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Iron oxygenases: structure, mechanism and modelling

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Abstract. Recent advances in the field of studies of the structures and the mechanisms of action of iron-containing oxygenases are considered. Examples of chemical modelling of these enzymes are given, which is very important for the design of efficient bio-inspired catalysts for selective hydrocarbon oxidation and directed oxygenation of non-activated C−H and C=C bonds in multifunctional molecules. The bibliography includes 253 references.

I. Introduction

Oil and gas are accessible and cheap natural sources of hydrocarbons. Though new oil and gas fields are permanently being discovered, it is obvious that their reserves on Earth are limited. This valuable chemical raw material is preferentially used as fuel, however, even this application of natural gas is problematic due to remoteness of gasfields from populated areas and difficulties in transportation. The initial stage of the usage of hydrocarbons of petroleum and natural gas as chemical raw materials is their functionalisation, which faces certain difficulties, because non-activated C−H bonds are inert to the action of common reagents. This is also true, with minor reservations, for non-activated multiple C=C bonds. The most acceptable, from the environmental and economical viewpoints, way of functionalisation of hydrocarbons is their oxidation with dioxygen, despite the fact that latter is also rather inert under ambient conditions.

At present, there are two approaches to the solution of this problem. On the one hand, inert molecules of alkanes and alkenes can be activated by transition metal complexes [1] to form species that possess reactivity sufficient for their direct oxidation with dioxygen or its stable derivatives, *e.g.*, water. An example of this approach is the Wacker process (catalytic oxidation of alkenes to aldehydes in the presence of palladium salts) and activation of alkanes by platinum-group metal complexes, which is being extensively studied at present in the hope to create similar process for alkanes.[1,2] On the other hand, it is also possible to activate dioxygen to obtain its more reactive forms capable of reacting with alkanes and alkenes.[1] It is in this way that numerous processes of biological oxygenation occur in living nature.[3] Oxygenases that are used for oxidation of non-activated C−H and C=C bonds are metalloproteins, *i.e.*, metal complexes with highly ordered polypeptide chains as ligands.

By the end of the 20th century, the structure of the active site and mechanism of functioning of the main class of oxygenases, the cytochrome P450 family, *viz.*, mononuclear iron-containing haem-type enzymes, have been roughly established and the outstanding role of iron in biological activation of O_2 has been shown. At that time, studies on other classes of iron-containing oxygenases were initiated. Copper-containing oxygenases have also been found.

Cytocromes P450 play a key role in oxidative transformations of endogenous and exogenous molecules. This is one of the most abundant and important enzymes in nature.[4] By June 2006, more than 6000 isoforms of cytochrome P450 have been established, which are present in different tissues of various organisms of one or another branch of the 'tree of life' and differ in details of the structure of the active site and biological functions. The interest in the chemistry of cytochrome P450 aroused from the desire to understand how the enzyme, being in the aqueous medium at neutral pH and physiological temperatures can selectively perform one of the most important reactions in organic chemistry, *viz.*, hydroxylation of non-activated C−H bonds. High organisation of the enzyme active site is also evidenced from the ability of ω-hydroxylating isoforms of oxidising predominantly terminal methyl groups of a hydrocarbon chain in the presence of

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more easily oxidisable methylene groups. Comprehensive studies of the mechanism of action of cytochrome P450 during the last three decades allowed the elucidation of the role of biological activation of O_2 in regio- and stereoselective oxygenation of complex molecules. The importance of this problem is obvious, because at present, general direct methods for specific hydroxylation of non-activated C—H bonds are lacking in synthetic chemistry.

