Reviews

Structure and catalytic mechanism of methane monooxygenase and approaches to its modelling

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The mechanisms of activation of O_2 and CH_4 by methane monooxygenase (MMO) are discussed. A new concept for the catalytic cycle of MMO is suggested, and approaches to its chemical modelling are considered.

Key words: methane monooxygenase, alkane oxidation, iron complexes, alkane activation, O_2 activation.

Methane is the most inert and abundant alkane. On the other hand, the stability of the whole organic world toward an oxygen-containing atmosphere is caused by the kinetic inertness of O_2. However, there are enzymes in nature, monooxygenases, which activate CH_4 and O_2 to such an extent that a rather fast reaction becomes possible between them at ambient temperature and pressure. This reaction occurs *via* the transfer of one atom of oxygen molecule to the C—H bond of a methane molecule to form methanol and water:

$$CH_4 + O_2 \xrightarrow[m\text{-monooxygenase}]{2\bar{e},\ 2H^+} MeOH + H_2O.$$

Owing to the use of the energy stored by the reducing agent, the reaction occurs at low temperature with extremely high selectivity. A fairly energy-saving expenditure of the reducing agent is achieved due to a perfect structural organization of the active center and the specific mechanism, which provides a highly efficient process.

Studies of the structure and mechanism of action of methane monooxygenase (MMO) have progressed considerably in recent years. The structure of the soluble form of MMO is established by X-ray diffraction analysis. The first data on the nature of active intermediates are available now. Attempts at chemical modelling of structural and functional features of MMO are being made. To advance in this direction, it is very important to develop concepts about the methods for activation of molecular oxygen and methane and about the nature of the active form of an oxidant on the basis of new data on the structure and functioning of MMO. The purpose of this work is to consider briefly the newest data on the structure and mechanism of action of MMO, to understand what factors of the structure or mechanism are determining for mild and selective oxidation of methane

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to methanol, and to analyze the possibilities of the chemical modelling of this great creation of nature.

MMO can be isolated from methanotrophic bacteria, which use methane for their vital activity by the unique transformation of methane into methanol by means of this enzyme at the first stage.[1,2] Depending on the conditions of microorganism growth, MMO can be obtained in the membrane-bound or soluble forms. Iron-containing soluble MMO from *Methylococcus capsulatus (Bath)*[3] and *Methylosinus trichosporium*[4] are the most studied. Although enzymes isolated from these cultures have many common features, they also have significant differences in activity, selectivity, spectral and other parameters (up to the mechanism) that remain to be studied. Enzyme systems can be arranged in the following series by their activities in methane oxidation (in mmol min^{-1} mg^{-1} of protein): *Ms. trichosporium* (1700) > *Mc. capsulatus* (150—36) > *M. bacterium* (93). The substrate selectivity for water-soluble MMO from *Mc. capsulatus* is the most studied. In the oxidation of *n*-alkanes (ω- and ω-1-hydroxylation), a terminal methyl group and adjacent methylene group are oxidized. When branched alkanes are oxidized, the part of a molecule that is the most distant from the branching point is oxidized evidencing significant steric hindrances near the active center. In other cases, the weakest ternary C—H bond is oxidized (*iso*-butane, adamantane, *cis*-1,4-dimethylcyclohexane).[5] In addition to the oxidation of C_1—C_8 alkanes, MMO can hydroxylate aromatic compounds and epoxidize olefins.

MMO consists of three tightly connected proteins: **A** (monooxygenase itself or hydroxylase consisting of two halves each of which contains two iron atoms), **B** (regulatory protein), and **C** (reductase).[3,4] The transfer of electrons from NADH to hydroxylase is performed by reductase and controlled by protein **B**.

Until recently, the information on MMO structure has been based on the spectroscopic and magnetic studies of this enzyme.[2] It was shown that the active center of MMO included a binuclear iron μ-oxo-μ-carboxylate complex and was similar to the active centers of the oxygen-binding protein hemerythrin (Hr), which is a non-heme analog of hemoglobin, and the redox-enzyme ribonucleotide reductase (RNR), which participates in the synthesis of RNA. It is known that strong antiferromagnetic exchange interaction *via* the O-bridge with the constant $-J = 120$ to 130 cm^{-1} ($H = -2J\ S_1 \cdot S_2$) exists between the iron atoms in these latter centers. This interaction results in the shortening of the Fe—O distance and appearance of a characteristic absorption in the visible region of the optical spectra. In addition, the characteristic ν_s(Fe—O—Fe) line in the Raman spectra is related to the μ-oxo bridge.

The presence of the Fe_2O center, similar to Hr and RNR, follows first of all from the ESR studies of reduced hydroxylase. Hydroxylase is usually isolated from cell structures in the oxidized $Fe^{III}Fe^{III}$ form. The reduced $Fe^{II}Fe^{II}$ and semireduced $Fe^{II}Fe^{III}$ forms can be obtained by reduction of the oxidized form by dithionite in the presence of the corresponding mediators. The action of dithionite results primarily in the appearance of three intense signals with $g < 2$ ($g_{av} = 1.85$) related to the partially reduced $Fe^{II}Fe^{III}$ state,[6] which then transforms into the $Fe^{II}Fe^{II}$ state with $g = 16$ that originates from the ferromagnetic coupling of two Fe^{II} ($S = 4$). A similar signal is observed for azidodesoxy Hr, which also exists in the $Fe^{II}Fe^{II}$ ferromagnetically coupled state.

