spectral changes over the reaction time or with substrate/solvent substitution was facilitated by normalization to the intensity of a 756 cm−1 protein mode as an internal standard. Further details of the spectral analysis are described in the supplementary text of reference 12.

Extended Data



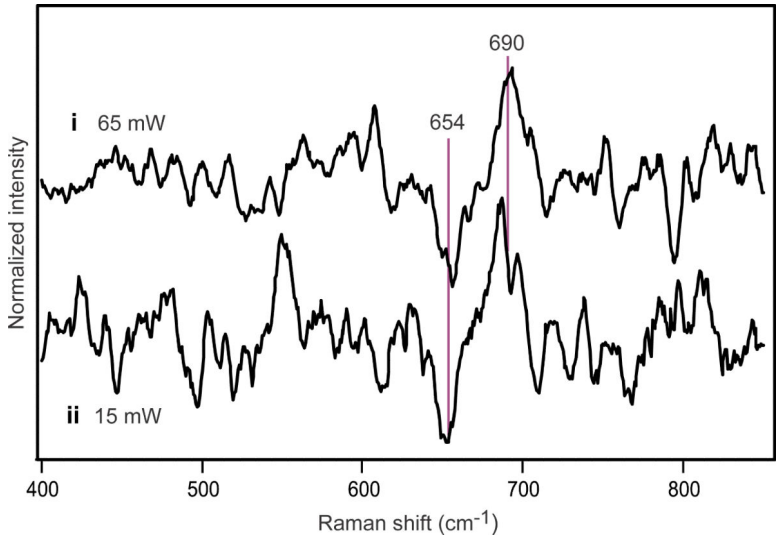
Extended Data Figure 1. Speciation plots of the sMMO reaction with O_2

Plots were computed using known rate constants of the catalytic steps[1,8,32,33] for the following conditions. **a**, No added substrate, **b**, presence of 0.45 mM CH_4, **c**, presence of 3.5 mM furan and **d**, presence of 0.45 mM CD_4 at pH 7.0, 4 °C. Rate constants used in simulation of individual conditions are shown for each step. All rate constants are first order (s^{-1}) except for the **Q** to **T** step, which is first order in both **Q** and substrate (M^{-1} s^{-1}) but is given as a pseudo first order constant for the current substrate concentration. The rate constant for the formation of intermediate **O** (oxygen binding) is unknown, but it is assumed to be fast based upon typical rates for metalloenzymes of the MMO type. It is irreversible because the rate constant of the next step (**O** → **P***) is independent of O_2 concentration.



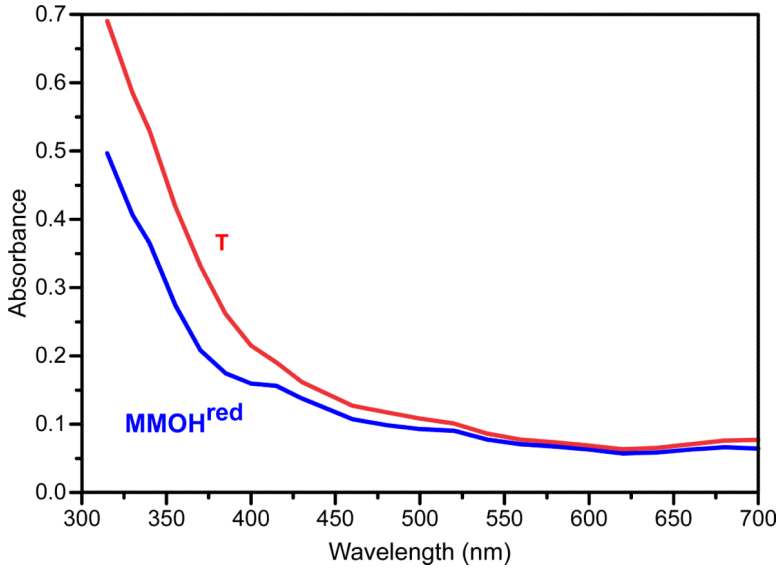
Extended Data Figure 2. Absolute TR^3 spectra of the sMMO reaction with O_2

Top, reaction using $^{16}O_2$-containing buffer (blue), $^{18}O_2$-containing buffer (red) or buffer background in the absence of MMOH/MMOB (black). Middle, $^{16}O_2 - ^{18}O_2$ difference spectra of the MMOH^{red}/MMOB reaction with O_2 at pH 7.0, 4 °C and $\Delta t \approx 3.0$ s. Bottom, $^{16}O_2 - ^{18}O_2$ difference spectra of the oxygenated buffers in the absence of MMOH and MMOB proteins. Intensities of sMMO spectra were normalized to protein vibration. In the absence of protein, relative intensity was normalized using buffer vibrations.