On the border of the 20th and 21st centuries, joint efforts of biologists, chemists and physicists resulted in a breakthrough in the studies of activation of O_2 in living systems and enzymatic oxygenation. First, it is concerned with the evaluation of the structure of highly reactive key intermediates in the catalytic cycle of cytochrome P450. The elucidation of the structure of the dinuclear active site of methane monooxygenase (MMO) containing two bound non-haem iron atoms was extremely important, as well as the registration of spectral characteristics of key intermediates in its catalytic cycle. The outstanding role of this bacterial enzyme consists in its ability to perform selective oxidation of extremely inert hydrocarbon, methane, under physiological conditions. During the last decade, a novel class of oxygenases, mononuclear iron-containing non-haem enzymes, have been discovered and comprehensively studied; the range of catalytic reactions of these oxygenases appeared to be wider than for cytochrome P450. Finally, the progress in the field of precise quantum chemical calculations for multiatomic systems containing transition metals significantly favoured the establishment of the mechanism of action of a large number of metalloenzymes including oxygenases.[5]

In parallel with the development of bioinorganic chemistry of oxygenation processes, currently a bio-inspired approach to catalysis is being emerged, *i.e.*, the principles of organisation and functioning of natural catalysts, enzymes, are used for the design of highly efficient and selective chemical catalysts for oxidation reactions (Refs 6 and 7).†

Numerous conferences and annual appearance of thousands of publications, as well as monographs and reviews (see, for example,[3–39]) devoted to particular oxygenases and their models reflect adequately the dynamic development of this field. In the present review, an attempt has been made to offer a general concept on iron-containing oxygenases and their models on the basis of the unified mechanism of action.

II. Activation of O_2 in biological systems. Cytochrome P450 as a catalyst for an oxygen atom formation and transfer

Enzymes with different mechanisms of catalytic action are involved in natural processes of activation of dioxygen molecule. Different substrates are oxidised with oxidases and oxygenases. The latter differ in their ability to oxygenate substrates, *i.e.*, to introduce one or both atoms of dioxygen into the substrate. The most difficult oxidation reactions, for instance, oxidation of non-activated C—H bonds, are performed by metal-containing oxidases and oxygenases.[3] Peroxidases and peroxygenases utilise hydrogen peroxide as the oxidant. Dioxygen can also be reducted on a metal site to peroxide, which is further activated on this site to form ultimately a significantly stronger oxidant, *viz.*, an oxo complex of a metal in a higher oxidation state. Monooxygenases are enzymes that catalyse oxygenation of a substrate by dioxygen in the presence of an endogenous reductant. In this case, one oxygen atom is incorporated into the substrate and the other forms water according to the stoichiometric equation:

$$RH + O_2 + 2H^+ + 2e^- \longrightarrow ROH + H_2O.$$

In the case of dioxygenases, both oxygen atoms are incorporated into substrate(s).

Many oxygenases contain transition metals, first of all iron. Copper-containing oxygenases are less abundant. Two electrons, which are required for the monooxygenase cycle to occur, are generated from an organic reductant that is necessarily present in the system under the action of reductase. Gaining electrons, the metal atom passes to the reduced state and acquires the ability to react with dioxygen thus activating it for the subsequent reaction with the substrate. The background for catalytic action of oxygenases is activation of dioxygen to form metal peroxo and oxo complexes. Haem and non-haem oxygenases differ in the structure of the catalytic site. The former contains iron porphyrin complex (haem); in the latter, the Fe atom of the active site is bound to the imidazole and carboxylate fragments of amino acids of the polypeptide chain.

Biological oxidation of different organic compounds under the action of haem oxygenase, cytochrome P450, has been studied in most detail. Lately, two families of non-haem monooxygenases have also been actively studied. Dinuclear non-haem oxygenases contain in the active site two iron atoms linked with one another, while mononuclear monooxygenases have one iron atom coordinated to two histidine and one glutamate or aspartate residues.