The most detailed information about the structure of the MMO active center is obtained by the EXAFS method, which allows one to establish the mutual arrangement of iron atoms in the binuclear cluster and to estimate the number of types of atoms near the iron atoms and their distances from the iron atoms. These measurements confirmed the existence of two iron atoms linked by μ-oxo-μ-carboxylate bridges as in Hr and RNR. According to the EXAFS data,[7,8] each iron atom in MMO has four to six O or N ligands. The Fe...Fe distance is equal to 3.4 Å both in oxidized and partially reduced hydroxylase. The absence of any indications of a short Fe—O distance, as in the case of Hr, testifies that there is no oxo bridge between the iron atoms. The OH bridge, whose formation should be accompanied by weakening of the antiferromagnetic interaction of the iron atoms, is suggested as an alternative. This is confirmed by the absence of any changes in the range >300 nm in the absorption spectra of oxidized hydroxylase and the absence of ν_s(Fe—O—Fe) in the Raman spectrum. The value of the constant of antiferromagnetic interaction found from the saturation curve of the ESR signal of the $Fe^{II}Fe^{III}$ form is $J = -32$ cm^{-1}. The ESR signal at $g = 8$ of the excited ($S = 2$) state of the Fe_2O cluster has recently been observed in the oxidized hydroxylase.[9] The analysis of the temperature dependence of this signal gives the value $J = 8$ cm^{-1}, which is more than an order of magnitude lower than those for Hr and RNR and agrees with the existence of the OH bridge. The parameters of the NGR spectra,[8] ΔE_Q = 1.05 mm s^{-1} for oxidized and 3.014 mm s^{-1} for reduced MMO, agree well with the assumption about the existence of both O and OH bridges. The bridging OH ligand in the $Fe^{II}Fe^{III}$ form is identified unambiguously and the terminal H_2O molecule is found on the basis of the spectra of proton ENDOR of semireduced hydroxylase.[10] Since Fe...Fe distances in the oxidized and partially reduced forms of hydroxylase are the same, one can believe that the OH bridge is retained in the oxidized form as well. At the same time, its retention is hardly probable in the reduced form in which the H_2O molecule can be expected to act as a bridge or any bridge other than a carboxylate one is absent. Indirect data are available that one imidazole group of histidine is coordinated to each iron atom in the active center of MMO.[11,12]

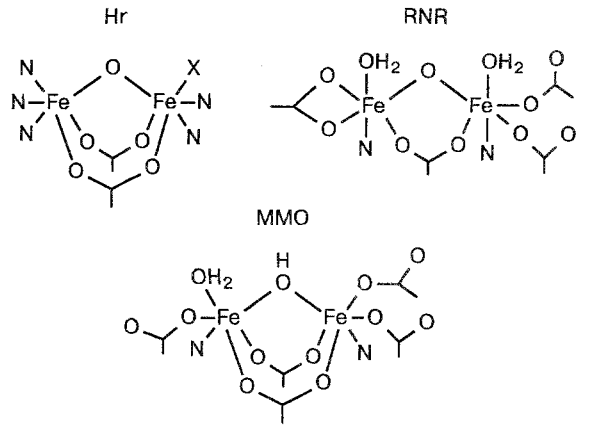
While the existence of the OH bridge in the $Fe^{II}Fe^{III}$ state is beyond doubt, the spectral and magnetic data for the $Fe^{III}Fe^{III}$ state can also be interpreted in terms of

the assumption about the formation of a strong intramolecular H bond involving the water molecule at the α-position of the μ-oxo bridge of the μ-O...H—O—Fe or μ-O...H—O...H—O—Fe type.[13] In fact, a considerable weakening of antiferromagnetic exchange interaction of the iron atoms occurs as the result of the H-bonding of the water molecule with the μ-oxo bridge of the binuclear Fe^{III} complex.[14]

A comparison of the sequence of amino acids in MMO and RNR based on the conception about the resemblance of their active centers makes it possible to advance some suggestions concerning the structure of hydroxylase,[15] which agree with the conclusions drawn from the spectral and magnetic data and confirmed by the X-ray diffraction analysis of MMO.

The data of the X-ray diffraction analysis on the structure of MMO from *V. capsulatus* (*Bath*)[16,17] not only confirmed the conclusions based on the spectral and magnetic studies, but also demonstrated the reliability of these methods at the modern level for obtaining data on the structures of active centers of enzymes. As expected, the structures of MMO and RNR turn out to be rather similar, including the same NO_5 coordination of ligands around each iron atom, where the N atom belongs to the imidazole group of histidine and the O atoms belong to the carboxylate groups of glutamic or aspartic acids. The X-ray data show unambiguously the existence of two Fe_2O centers in the hydroxylase component **A**. They are spaced symmetrically at 45 Å from one another, and each of them is in the four-spiral package, which supplies amino acid groups for coordination with the iron ions. The distance between the iron atoms in cluster Fe_2O is 3.4 Å, in agreement with EXAFS data. The iron atoms are bonded to four glutamine carboxylate groups, one of which acts as the bridge between the iron atoms and the three others act as monodentate ligands, and three imidazole groups of histidine (one group is at each iron atom). In addition, exogenic HO^- and AcO^- anions act as bridges, and an H_2O molecule at one of the iron atoms is at the *cis*-position relative to them. Each iron atom is in pseudo-octahedral coordination surroundings. The binuclear iron complex occurs in the hydrophobic cavity, which is suitable for binding CH_4. The regions of binding reductase and regulatory protein **B** in the immediate vicinity of the Fe_2O cluster are also identified. This allows one to explain the effect of protein **B** on the ESR spectrum of hydroxylase. Exogenic acetate bonded to the Fe_2O center can indicate the point of interaction of O_2, methane, or the methoxide formed with the core of the enzyme.

The structures of MMO and RNR molecules can be compared on the basis of X-ray data. The hydrophobic cavity in MMO is considerably larger than that in RNR, in agreement with the capability of the first enzyme to bind hydrophobic substrates. The ε-N atoms of the



imidazole groups are bonded by a hydrogen bond to the anions of aspartic acid, increasing the pK_a of the bridge hydroxide ligand in MMO compared to that in RNR, which has only one H-bonded residue of aspartic acid. The site occupied by tyrosine in RNR is occupied by cysteine in MMO, which allowed the authors[18] to advance an unconventional hypothesis about the catalytic mechanism of MMO involving the SH group. Thr 213 is the only protonated group except cysteine in the vicinity of the hydrophobic cavity. It is probable that Thr 213 is necessary for proton transfer during the catalytic cycle, similar to the so-called charge-transfer system in cytochrome P450.

The kinetic analysis of methane oxidation involving soluble MMO[19] indicates that the reduced enzyme-substrate complex binds an O_2 molecule to form a triple complex, which decomposes at the limiting stage to yield methanol and water.

$$E \overset{CH_4}{\rightleftharpoons} E{\cdot}CH_4{\cdot}NADH \overset{O_2}{\rightleftharpoons} EH_2{\cdot}CH_4 \rightleftharpoons EH_2{\cdot}CH_4{\cdot}O_2 \longrightarrow P$$

It is still impossible to explain the capability of MMO to bind methane efficiently (Michaelis constant $K_M = 3 \cdot 10^{-6}$ mol L^{-1}). The measure of the enzyme affinity to a substrate, which is determined by the ratio of the maximum rate to the Michaelis constant, is ~10 times higher for methane than for other alkanes. This shows that MMO is highly suitable for the oxidation of CH_4. Although the active center of MMO is highly hydrophobic, the hydrophoby itself cannot explain the stronger binding of methane as compared to other alkanes. In addition, the Michaelis constant for CH_4 increases with temperature and decreases for the hydrophobic binding (for example, in the case of cytochrome P450). In this context, the binding of CH_4 with the metal ion in the active center of MMO was suggested but has still not been confirmed.