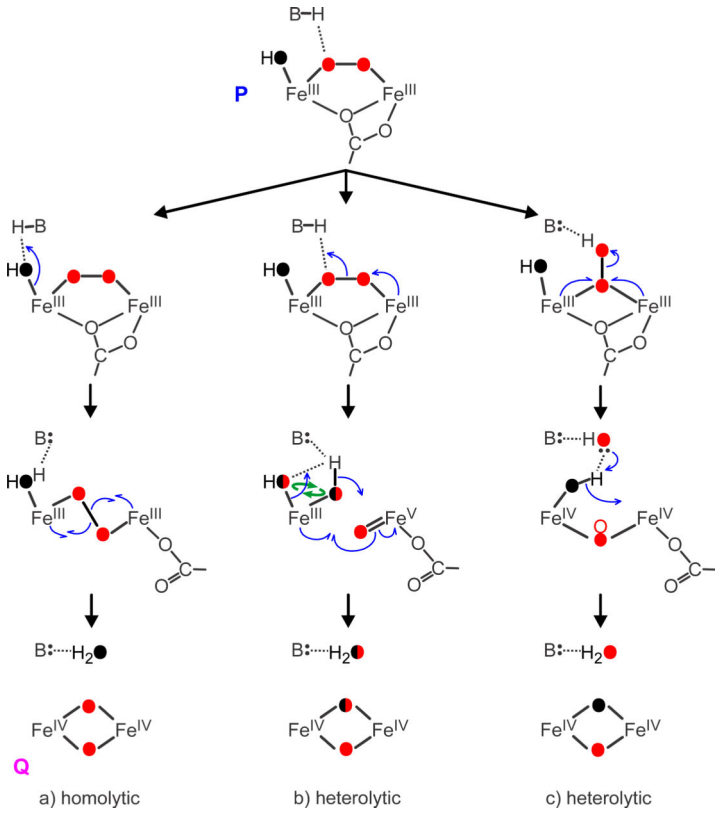
Extended Data Figure 3. Power dependence of TR^3 spectra of sMMO

$^{16}O_2 - ^{18}O_2$ difference spectra obtained using 65 mW (i) and 15 mW (ii) excitation laser power show the same normalized intensity of oxygen vibrations in **Q**, indicating that no detectable photodecomposition is taking place under current conditions.



Extended Data Figure 4. A comparison of the electronic absorption spectra of compound T (red trace) and MMOH^{red} (blue trace)

T exhibits an absorption band in the near-ultraviolet region, giving rise to its resonance Raman enhancement. Single wavelength time courses of the reaction of 25 μM MMOH^{red}/ MMOB with a 450 μM solution of CH_4 and 450 μM O_2 at 4 °C, pH 7 were recorded throughout the visible region (concentrations after mixing). The absorbance at each wavelength at the time of maximal **T** formation given by the speciation plot shown in Extended Data Fig. 1b was extracted and used to make the red trace shown.



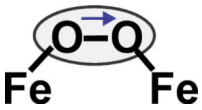
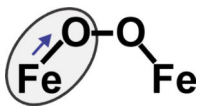
Extended Data Figure 5. Potential O-O bond cleavage mechanisms in the dinuclear centre of MMO