Cytochrome P450 is present in all living organisms, from bacteria to mammals, and bears different functions.[4,8–11] This versatile biological oxidation catalyst, along with hydroxylation of alkanes and other compounds containung C—H bonds, is able to perform dehydrogenation of saturated systems, epoxidation of alkenes, formation of *S*- and *N*-oxides, *etc*. However, selective hydroxylation of non-activated C—H bonds is considered to be the unique property of cytochrome P450 and other iron-containing oxygenases, which accounts for frequent use of the term 'hydroxylases' in relation to them. High organisation of the active sites of some isoforms of cytochrome P450 named ω-hydroxylases allow selective oxidation of the strongest C—H bonds of terminal methyl groups in the presence of weaker C—H bonds of methylene groups of n-alkanes and activated α- and β-methylene groups of normal fatty acids.[40]

The active site of the enzyme (Fig. 1) contains the octahedral iron complex incorporated into a protein. Four equatorial coordination sites are occupied by the porphyrin ligand, and one of the axial sites (fifth or proximal) is occupied by the thiolate group of the cysteine residue.[41,42] The second axial site (distal) in the 'resting' state of the enzyme (ferri-state) is occupied by a water molecule. This coordination site on the distal side of the porphyrin has in its environment predominantly lipophilic amino-acid residues comprising the so-called 'hydrophobic pocket' to bind substrates. In the course of substrate binding due to non-covalent interactions in the hydrophobic cavity of the

† See also recent reviews: L Que Jr, W B Tolman *Nature (London)* **455** 333 (2008); J Piera, J-E Bäckvall *Angew. Chem., Int. Ed.* **47** 3507 (2008).

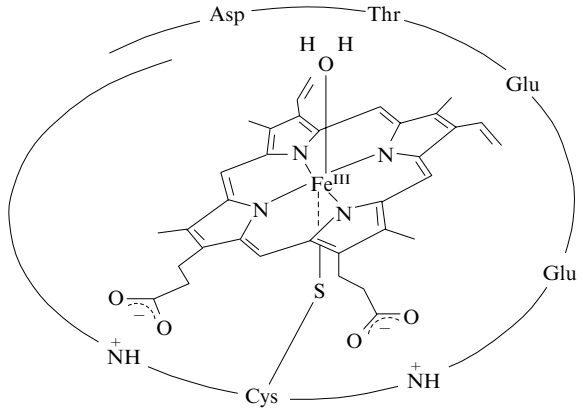


Figure 1. The active site of cytochrome P450.
Here and in Figs 3 and 5, the protein environment is conventionally presented as a helix turn about the metal centre.

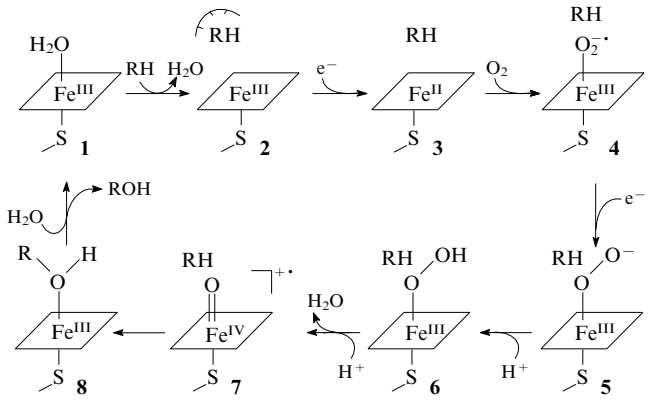


Figure 2. The catalytic cycle of cytochrome P450.
RH is a substrate.

enzyme, a water molecule is released liberating the coordination site for further addition of dioxygen after reduction of iron to the ferro-state. The structural data indicate the presence of the channel for the substrate delivery from the enzyme surface to the active site inside protein globule.[43]

In spite of the fact that the controlled proton transfer in the hydrophobic active site of cytochrome P450 presents a certain problem, it is the necessary stage of molecular oxygen activation. Studies by the methods of directed mutations[44] and cryocrystallography[45] showed that polar amino acid residues in the distal region of the haem are involved in the proton transfer from the surface of the protein globule to the iron-bound oxygen to form, together with several water molecules, a chain of hydrogen bonds, which operates by a shuttle mechanism.

A highly nucleophilic thiolate axial ligand favours the heterolytic O—O bond cleavage through electron donation.[8,46] Replacement of the iron-coordinated cysteine residue in the active site by serine leads to a sharp decrease in the monooxygenase activity.[47] In the heterolytic O—O bond cleavage, the axial thiolate ligand and protons act in the so-called 'push-pull' mechanism.