Hydroxylase reduced by dithionite can oxidize methane with oxygen stoichiometrically (one cycle) in the absence of reductase. This proves that the function of the latter is only the transfer of electrons to hydroxylase.

Hydroxylase can catalyze the oxidation of hydrocarbons by hydrogen peroxide in the absence of a reducing agent, but with a lower efficiency.

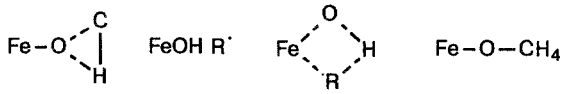
Regulatory protein **B** plays an important and in many respects puzzling role in the oxidation process.[17–19] This protein controls the transfer of electrons from reductase to hydroxylase in such a way that the transfer occurs only in the presence of substrates. It also shifts the redox potential of the enzyme-substrate complex, favoring two-electron reduction of hydroxylase. And finally, it increases the rate of substrate oxidation and changes substantially the regioselectivity of the oxidation. The X-ray diffraction studies show that protein **B** binds the part of hydroxylase that is in the immediate vicinity of the Fe_2O center and probably changes the coordination surroundings of iron atoms. This conclusion is confirmed by the observed perturbation of the ESR spectrum of semi-reduced hydroxylase when it binds protein **B**.

The considerable success achieved in recent years in studying heme monooxygenase of cytochrome P450 and its structural functional modelling by metalloporphyrin complexes[20] evoked a certain interest to extend to MMO the concepts about the mechanism of functioning of cytochrome P450, especially as similar selectivities in the hydroxylation of some substrates were observed. In fact, despite sufficient differences in the structures, cytochrome P450 and MMO have certain functions that resemble those of monooxygenase and a series of common substrates. However, there are obvious differences in the mechanisms of binding and activation of O_2. In addition, methane cannot be oxidized by cytochrome P450 and its iron porphyrin models. Therefore, one may assume that the binuclear nature of the active center plays a significant role in the mechanism of MMO functioning. It is accepted for cytochrome P450 (and it is reliably proven for its metalloporphyrin models)[20] that the heme monooxygenase oxidation of hydrocarbons involves ferryl intermediates. The mechanism of the activation of O_2 by iron porphyrin complexes[20,21] involves the formation of the $Fe—O_2$ complex and its stepwise reduction to a peroxide state followed by heterolytic elimination of O^{2-} to form an active intermediate bearing an oxene O atom.

$$PFe^{III} \xrightarrow{\bar{e}} PFe^{II} \xrightarrow{O_2} PFe^{III}O_2^{-\cdot} \xrightarrow{\bar{e},\,H^+} PFe^{III}—OOH \xrightarrow{H^+}$$

$$\longrightarrow P^{+\cdot}Fe^{IV}=O$$

The transformation of the peroxide intermediate into the active carrier of the O atom requires an acceptor of O^{2-}. The ions M^{n+}, Ac^+, or H^+ can act as such acceptors. Thus, the stage of activation of $O^2{}_2{}^-$ accompanied by detachment of O^{2-} precedes the key reaction of monooxygenase enzymes, the transfer of the O atom to the substrate: O^{2-}/O.



1 **2** **3** **4**

The problem of the reaction between the ferryl intermediate and the C—H bond of alkane has long been discussed in the literature. The early views about the synchronous insertion of the O atom *via* a mechanism including the three-centered transition state **1**, which is in accordance with the predominant retention of the configuration at the carbon atom, has later given way to the idea of a detachment-recombination mechanism with the intermediate formation of radical pair **2**. This made it possible to explain the partial racemization and the large isotope effect. Based on the analogies with alkane activation by complexes of high-valence metals, similar high negative values of the activation entropies, large isotope effects, and probable tunneling, the reaction route *via* four-centered transition state **3** has been suggested.[21] This route is characterized by an important role of the metal in the activation of the C—H bond. In this mechanism, unlike **2**, the formation of the radical pair as one of the reaction routes after the transition state is possible, which is realized in the case of a relatively weak C—H bond and more stable radicals. Recently[22] all these mechanisms have been critically revised, and the mechanism of synchronous insertion of the oxygen atom supplemented with the preliminary formation of ferryl—alkane complex **4** containing the pentacoordinate carbon atom has been preferred. In this latter mechanism, the metal acts as the ligand at the O atom rather than the center of a complex formation.

The following mechanism of alkane oxidation involving MMO is presently accepted as the most probable.[5,23] Alkane binding is followed by fast two-electron reduction of μ-hydroxo-Fe_2^{III} to the μ-hydroxo-Fe_2^{II} state. One-electron reduction can result in the formation of the μ-hydroxo-$Fe^{II}Fe^{III}$ intermediate, which can be detected under special conditions. It is shown that this intermediate reacts slowly with oxygen. On the contrary, the Fe_2^{II} state rapidly binds O_2 to form a hydroperoxide intermediate, which is probably similar to oxyhemoerythrin. Then the heterolytic breaking of the O—O bond can take place to form the active $Fe^0Fe{=}O$ intermediate. The homolytic breaking of the O—O bond to form the ·OH radical has not previously been ruled out at this stage, but the absence of an ·OH radical in the catalytic cycle of MMO has recently been proved by ESR traps.[24] It is also noteworthy that the active intermediate includes an O atom from molecular oxygen almost without an exchange with water, which is typical of ferryl. It is assumed that the binuclear ferryl intermediate formed is capable of reacting with alkanes *via* a detachment-recombination mechanism, and the second

stage (recombination) is rather slow, which leads to the lack of complete retention of the configuration in the oxidation of stereoisomers of dimethylcyclohexane and makes it possible to detect alkyl radicals by ESR traps. In fact, the study of the oxidation of (*S*)- or (*R*)-[1-$^2H_1$, 1-$^3H_1$]ethane to ethanol catalyzed by MMO from *Ms. trichosporium* shows[24] that the hydroxylation occurs with the predominant retention of configuration. At the same time, the relatively high degree of inversion (35 %) agrees well with the intermediate formation of ethyl radical. It should be mentioned that these results can also be explained[22] by mechanism (4), taking into account the possibility of rearrangement in the intermediately formed complex with pentacoordinate carbon. The intramolecular kinetic isotope effect k_H/k_D is equal to 4.2 to 0.2.