The most divergent mechanisms are shown along with expected isotopic composition of oxygen derived from O_2 (red) and solvent (black). All mechanisms are triggered by proton-dependent rearrangement of **P**[10,33]. The monodentate carboxylate bridge (E243) found in the diferrous enzyme[34] is likely to maintain this position in **P**, but return to the non-bridging position in **Q**, as found in the resting enzyme, to accommodate the diamond core structure. The catalytic base B, which mediates proton dependency, has not been definitively identified. Based on structural similarity to other di-iron O_2-activating enzymes and DFT computations for **P**-analogues in those systems[11,35,36], we have proposed[11] that E114 (a ligand to solvent-coordinated iron in **P**), is this base. Other ligands not directly involved in cleavage are omitted for clarity (see Fig. 3). Equal intensities of **Q**-$^{16}O_2$ and **Q**-$^{18}O_2$ modes, and the absence of **Q**-$^{16}O^{18}O$ mode in Fig. 2c, (i) argue against isotope scrambling in **Q** formation. This and all other experimental results reported to date are in full accord with the nominally concerted homolytic cleavage mechanism. We postulate that the loss of E243 bridge facilitates the conversion of *cis*-μ-peroxo adduct in **P** to the *trans*-μ-peroxo conformation and the ensuing O-O bond cleavage (**a**) to form the diamond core structure detected here. This transition is supported by DFT computations[37]. In contrast, the stepwise, end-on heterolytic cleavage mechanism (**c**) (analogous to formation of compound I in cytochrome P450) leads to the mixed isotope cluster in **Q**-$^{18}O_2$ and can be ruled out. Heterolytic cleavage of *trans*-μ-peroxo is essentially isoelectronic to and experimentally indistinguishable from the homolytic mechanism (**a**). The proton-assisted heterolytic cleavage of *cis*-μ-peroxo bridge (**b**) cannot be ruled out yet, but several observations argue

against it. (1) We did not observe isotope scrambling (curved green arrows), which is expected upon formation of two terminal oxygenic ligands on the same iron. While scrambling may not occur if ligands are highly stabilized, structural basis for such putative stabilization is not apparent. Scrambling may also not be observed if formation of diamond core is fast following bond cleavage, in which case mechanism (**b**) becomes, in essence, a stepwise, proton-assisted homolytic cleavage, also indistinguishable from (**a**). (2) Two iron atoms in di-ferric **P** and di-ferryl **Q** are in the same oxidation states and in indistinguishable electronic environments[2,20]. Such symmetry is unfavourable for O-O bond polarization and charge separation in the Fe^{III}/Fe^{V} state during heterolytic cleavage. The deprotonated state of the peroxo bridge in **P** also argues against overall polarity of the site that would aid heterolytic cleavage.

Extended Data Table 1

Additional vibrational modes of metal centres relevant to Q and T

Vibrational structure	System	$\nu(\Delta^{18}O)$ cm^{-1}	$\nu^{16}O^{18}O$ cm^{-1}	$\Delta^{2}H$ cm^{-1}	Ref
	sMMO Q	690 (−36)	673	0	This work
	sMMO T	556 (−23)	-	0	This work
	$[Fe^{IV}(O)(TMG_3tren)]$	843 (−33)	-	-	38
	TauD-F4	825 (−37)	-	0	12
	$[Fe^{IV}(O)(TMC)(NCCH_3)]^{2+}$	834 (−34)	-	-	39
	$hDOHH_{peroxo}$	855[a] (−44)	833	-	15
	Peroxo-Δ^9D	898 (−53)	874	-	40
	Frog M ferritin peroxo	851 (−51)	-	-	41
	$hDOHH_{peroxo}$	473 (−16)	465	-	15
	Peroxo-Δ^9D	442 (−17)	433	-	40
	Frog M ferritin peroxo	485 (−17)	477	-	41

References 38-41 are cited in this Table.

[a] represents the center of a Fermi Doublet

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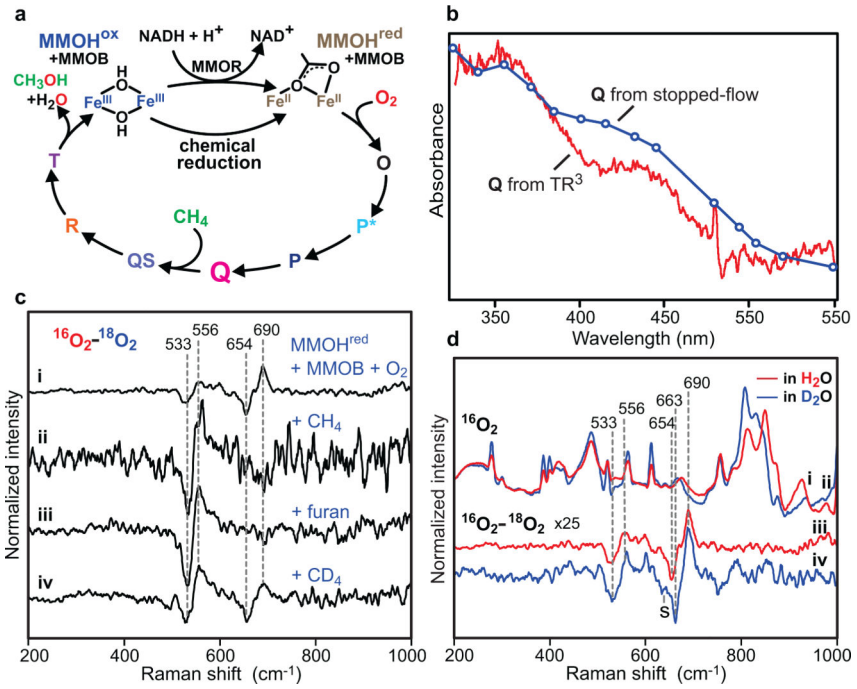