Activation of O_2 by haem oxygenases was studied using mainly the most available bacterial camphor cytochrome $P450_{cam}$ (CYP101 according to the modern classification) as an example. The intermediates of the catalytic cycle of cytochrome P450 are schematically presented in Fig. 2. Upon substrate binding in the active site, iron in the low-spin Fe^{III}-state (structure **1**) transforms to the five-coordinated high-spin state **2** due to conformational changes in the polypeptide chains adjacent to the hydrophobic region. Then one electron from the cytochrome P450 reductase is transferred to the enzyme–substrate complex. The reduced enzyme–substrate complex **3** rapidly reacts with O_2 to form the dioxygen complex **4**. The latter is formally similar to haemoglobin–oxygen complex, however, it differs in the electronic structure, reactivity and lower stability. At low temperatures, complex **4** is relatively stable and can be observed by spectral methods.[48] The transfer of the second electron determines the rate of the overall process and immediately affords the oxidation products. Till recently, no evidence of intermediate stages of this process has been found in the case of the enzyme itself, because the rate of transformations of intermediates is much higher than that of their formation.

The second electron transfer to complex **4** results in peroxo intermediate **5**, which seems to be a nucleophilic oxidant in some reactions of cytochrome P450 with substrates containing activated C—H bonds. The analysis of the spectrum of this intermediate obtained with a mutant cytochrome P450 in which the proton transfer is partially blocked due to replacement of definite amino acid residues, allowed the conclusion to be drawn that the second electron transfer in the limiting stage at first results in peroxo complex **5**. Good correlation was obtained between the calculated[12] by the density functional theory (DFT) and spectra of intermediates with the $Fe^{III}-O_2^{\bullet}$ and found experimentally[44] $Fe^{III}-O_2^-$ groups. Protonation of the latter intermediate at the distal oxygen atom[49] yields the hydroperoxo complex **6**, which can be detected at low temperature (see below).

Subsequent stages of the cycle have been suggested earlier by analogy with the cycle of related haem enzymes that use hydrogen peroxide as a substrate (peroxidases, catalases), which are better studied; however, only recently direct confirmations have been obtained. Second protonation at the distal oxygen atom[49] should result in the hydrated oxo intermediate **7** with simultaneous or consecutive elimination of a water molecule.[50] Investigations with isotopically enriched O_2 confirmed that it is the O atom directly bound to the haem iron of cytochrome P450 that is transfered to the substrate, whereas the terminal O atom gives water molecule.[12] Calculations confirm that the terminal O atom of peroxo intermediate **5** is doubly protonated, which facilitates the heterolytic O—O bond cleavage to form water and ferryl intermediate **7**.

It is supposed that the transfer of two electrons and two protons to dioxygen transforms it into peroxide. In favour of this supposition is the fact that, in the absence of a natural reductant, peroxides are able to perform cytochrome P450-catalysed oxidation of alkanes playing the role of a peroxide shunt. Other of oxygen atom donors, for example, iodosobenzene (PhIO) or hypochlorite ion (ClO^-) seem to form directly ferryl intermediate **7** in the shunted systems through the O atom transfer from the oxidant. Whereas intermediate **7** could not be isolated from the natural cycle of cytochrome P450, it was observed spectroscopically in the systems with a peroxide shunt. It is noteworthy that oxygenation of substrates with cytochrome P450 is essen-

tially a coupled process, which allows oxidation of oxidation-resistant substrates due to parallel oxidation of a readily oxidisable compound, *e.g.*, natural reductant, nicotinamide dinucleotide (phosphate), NAD(P)H.