The intramolecular isotope effect for the hydroxylation of *exo,exo,exo,exo*-2,3,5,6-d_4-norbornane by MMO from *Ms. trichosporium* is equal to or greater than 5.5.[25] The authors pointed out the resemblance of the distribution of products to cytochrome P450 of liver and assumed a similar mechanism.

Substrate probes, which are called radical clocks, are used to prove the presence of radical intermediates in reactions of O atom transfer. Such reagents can be rapidly rearranged after the elimination of the H atom to yield racemized products. The use of the fastest radical clock tested for cytochrome P450 and other monooxygenases[18,26] shows no products, which is in agreement with the formation of hydrocarbon radicals, in the case of MMO from *Mc. capsulatus,* although these products are found for MMO from *Ms. trichosporium.* The authors[26] conclude that no unified mechanism can explain all data on MMO and it is likely that different mechanisms are probable, depending on the origin of MMO, steric and energy requirements of the substrate, and probably other factors. Based on studying the products of oxidation of various substrates, including alkanes, other authors[23] also arrive at the same opinion about the dual mechanism of MMO, which includes both two- and one-electron routes.

The absence of an intermolecular isotope effect in alkane hydroxylation shows that the breaking of the C—H bond is not the limiting stage in the overall enzymatic reaction. At the same time, a considerable intramolecular isotope effect indicates a substantial stretching of the C—H bond in the transition state or the tunneling of a proton. Taking into account that the tunneling is observed in the reactions of carbenes with alkanes, it is also possible for similar processes of the transfer of the O atom to the C—H bond. The intramolecular isotope effect k_H/k_D is equal to 5.1 for the oxidation of the methyl group of phenylmethylcyclopropane in the presence of MMO from *Mc. capsulatus* (*Bath*)[26] and 4.2 in the oxidation of ethane in the presence of MMO from *Ms. trichosporium.*[24] It should be mentioned that $k_H/k_D = 5$ for the oxidation of alkanes by dimethyloxirane *via* a three-centered transition state.[18]

The heme monooxygenase oxidation (with cytochrome P450 and its models) is considered to occur with the participation of ferryl complexes containing the terminally bonded O atom ($P^{+\cdot}M^{IV}=O$ or $PM^{V}=O$). It is assumed that the non-heme monooxygenase oxidation also includes the mononuclear ($Fe^{V}=O$) or binuclear ($Fe^{IV}—O—Fe^{IV}=O$ for MMO) analog of the heme complex.

The first ferryl complex with the radical cation of the non-heme ligand has recently[27] been characterized, and its reactions with alkanes have been described. In this mononuclear complex, pyridine ligands act as the electron buffer similar to porphyrin in cytochrome P450. Although the imidazole group of histidine or the second iron atom could play the role of such a buffer in MMO, it is hardly probable that they could provide sufficient stability for the existence of such a particle as a significant intermediate. The possibility of the formation of ferryl particles under biological conditions has been questioned in the literature for non-heme systems in the absence of a sufficiently polarized ligand. It is shown[28] that activated bleomycin, a natural antibiotic containing a non-porphyrin mononuclear iron complex, which is capable of oxidizing hydrocarbons with the transfer of an O atom similarly to cytochrome P450, is in fact the peroxide FeOOH complex rather than ferryl as assumed previously.

Various donors of an O atom such as PhIO, $NaIO_4$, and Bu^tOOH that are active in the case of cytochrome P450 and its models, are inefficient for MMO, although H_2O_2 can be used instead of O_2/NADH in the oxidation of *n*-alkanes, but with a low efficiency and different regioselectivity. These results cast some doubt on the possibility of including ferryl as the active intermediate of MMO. The studies[26] show that the peroxide precursor of ferryl FeOX containing the bridging O atom is sufficiently active to transfer this O atom to the C—H bonds of alkanes. These active intermediates seem to be considerably more preferable for functioning under physiological conditions than the energetically less favorable ferryl intermediates, especially in the case of non-heme systems.[13,21]

Based on the X-ray studies[16] and the comparison[15] of the sequence of amino acids in RNR and MMO, the presence of cysteine-151 is established at the same site relative to Fe_2O, which is occupied by the tyrosyl radical in RNR. Assuming that this is not accidental and taking into account the large resemblance of the structures and functions of MMO and RNR, the authors[18] proposed a new mechanism for methane oxidation involving the RS$^{\cdot}$ radical of cysteine at the stage of methane activation. They assume that O_2 binds a reduced hydroxylase to form η_2,η_2-peroxide, which reacts with the SH group of cysteine-151 to transform to the radical pair. The breaking of the C—H bond occurs *via* a concerted mechanism *via* the reaction with this radical pair to form a coordinated alcohol and regenerate the SH group of cysteine.

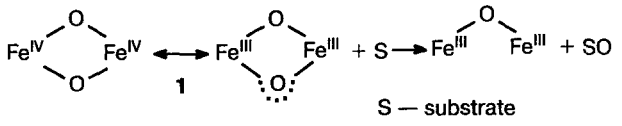
Modern concepts about the mechanism of MMO action cannot explain the oxidation of methane, nor the inverse series of reactivities of alkanes with maxima for methane and ethane, nor the unusual regioselectivity of the oxidation of alkanes.

Ferryl can be considered as the complex of an O atom with Fe^{II}, and perferryl can be considered as the complex with Fe^{III}.

$$Fe^{II}{:}O: \longleftrightarrow Fe^{III}-O^{\cdot} \longleftrightarrow Fe^{IV}=O$$

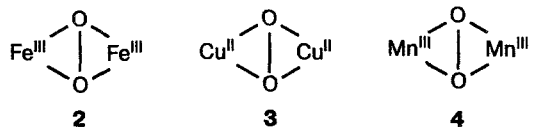
$$Fe^{III}{:}O: \longleftrightarrow Fe^{IV}-O^{\cdot} \longleftrightarrow Fe^{V}=O$$

In the case of binuclear iron complexes, the O atom can be bonded to two Fe atoms in the structure of the bis-μ-oxo complex of Fe^{IV} **1**, which is an oxenoid reagent in this case, similar to peroxides and peracids, and is an alternative to ferryl. It is likely that carboxylate should be the third bridge connecting two iron ions, and the bis-μ-oxodiferryl fragment can be nonplanar. The process of the transfer of the O atom from **1** should be favored by the extreme stability of binuclear μ-oxo complexes of iron(III) at physiological pH.