Figure 1. Reaction of sMMO with O_2

a, The catalytic cycle includes stable $MMOH^{ox}$ and $MMOH^{red}$ and detectable transient species **O**, **P***, **P**, **Q** and **T**; transient states **QS** and **R** were predicted from kinetic, spectroscopic, and chemical studies. **b**, An *in situ* transient difference electronic absorption spectrum of the reaction mixture (vs anaerobic $MMOH^{red}$) in the TR^3 instrument at $\Delta t \approx 3.0$ s (red, 1.0×0.1^2 mm probe volume) in comparison with spectrum of **Q** reconstructed from stopped-flow kinetic traces (markers, blue). **c**, Transient $^{16}O_2 - {}^{18}O_2$ difference resonance Raman spectra of sMMO reveal vibrations of iron-bound oxygen atoms. Measurement conditions: pH 7.0, 4 °C, $\Delta t \approx 3.0$ s. Spectra recorded without the substrate (i) show marked changes when 0.45 mM CH_4 (ii), 3.5 mM furan (iii) or 0.45 mM CD_4 (iv) is added. **d**, Solvent vibrations in the absolute resonance Raman spectra are sensitive to H_2O (i)/D_2O (ii) substitution. $^{16}O_2 - {}^{18}O_2$ difference spectra of sMMO recorded in H_2O (iii) show little sensitivity to D_2O (iv) substitution. The upshift of ^{18}O vibration of **Q** and the appearance of a low-frequency shoulder (marked with S) in D_2O is attributed to Fermi resonance with a protein-derived metal ligand.

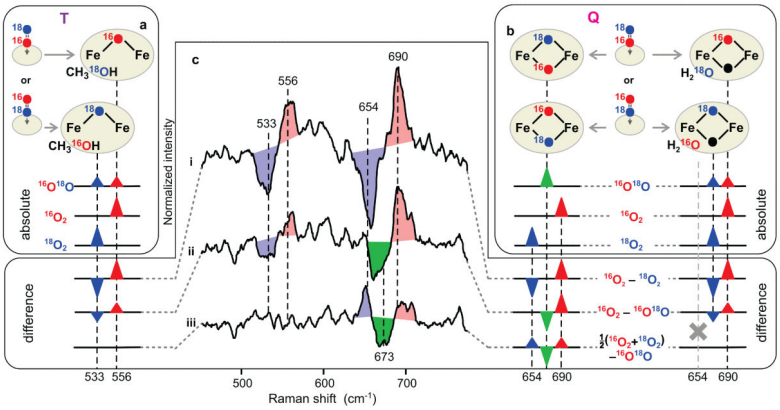


Figure 2. Fingerprinting cluster structure using $^{16}O^{18}O$ mixed oxygen isotope

a, **b**, Asymmetrically labelled $^{16}O^{18}O$ can initially bind in two equiprobable orientations, yielding an even mixture of two isotopomers in **T** or **Q** (panels **a** and **b**, top) exhibiting characteristic vibrations (panels **a** and **b**, bottom). Two scenarios are possible for **Q**, depending on whether two (left) or one (right) O_2-derived atoms (red and blue) are incorporated into the cluster. If only one atom is incorporated, then the second oxygen atom in the core structure would derive from solvent (black). Water and methanol exemplify a departing oxygen atom while other ligands are omitted for simplicity. Since the two