An attack of the oxygen atom proximal to the metal by the second proton should yield a complex with hydrogen peroxide. Elimination of hydrogen peroxide should be considered as a violation of the normal coupled oxidation process. In the shunted reaction, the complex with hydrogen peroxide is the first oxygen intermediate, similar to that of peroxidases. The latter contain a histidine residue as a proximal ligand and usually perform one-electron oxidation of phenols, *i.e.*, much more reactive compounds than alkanes. The reactive oxidising intermediate of peroxidases was isolated and characterised as a high-valent iron-oxo complex having the structure $P^{+\bullet}Fe^{IV}=O$ ($P^{+\bullet}$ is a porphyrin radical cation) and called 'Compound I' (Cpd I).[51] Heterolytic cleavage of H_2O_2 by peroxidases to form Cpd I and water models the corresponding stage of the catalytic cycle of cytochrome P450. One-electron reduction of Cpd I results in another intermediate, 'Compound II' (Cpd II).

By analogy with peroxidases, oxidative ferryl intermediate **7** containing formally the group $PFe^{V}=O$ is often described as a species $P^{+\bullet}Fe^{IV}=O$ and is denoted Cpd I of P450. Analogues of Cpd I were isolated and characterised in the alkane oxidation and alkene epoxidation in shunted systems catalysed by simple iron porphyrin complexes. Other intermediates shown in Fig. 2 were also identified in model systems using synthetic analogues like iron *meso*-tetraarylporphyrins (see below).

Chloroperoxidase[52] occupies a special place among peroxidases; in addition to one-electron oxidation, it can effect two-electron oxidation similar to those known for cytochrome P450, *i.e.*, play the role of peroxygenase. It is associated with the fact that, unlike typical peroxidases, chloroperoxidase contains the thiolate group of the cysteine residue as a proximal ligand, as is the case of cytochrome P450. Chloroperoxidase catalyses substrate halogenation in the presence of halide ions; in the absence of halide ions, hydroxylation and epoxidation occur, which is typical of cytochrome P450.[53] 'Compound I' of chloroperoxidase is the most comprehensively studied enzymatic model of intermediate Cpd I of P450. It is noteworthy that there are several important differences in the mechanisms of action of chloroperoxidase and cytochrome P450. First, in the case of chloroperoxidase, as well as other peroxidases, the distal region of the haem in which the substrate difuses after the formation of Cpd I has a polar character, whereas in the case of cytochrome P450, the substrate is bound to the hydrophobic region in the proximity of the haem, which initiates the catalytic cycle resulting in Cpd I. Second, in the case of peroxidases, the formation of Cpd I requires the rearrangement of protons in iron-coordinated hydrogen peroxide with the participation of the proximal imidazole ring of histidine; proton delivery to the non-polar catalytic site of cytochrome P450 requires a special channel binding the enzyme hydrophilic surface to its hydrophobic active site. These facts explain higher activation energy for the formation of Cpd I of P450 (14 kcal mol^{-1}) as compared to that for the formation of analogous intermediate of peroxidases (2–5 kcal mol^{-1}).[54]

An analysis of the crystal structure of cytochrome P450 shows that from the haem proximal side there is also a hydrophobic region called a 'cysteine pocket'. Conformational changes occuring in the adjacent α-helix part of the polypeptide chain through changing of hydrogen bonding to the coordinated thiolate group control redox properties of the haem-thiolate site.[50] Investigation of haem-thiolate model complexes demonstrated that the thiolate ligand increases the rate of the O–O bond cleavage in these complexes and favours its cleavage according to the heterolytic rather than homolytic mechanism.[55]

Iron porphyrin having an attached thiolate group and a hydrophobic cavity from the distal side has been suggested as a model of the active site of cytochrome P450.[56,57] This complex exhibits high stability and maintains its structure including the thiolate ligand in the course of alkane catalytic oxidation in benzene, which has polarity similar to that of the haem environment in the active site of cytochrome P450. It is shown[57] that the thiolate ligand not only considerably increases the rate of O–O bond cleavage in peroxy acids, but favours heterolysis of this bond even in a highly non-polar medium without the action of acid or base. The ferryl intermediate formed in this model system possesses high electrophilicity and is able to hydroxylate alkanes and epoxidate alkenes. In the presence of the thiolate ligand, the attack of the ferryl group (Fe=O) on a C–H bond is more favourable as compared to the addition to unsaturated systems.[55] It is usually explained by an increased contribution of the structure with the coordinated thiyl radical, $PFe^{IV}=O(RS^{\bullet})$.[58] Actually, DFT calculation showed that the RS^{-} anion stabilises the iron high-valent state, favours the O–O bond heterolytic cleavage and lowers the efficient barrier to the reaction of alkane hydroxylation to a larger extent than the imidazole ring (Im) or Cl^{-} anion do.[59] There is reason to hope that similar haem-thiolate complexes[55] will allow modelling of genuine Cpd I of P450.