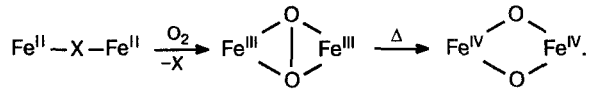
Similar binuclear complexes of Mn^{IV} are strong oxidants capable of evolving O_2 from water or hydrogen peroxide and the oxidizing alkanes.[29] These complexes are still unknown for iron, probably due to their high reactivities as oxidants. Although **1** should be thermodynamically more stable than ferryl, especially in nonpolar media, its chemical reactivity can be even higher due to the participation of two Fe atoms in the transition state of the transfer of the O atom. In fact, it is shown for the analogs of molybdenum O-transferases[30] that the rate of transfer of the O atom from $Mo^{VI}=O$ to the substrate increases when the terminal O atom is bonded by one more Mo atom to become a bridging atom, Mo=O:→Mo. This is clear, because the electrophilicity of the O atom is enhanced by two electron-withdrawing iron ions. Similarly to ferryl, **1** can react with the C—H bond *via* a two-electron insertion or one-electron elimination of the H atom. The latter process is more probable in the case of sterically hindered and weak C—H bonds, *i.e.*, its probability increases in the series CH_4 < primary CH < *sec*-CH < *tert*-CH.

Active intermediate **1** can be obtained from molecular oxygen *via* the stage of μ-1,2-peroxide, Fe—O—O—Fe. However, the η_2,η_2'-peroxide complex (**2**) is its most reasonable precursor, and we assumed it to be the first product of the reaction of reduced MMO with O_2, while other authors[26] consider it as one of the probable active intermediates.



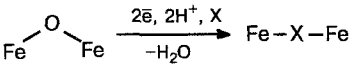
2 **3** **4**

The oxygen complex of hemocyanine (**3**) has a similar structure,[31] and it follows from the theoretical calculations of manganese complex **4** that the binding of O_2 to form this complex is energetically favorable and probably irreversible.[32] The side binding of peroxide is often realized in the case of mononuclear peroxide complexes of Fe^{III}, and there are no grounds to deny the possibility of similar structures for binuclear complexes. Compound **2** is attractive as the first product of the reaction with O_2, because **1** can be formed from **2** by a simple electron transfer without a substantial rearrangement of nuclei. Thus, a fundamentally new mechanism of O_2 activation that is possible only for binuclear complexes can be suggested.



In fact, in the case of iron porphyrin complexes, the peroxide intermediate should react at first with the external electrophilic acceptor (H^+, CH_3CO^+) [24] to remove O^{2-} and form an active intermediate.

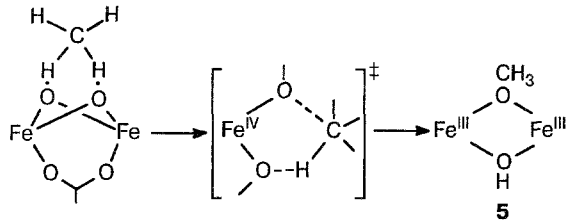
In the case of binuclear complexes, two iron ions are likely to be capable of accepting the O^{2-} anion without the participation of an external electrophile, as occurs in the case of Ph_2SnO_2.[33] The accepted O^{2-} anion leaves the reaction center in the form of H_2O at the subsequent stage of the reduction of the Fe_2O complex, after the completion of the substrate oxidation, or it is displaced by the O_2 molecule at the next stage of the catalytic cycle.



Thus, the different sequence of the detachment of the fragments of the activated oxygen molecule is assumed in the new mechanism: O^{2-}/O in the case of iron porphyrin complexes and O/O^{2-} in the case of binuclear iron complexes. A similar mechanism can also act in the case of RNR, which has many common features with MMO.

The inclusion of bis-μ-oxo complex **1** in the catalytic cycle of MMO makes it possible to explain the unusual regioselectivity and specificity of the oxidation of al-

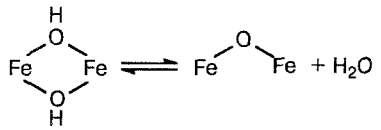
kanes by this enzyme. Taking into account the capability of the C—H bond of forming H bonds with the O atom of ether,[34] one can assume the occurrence of H-bonding of methane and other alkanes with the μ-oxo bridge, which can exhibit a noticeable basicity in the non-solvating hydrophobic cavity of the active center of MMO. A stronger binding of methane in the active center of MMO as compared to other alkanes can be explained by the formation of such an enzyme-substrate complex due to one or two μ-oxo bridges, because the acidity of methane is the highest: CH_4 > primary CH > *sec*-CH > *tert*-CH. The O atom can be transferred to the C—H bond *via* a concerted mechanism by the electrophilic attack of one of the bridge atoms, as assumed previously[35] for solvated oxenoid. The process as a whole can be considered as an electrophilic attack on the C—H bond with nucleophilic assistance *via* the multi-centered cyclic transition state.[36]



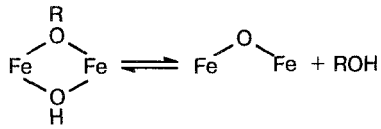
5

The determining role of nucleophilic assistance and/or taking into account steric hindrances (see below) make it possible to explain the highest value of the rate constant in the case of methane compared to other alkanes.

Complex **5** between the enzyme and the reaction product is similar to the well-known bis-μ-hydroxo complexes of iron(III). These complexes are typical of equilibrium (1).



(1)



(2)

Similar equilibrium (2) can also result in the formation of the same reaction product.

The analysis by molecular models shows that the mechanism involving the transfer of an O atom is suitable only for CH_4 and a terminal CH_3 group (the primary C—H bond). For secondary and ternary C—H bonds this mechanism is less probable due to steric hindrances of adjacent alkyl groups. In this case another mechanism is probable, which includes electron transfer, with the assistance of a proton to form an alkyl radical at the initial stage.[36] Although the reaction of intermediate **1** with alkane is related to spatial restrictions, the estimations show that they are not so pronounced as to make this mechanism improbable. On the other hand, the existence of steric hindrances explains well the selectivity of alkane oxidation by MMO and the possibility of two mechanisms.