isotopomers of **T** (**a**) and those of singly labelled **Q** (**b**, right) have identical composition as corresponding $^{16}O_2$ and $^{18}O_2$ derivatives, they will exhibit both vibrations simultaneously at half the intensity. Doubly labelled **Q** (panel **b**, left) will be different from both symmetrically labelled derivatives and thus, **c**, will exhibit a new vibration (green), as illustrated by isotope difference spectra (**c**). The $^{16}O_2 - {}^{16}O^{18}O$ difference (ii) in singly labelled cluster (**a**, **b** right) will appear as the $^{16}O_2 - {}^{18}O_2$ difference (i) with reduced intensity. The $^{16}O^{18}O$ derivative will be identical to the average of symmetrical isotopomers, yielding no signal in trace iii, as observed experimentally for **T**. The new frequency in doubly labelled **Q** should appear in both difference (ii) and (iii), and can, indeed, be seen in experimental data at 673 cm^{-1}. Spectral superimposition inflates the apparent isotopic shift when frequencies of isotopomers are close (traces (ii) and (iii), right), but not when bands are well separated (trace (i)).

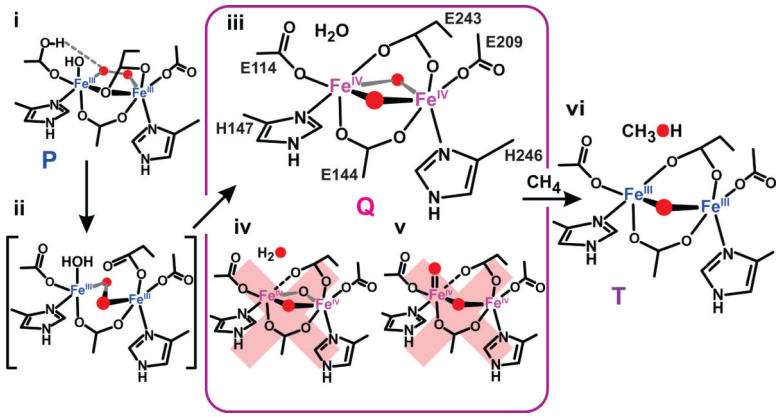
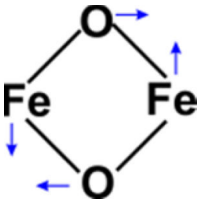
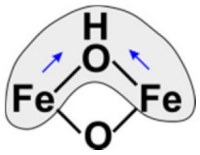
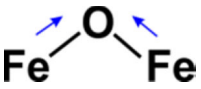


Figure 3. Formation of compound Q and its reaction with methane

Homolytic mechanism of O-O bond cleavage (ii) upon formation of **Q** from **P** (i) follows from the structure of **Q** (iii) presented here. Alternative structures of **Q** (iv, v) discounted by current results are also shown. The iron shown on the left in the **Q** structure (iii) may retain the solvent found in **P**. In this case, E243 would not bind to this iron, but would be likely to hydrogen bond with the bound solvent[4,5]. **T** (vi) contains a single atom from O_2 while another is incorporated in the product.

Table 1

Vibrational modes of metal centers relevant to Q and T

Vibrational structure	System	$\nu(\Delta^{18}O)$ cm^{-1}	$\nu^{16}O^{18}O$ cm^{-1}	$\Delta^{2}H$ cm^{-1}	Ref.
	[4-OMe-3,5-Me-TPAFe^{IV}(μ-O)]$_2$	674 (−30)	665	-	7
	[(TPA)$_2Fe^{III}$(μ-O)2Fe^{IV}]	666 (−28)*	644	-	19
	[(5-Me-TPA)$_2Fe^{3+}$(μ-O)$_2Fe^{IV}$]	668 (−32)	-	-	19
	sMMO **Q**	690 (−36)	673	0	This work
	sMMO **T**	556 (−23)	-	0	This work
	[Fe_2(μ-O)(μ-OH)(4,6-Me_6-TPA)$_2$]^{III}	497 (−13)	-	−3	25
	[Fe_2-(μ-O)(μ-OH)(BPEEN)$_2$]^{III}	504 (−16)		−4	25
	Ribonucleotide reductase-R2	496 (−15)	-	0	26
	Δ^9-desaturase	519 (−18)	-	0	27
	Urease UreA2B2	497 (−21)	-	-	28
	Hydroxomet-hemerythrin	565 (−27)	-	−5	16
	Chloroperoxidase Compound-II	565 (−22)	-	−13	17
	Horseradish peroxidase	503 (−19)	-	+6	29

‡ Additional cases are considered in references 12, 15 and as noted in Extended Data Table 1.

* represents center of a Fermi Doublet