Studies of the reactions at low and superlow temperatures, as well as application of modern spectral methods for investigation of fast reactions such as a stopped flow technique (SF) and rapid freeze-quench (RFQ) were important in attempted trapping of short-living intermediates of the catalytic cycle of cytochrome P450.[13]

It is difficult to observe the active oxidising intermediate Cpd I of P450, because it is not accumulated at a significant concentration in native system due to its extremely high reactivity. Cryoreduction of cytochrome $P450_{cam}$ in aqueous glycerol in the presense of camphor by electrons generated upon γ-radiolysis affords intermediate **3** in good yield, which react with O_2 to give complex **4** (see Fig. 2).[60] Further, the latter is reduced to peroxo intermediate **5**, which is sufficiently stable at very low temperatures and can be characterised by the ESR and ENDOR (Electron-Nuclear Double Resonance) methods.[60] Careful annealing of the sample allows proton transfer due to unfreezing of some degrees of freedom and leads to the appearance of the hydroperoxide intermediate **6**, which was also characterised by spectral methods. However, after gradual annealing of the sample to a higher temperature, the only intermediate observed was the strained complex in which the oxygen atom of the alcohol formed was bound to the iron atom significantly stronger than in the equilibrium complex **8**.[60] Upon further heating, this complex transforms into intermediate **8**. The failure to observe active intermediate **7** indicates that, under these conditions, it reacts with the substrare an order of magnitude faster than is formed. This result allows the estimation of the rate constant for the preuso-first order reaction of camphor hydroxylation to be $> 1000\ s^{-1}$ at phisyological temperatures.[61]

Recently the structures of intermediates **3** and **4** have been established by the cryocrystallography technique; the catalytic pathway of cytochrome $P450_{cam}$ has been demonstrated at atomic resolution.[45] Upon binding of O_2 to intermediate **3**, the distal oxygen atom forms hydrogen bonds with hydroxy groups of the amino acid residue threonine (Thr) and water. Soft X-ray irradiation of crystals of complex **4** at 100 K results in electron transfer from water to the iron atom in the water radiolysis. In this case, Cpd I of P450 is probably formed, because the Fe–O distance of 1.65 Å determined for this complex is in accordance with the ferryl nature of this intermediate and the distal O atom cannot be detected.[51] In the proximity of the supposed ferryl oxygen atom, a water molecule was found, which is probably formed from the distal O atom following the O–O bond cleavage. As was expected, in this intermediate the thiolate ligand is retained, the Fe–S distance being close to that in complex **4**. Upon short-term thawing, the stereospecific O atom transfer to the position 5 of camphor occurs to form the known structure, the ferri-state P450 (Fig. 1). The protons required for this process are delivered to the hydrophobic active site of cytochrome P450 through the system of hydrogen bonds according to the shuttle mechanism with the participation of amino acid residues Thr and Asp and several ordered water molecules. In the case of a mutant cytochrome P450 in which this chain of hydrogen bonds is violated the formation of hydroperoxo intermediate **6** was observed. Thus, the X-ray data confirm that the transfer of the second electron and two protons to intermediate **4** results in the O–O bond cleavage to form the ferryl intermediate **7**. The fact that the ferryl intermediate was found in a crystal rather than in a frozen solution can be explained by the limited mobility of camphor in the crystal lattice of the enzyme–substrate complex, which prevents its reaction with the ferryl intermediate.[9] Although the technique described above did not provide characterisation of Cpd I of P450, the results obtained evidence in favour of involvement of this intermediate in substrate hydroxylation.