The hypothesis about the new type of O_2 activation can be verified by two cycles of the reaction with MMO using labeled oxygen under the conditions when the exchange with the μ-bridge is impossible. As a result of the first cycle, the label should be equally distributed between the product (alcohol) and the μ-oxo bridge. The label should go into water after the second cycle. A similar experiment, which has recently been carried out for RNR, shows[37] that molecular oxygen is the source of the μ-oxo bridge in the reaction between $Fe^{II}Fe^{II}$ of RNR and O_2. The authors assumed that μ-1,1-peroxide and ferryl are intermediates. However, taking into account the aforesaid, the scheme involving μ-η_2,η_2-peroxide and bis-μ-oxo complex seems to be more reasonable. In principle, the aromatic C—H bond has no serious steric hindrances to its reaction with the μ-oxo center followed by the insertion of the O atom. However, possible positions of the aromatic ring of tyrosine-122 in RNR are restricted by the requirements imposed by the protein structure. Therefore, its reaction with the suggested bis-μ-oxo intermediate can involve only an electron transfer rather than the insertion of μ-O into the aromatic C—H bond. The μ-O$^{\cdot}$ intermediate formed has previously been observed by spectral methods.[38]

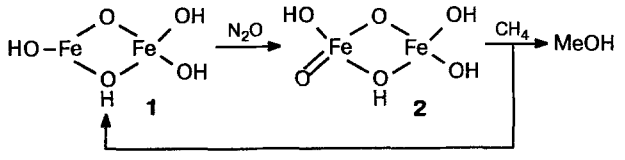
Let us consider some recent observations which testify in favor of the new concept of the catalytic cycle of MMO. The formation of three intermediates **P**, **Q**, and **T** during one cycle of MMO from *Ms.trichosporium* has been detected[39] by kinetic methods of studying fast reactions. A colorless intermediate P is formed immediately after mixing reduced MMO with O_2, and then it is transformed slowly to **Q** (λ_{max} = 330, 430 nm, ε=8000 $M^{-1}cm^{-1}$). The rate of disappearance of **Q** depends on the nature and concentration of a substrate, thus indicating that **Q** is an active intermediate. Finally, the intermediate **T** formed in the reaction between **Q** and a substrate gives the product at that stage, which determines the rate of the whole process. According to NGR,[40] compound **Q** is a diamagnetic binuclear complex containing two indistinguishable Fe^{IV} atoms (S=2). This means that the active oxygen occupies the symmetric position in the cluster and the iron atoms are antiferromagnetically bonded. The value of the exchange coupling constant J>−60 cm^{-1} ($H = J \cdot S_1 \cdot S_2$) corresponds to the μ-oxo bridge between the iron atoms and agrees with structure **1** for intermediate **Q**. Structures **2** and **5** can be assigned to intermediates **P** and **T**. In fact, it is unlikely that the highly symmetric and weakly polarized complex **2** can absorb noticeably in the visible spectral region, and the decomposition of complex **5** to

the final product is related to the drastic rearrangement of the structure and, hence, should be a relatively slow process.

These results were developed[41] for MMO from *Mc. capsulatus (Bath)* using the same methods. An intermediate similar to **Q** with λ_{max} 350, 420, and 520 nm, which, however, has non-equivalent iron atoms, is characterized in this work. The authors mention that the average isomeric shift δ in the NGR spectrum of this intermediate, which characterizes the oxidation state of iron, is close, but nevertheless, differs from the values characteristic of both heme and non-heme Fe=O. The information for intermediate **L** is the most interesting. This intermediate has one quadruple doublet in the NGR spectrum, which indicates that two iron atoms are in identical coordination surroundings. The parameters of the NGR spectrum for **L** (δ = 0.66 mm s^{-1} and ΔE_Q = 1.51 mm s^{-1}) are very unusual for carboxylate-bridge Fe_2 clusters. These parameters agree well with structure **2** for **L**.

The stoichiometric oxidation of methane, CO, and benzene has been found[42] at room temperature on the so-called α-centers of iron-containing zeolites ZSM-5, which, according to theoretical estimations,[43] are surface hydroxide iron clusters with structure **1**.

The dissociation of N_2O on the α-centers results in the addition of an O atom to form an active oxidant of the suggested structure **2**, which stoichiometrically oxidizes methane to methanol at room temperature:



Taking into account the results in Ref. 30, a bridging oxygen should be active rather than a terminal one. However, if the bridging OH is more acidic than the terminal oxygen, the real structure of the active center should rather correspond to the isomeric bis-μ-oxo complex. It is likely that this is the best functional model of MMO known at the present time. It is interesting that no other transition metals except iron can form similar α-centers.

The first $Fe_2^{III}(\mu\text{-OH})(\mu\text{-O})$ iron complex, which is capable of dehydrogenating 1-Me-1,4-cyclohexadiene to toluene and transferring an O atom to transform PPh_3 into phosphine oxide, has recently been synthesized.[44] There are no grounds to assume that the change in the coordination surroundings of iron cannot make such a complex capable of transferring the μ-oxo O atom to the C—H bond of an alkane molecule.

The existence of monooxygenase processes of alkane oxidation, especially the oxidation of methane, which is the most inert alkane, is a serious challenge for chemists in the development of similar chemical systems that can selectively oxidize these hydrocarbons under ambient conditions.* The attempts to model biological methane oxidation have failed so far. Further study of methane oxidation on the α-centers of iron-containing zeolites ZSM-5 should show whether this system is a structural model of the active center of MMO. In the past 10 years many μ-oxo-μ-carboxylate iron complexes have been synthesized,[45] which satisfactorily model structural, spectral, and magnetic properties of Hr, but they model to a lesser extent those properties of RNR and MMO. It is assumed that many of these complexes are unable to catalyze oxidation processes due to the absence in the coordination sphere of free or labile sites that bind O_2 or donors of oxygen. In this connection, the synthesis of similar complexes has been carried out recently.[46] However, it becomes clear that many other factors should also be taken into account. Since high-valentce iron complexes formed in the reduction of activated O_2 are strong oxidants, they can react with the Fe^{II} and Fe^{III} forms that simultaneously exist in solution and thus lose the capability of oxidizing of alkanes. To use these active intermediates only in reactions with alkanes, bimolecular reactions of iron complexes should be ruled out, *e.g.*, by supporting the complexes onto a surface. This possibility has recently been realized.[47] It is shown that methane can be oxidized by molecular oxygen with low efficiency with the participation of complexes obtained by self-assembly on the surface of silica gel modified by imidazole groups.

The oxidation of methane and other alkanes by hydrogen peroxide catalyzed by the complex $[Fe_2O(bpy)_2(OBz)_2(H_2O)_2](ClO_4)_2$ in acetonitrile has been studied.[48] The mechanism of oxidation includes the replacement of a water molecule by an H_2O_2 molecule followed by the heterolytic decomposition of a peroxide intermediate. The observed value of KIE (k_H/k_D = 3.0) and selectivity in the oxidation of hexane, cyclopentane, and cyclohexane allow one to assume the participation of FeO, which is formed in the decomposition of the FeOOH intermediate. Similar complexes with labile coordination spheres are more active in alkane oxidation than complexes with closed coordination spheres. Reliable proofs for the metal complex nature of the active intermediate in alkane oxidation by *tert*-butyl hydroperoxide in MeCN, involving binuclear complexes of the $[Fe_2(TPA)_2O(OAc)](ClO_4)_3$ type, have recently appeared.[49] The replacement of tris-2-pyridylmethylamine in the complex by similar tridentate ligands affects the KIE value in the functionalization of the C—H bond, which directly indicates that the iron complex participates in the transfer of an O atom. The authors succeeded in distinguishing the contributions of

* The simple and rather efficient models of non-heme monooxygenases (so-called Gif systems) for selective alkane oxidation have recently been found. For detailed information see the review by D. Barton and D. Taylor in *Izv. Akad. Nauk, Ser. Khim.*, 1995, 595 [*Russ. Chem. Bull.*, 1995, **44**, 575 (Engl.Transl.)].

the radical and oxenoid reactions in the formation of the reaction products. The addition of dimethyl sulfide completely suppresses the formation of ketone and alcohol but exerts no effect on the formation of $Bu^tOO—C_6H_{11}$. In addition, KIE, which is equal to 7.7±0.5 in the formation of $Bu^tOO—C_6H_{11}$, is independent of the nature of the complex used, *i.e.*, in this case the C—H bond breaking is not related to the metal complex intermediate.

Binuclear μ-oxo iron complexes $[Fe_2OL_4(H_2O)_2](ClO_4)_4$, where L = bpy, phen, or their substituted derivatives, which do not contain carboxylate bridges but have labile water molecules in the coordination sphere, also catalyze the transfer of an O atom from Bu^tOOH or H_2O_2.[50,51] The yield of the products, turnover number, and stability of the catalyst depend on the nature of ligand **L**. The observed rate of oxidation of cyclohexane (16 turnovers/min) is one of the highest for similar systems. KIE in the oxidation of cyclohexane (k_H/k_D) is equal to 3 to 4.8, depending on the ligand. Epoxide is the main product in the oxidation of *trans*-stilbene. The common disadvantage of similar systems is the instability of binuclear iron complexes in the catalytic transformation, which prevents reliable information on the nature of active intermediates from being obtained. In addition, the ligand surroundings of the iron atoms in these complexes are established much less reliably than those of the active center of MMO.

Thus, only the first steps in modelling of MMO have been taken. The problem as a whole is still far from solution. There are few systems in which the participation of well characterized binuclear complexes in alkane oxidation is proved, and reliable proofs of the nature of active intermediates are almost lacking. At the same time, the available information shows that the creation of efficient analogs of MMO is a problem of the nearest future. The stabilization of binuclear complexes during the catalytic cycle is one of an important problems. The preliminary data show that great progress can be expected for the immobilization of complexes on a solid substrate. The large amount of new information on MMO accumulated in the last several years and the appearance of new hypotheses concerning methane and oxygen activation allow a new mode of thought with regard to this problem. In particular, it is of interest to synthesize bis-μ-oxo complexes and η_2,η_2-peroxides, which are still unknown for iron, and to study their reactivity. The establishment of the structure of MMO provides reliable grounds for its structural functional modelling. However, the proficiency of chemists will be required for designing efficient structural functional analogs. It is likely that, as in the case of cytochrome P450, the design and synthesis of more complicated model complexes, which take into account more precisely the structure of the binuclear iron complex and the character of its surroundings in the protein globule, will be significant.