First spectral characteristics of Cpd I of P450 were obtained by the stopped flow method.[62] Fast mixing of solutions of cytochrome P450 and *m*-chloroperoxybenzoic acid yields a short-living active intermediate the absorption spectrum of which is close to that of the more stable and better-characterised Cpd I of chloroperoxidase[63] (Table 1). Similar results were obtained for thermophilic cytochrome P450 (CYP119)[54] the Cpd I of which appeared to be more stable and compared in stability with the Cpd I of chloroperoxidase (Table 1). Note that quantum chemical calculations are in agreement with the experimental spectra of Cpd I.[11,12]

Table 1. The optical spectra and lifetime for the ferryl intermediates of chloroperoxidase and cytochrome P450 at 20 °C and pH 7.

Enzyme	Absorption maximum /nm		Life-time /s	Ref.
	Cpd I	Cpd II		
Cloroperoxidase	367, 610, 688	433, 542, 571	0.03	63
P450 CYP101	367, 694	429, 536, 568	–	63
P450 CYP119	370, 608, 690	–	0.03	63
	367, 416, 650	429, 540, 570	0.2	64 [a]

[a] Photochemical oxidation, in other cases, oxidation with a peroxy acid.

According to the spectral data, intermediates Cpd I of chloroperoxidase and cytochrome P450 have the structure $P^{+\bullet}Fe^{IV}{=}O(RS^{-})$. Quantum chemical calculations show that due to the interaction of two unpaired electrons of the ferryl group with an unpaired electron of the porphyrin ring the doublet (ground) state of this system is very close in energy to the quartet (excited) state.[11] The state with the unpaired electron being on the sulfur atom of cysteine is also very close. According to the data from ESR and ENDOR spectroscopy, the unpaired electron of Cpd I of chloroperoxidase is predominantly localised on porphyrin[65] and only partially delocalised on the thiolate ligand. However, this effect strongly depends on the degree of hydrogen bonding of the thiolate fragment with the N–H groups of the polypeptide chain.

In the majority of works, two-electron oxidation of the ferri-cytochrome by strong oxidants, for example, peroxy acids, was used for the formation of Cpd I. Another approach makes use of the photochemical method; in one of the versions, controlled laser pulse causes the abstraction of an electron from porphyrin in Cpd II (Table 1):[64]

$$PFe^{IV}{=}O \longrightarrow P^{+\bullet}Fe^{IV}{=}O.$$

Under these conditions, the lifetime of the active intermediate increases, which allows studies of its reactivity with respect to common substrates of CYP119. The intermediate obtained in this way appeared to react with the non-activated secondary C–H bond of lauric acid more slowly (> 1000 times) than the active intermediate of cytochrome P450 with the similar bond in camphor.[64] It is believed[64] that the high-energy isomer of Cpd I in cytochrome P450 is used for oxygenation of the most inert substrates. This intermediate contains both oxidation equivalents on the iron atom[66] and is a precursor of Cpd I:

$$Fe^{III}OOH_2 \longrightarrow PFe^{V}{=}O \longrightarrow P^{+\bullet}Fe^{IV}{=}O.$$

This might also explain the fact that chloroperoxidase, which reacts with the substrate after Cpd I is formed, cannot oxidise C–H bonds that are stronger than those in the benzyl group, oxidation of the latter being also inefficient.[67]

In spite of the fact that chemical models of Cpd I of P450, oxo complexes of iron porphyrins, catalyse many reactions of this enzyme, their activities are several orders of magnitude lower.[68] However, it is not related to catalytic oxygenation with the involvement of iron porphyrins in which complexes $PFe^{V}{=}O$ might be formed and react with the substrate before transformation into intermediates $P^{+\bullet}Fe^{IV}{=}O$. Since oxidation process involving cytochrome P450 is initiated by substrate binding into the hydrophobic cavity in the proximity of haem, oxygenation can be performed by the superactive intermediate $PFe^{V}{=}O$ prior to its relaxation into a more stable isomer.[68] This hypothesis seems quite reasonable, but it requires additional studies for its proof.