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References

1. C. Antony, *The Biochemistry of Methylotrophs*, Acad. Press, New York, 1982.
2. J. B. Vincent, G. L. Olivier-Lilley, and B. A. Averill, *Chem. Revs.*, 1990, **90**, 1447.
3. J. Colby and H. Dalton, *Biochem. J.*, 1978, **171**, 461.
4. B. G. Fox, W. A. Froland, J. E. Dege, and J. D. Lipscomb, *J. Biol. Chem.*, 1989, **264**, 10023.
5. J. Green and H. Dalton, *J. Biol. Chem.*, 1989, **264**, 17698.
6. M. R. Woodland, D. S. Patil, R. Cammack, and H. Dalton, *Biochim. Biophys. Acta*, 1986, **873**, 237.
7. R. C. Prince, G. N. George, J. C. Savas, S. P. Cramer, and R. N. Patel, *Biochim. Biophys. Acta*, 1988, **952**, 220.
8. J. G. Dewitt, J. G. Bentsen, A. C. Rosenzweig, B. Hedman, J. Green, S. Pilkington, G. C. Papaefthymiou, H. Dalton, K. O. Hodgson, and S. J. Lippard, *J. Am. Chem. Soc.*, 1991, **113**, 9219.
9. B. G. Fox, M. P. Hendrich, K. K. Surerus, K. K. Andersson, W. A. Froland, J. D. Lipscomb, and E. Muenk, *J. Am. Chem. Soc.*, 1993, **115**, 3688.
10. V. DeRose, K. E. Liu, S. J. Lippard, and B. Hoffman, *J. Am. Chem. Soc.*, 1993, **115**, 6440.
11. M. P. Hendrich, B. G. Fox, K. K. Anderson, P. G. Debrunner, and J. D. Lipscomb, *J. Inorg. Biochem.*, 1991, **43**, 550.
12. D. D. Smith and H. Dalton, *Eur. J. Biochem.*, 1992, **210**, 629.
13. A. A. Shteinman, *Dokl. Akad. Nauk*, 1992, **324**, 617 [*Dokl. Chem.*, 1992, **324** (Engl. Transl.)].
14. K. J. Oberhausen, J. F. Richardson, R. J. O'Brien, R. M. Buchanan, J. K. McCusker, R. J. Webb, and D. N. Hendrickson, *Inorg. Chem.*, 1992, **31**, 1123.
15. P. Nordlund, H. Dalton, and H. Eklund, *FEBS Letters*, 1992, **307**, 257.
16. A. C. Rosenzweig, C. A. Frederick, S. J. Lippard, and P. Nordlund, *Nature*, 1993, **366**, 537.
17. A. L. Feig and S. J. Lippard, *Chem. Rev.*, 1994, **94**, 759.
18. A. C. Rosenzweig and S. J. Lippard, *Acc. Chem. Res.*, 1994, **27**, 229.
19. J. Green and H. Dalton, *Biochem. J.*, 1986, **236**, 155.
20. A. M. Khenkin and A. A. Shteinman, *Kinet. Katal.*, 1989, **30**, 7 [*Kinet. Catal.*, 1989, **30** (Engl. Transl.)].
21. A. A. Shteinman, *Izv. Akad. Nauk, Ser. Khim.*, 1993, 272 [*Russ. Chem. Bull.*, 1993, **42**, 227 (Engl. Transl.)].
22. A. A. Shestakov and A. E. Shilov, *Zh. Obshch. Khim.*, 1995 (in press) [*J. Gen. Chem.*, 1995, in press (Engl. Transl.)].
23. H. Dalton, P. C. Wilkins, and Y. Jiang, *Biochem. Soc. Trans.*, 1993, **21**, 749.
24. N. D. Priestley, H. G. Floss, W. A. Froland, J. D. Lipscomb, P. G. Williams, and H. Morimoto, *J. Am. Chem. Soc.*, 1992, **114**, 7561.
25. M. J. Rataj, J. E. Kanth, and M. I. Donnally, *J. Biol. Chem.*, 1991, **266**, 18684.
26. K. E. Liu, C. C. Johnson, M. Newcomb, and S. J. Lippard, *J. Am. Chem. Soc.*, 1993, **115**, 939.

27. R. A. Leising, B. A. Brennan, L. Que, B. G. Fox, and E. Munck, *J. Am. Chem. Soc.*, 1991, **113**, 3988.
28. S. W. Sam, X.-J. Tang, and J. Peisach, *J. Am. Chem. Soc.*, 1994, **116**, 5250.
29. A. Sorokin, A. Robert, and B. Meunier, *J. Am. Chem. Soc.*, 1993, **115**, 7293.
29. J. E. Sarneski, D. Michos, H. H. Thorp, M. Didiuk, T. Poon, J. Blewitt, G. W. Brudvig, and R. N. Crabtree, *Tetr. Lett.*, 1991, **32**, 1153.
30. S. Bhattacharjee and R. Bhattacharyya, *J. Chem. Soc., Dalton*, 1993, 1151.
31. K. Magnus and H. Ton-Thot, *J. Inorg. Biochem.*, 1992, **47**, 20.
32. D. M. Proserpio, R. Hoffmann, and G. Ch. Dismukes, *J. Am. Chem. Soc.*, 1992, **114**, 4374.
33. N. Z. Muradov, A. E. Shilov, and A. A. Shteinman, *React. Kin. Cat. Lett.*, 1975, **2**, 417.
34. J. J. Novoa, P. Constans, and M.-H. Whrang, *Angew. Chem. Int. Ed.*, 1993, **32**, 588.
35. R. D. Bach, *J. Am. Chem. Soc.*, 1993, **115**, 5768.
36. E. S. Rudakov, *Reaktsii alkanov s okislitelyami, metallokompleksami i radikalami v rastvorakh* [*Reactions of Alkanes with Oxidants, Metal Complexes, and Radicals in Solutions*], Naukova Dumka, Kiev, 1985 (in Russian).
37. J. Ling, M. Sahlin, B. M. Sjoeberg, T. M. Loehr, and J. Sanders-Loehr, *J. Biol. Chem*, 1994, **269**, 5595.
38. J. M. Bollinger, D. E. Edmondson, B. H. Huynh, J. Filley, J. R. Norton, and J. Stubbe, *Science*, 1991, **253**, 292.
39. S. K. Lee, J. C. Nesheim, and J. D. Lipscomb, *J. Biol. Chem.*, 1993, **268**, 21569.
40. S. K. Lee, B. G. Fox, W. A. Froland, J. D. Lipscomb, and E. Munck, *J. Am. Chem. Soc.*, 1993, **115**, 6450.
41. K. E. Liu, D. Wang, B. H. Huynh, D. E. Edmondson, A. Salifoglou, and S. J. Lippard, *J. Am. Chem. Soc.*, 1994, **116**, 7465.
42. G. I. Panov, V. I. Sobolev, and A. I. Kharitonov, *J. Mol. Catal.*, 1990, **61**, 85.
43. M. J. Filatov, A. G. Pelmenschikov, and G. M. Zhidomirov, *J. Mol. Catal.*, 1993, **80**, 243.
44. Y. Zang, G. Pan, L. Que, B. G. Fox, and E. Muenck, *J. Am. Chem. Soc.*, 1994, **116**, 3653.
45. D. M. Kurtz, *Chem. Rev.*, 1990, **90**, 585.
46. A. A. Shteinman, *Mendeleev Commun.*, 1992, 155.
47. V. S. Be!ova, A. M. Khenkin, V. N. Postnov, V. E. Prusakov, A. E. Shilov, and M. L. Stepanova, *Mendeleev Commun.*, 1992, 7.
48. A. A. Shteinman, *Dokl. Akad. Nauk SSSR*, 1991, **321**, 775 [*Dokl. Chem.*, 1991, **321** (Engl. Transl.)].
49. R. A. Leising, J. Kim, M. A. Perez, and L. Que, *J. Am. Chem. Soc.*, 1993, **115**, 9524.
50. S. Menage, J. M. Vincent, C. Lambeaux, G. Chottard, A. Grand, and M. Fontecave, *Inorg. Chem.*, 1993, **32**, 4766.
51. S. Menage, J. M. Vincent, C. Lambeaux, and M. Fontecave, *J. Chem. Soc., Dalton*, 1994, 2081.

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