The mechanism of substrate oxygenation by cytochrome P450 has been discussed for a long time.[69] The most popular is the viewpoint that the only electrophilic inter-

mediate in this process is complex $P^{+\bullet}Fe^{IV}=O$, which reacts with C–H bonds according to the abstraction–recombination mechanism (oxygen rebound).[70] However, experimental data are not always in agreement with this concept. Some authors point out that hydrogen peroxide coordinated to iron or the hydroperoxo intermediate **6** can also be electrophilic oxidants,[69,71] which react through a concerted insertion of the O atom into C–H or C=C bonds. Recent calculations by the DFT method have shown that reactivity of Cpd I of P450 exceeds significantly that of intermediate **6** or the peroxo complex $Fe^{III}\cdot H_2O_2$, therefore, their involvement in the native system can be neglected.[72]

In principle, two mechanisms of substrate oxygenation with the participation of Cpd I of P450 can be considered. According to the oxygen rebound mechanism, the abstraction of the hydrogen atom from the C–H bond (or addition to the double bond) results in the carbon-centred radical, which further binds to the ferryl oxygen atom.[70] The concerted mechanism suggests synchronous insertion of O atom into C–H and C=C bonds.[73] Theoretical calculations indicate that these mechanisms can be realised by two spin states of $P^{+\bullet}Fe^{IV}=O$: low-spin intermediate inserts the O atom, whereas the high-spin intermediate reacts *via* abstraction of the H atom to form radical.[11] Since Cpd I of P450 has the ground state as two closely located states, a doublet and a quartet, the interaction with C–H bond occuring on two competitive surfaces was termed as two-state reactivity (TSR).[74]

In conclusion of this chapter, we will answer the question: why does the enzyme active intermediate oxidise the most inert compounds, but does not affect the labile protein structure? Recently proofs have been obtained[14] that the ability of metal-oxo compounds to abstract the H atom from the substrate is determined by the strengh of the O–H bond formed, which, in turn, depends on the potential of one-electron reduction (E°) of Cpd I and pK_a of Cpd II:[75]

$$D(O-H) = 23.06\,E^\circ + 1.37\,pK_a + 57 \pm 2\ (\text{kcal mol}^{-1}). \qquad (1)$$

The data of Mössbauer spectroscopy and DFT calculations showed that the ferryl group in Cpd II is protonated under physiological pH.[76] This fact points to the basic character of the ferryl oxygen atom in Cpd II caused by strong electron donation by the axial thiolate ligand An increase in the contribution of the second term in equation (1) allows a decrease in the contribution of the first term, *i.e.* it is possible to perform the abstraction of the H atom followed by hydroxylation at relatively low values of redox potentials of Cpd I, when the protein environment of enzyme is not destroyed.[77]

Thus, extensive experimental studies on the enzyme and its models, as well as quantum chemical calculations resulted in the creation of the precise picture of activation of O_2 by haem monooxygenase P450: after heterolytic O–O bond cleavage, the unique haem-thiolate ferryl intermediate is formed capable of incorporating the O atom into C–H bond of alkanes and epoxidating alkenes. These studies showed that cytochrome P450 is a well-organised biocatalyst for controlled oxidation of alkanes and other substrates and discovered a novel catalytic reaction for chemistry, monooxygenation of non-activated C–H bonds.

III. Non-haem mononuclear oxygenases as an ideal platform for catalysis

During the last decade, mononuclear non-haem oxygenases playing important role in metabolism and physiology of mammals and in biodegradation processes involving microorganisms have been studied in detail (Refs 15, 16, 78–80).‡ By January 2005, more than 125 structures of thirty different forms of this class of enzymes have been investigated.[79] These oxygenases catalyse the following oxygenation reactions: hydroxylation of aliphatic and aromatic C–H bonds, epoxidation of C=C bonds, *cis*-dihydroxylation of alkenes and arenes, formation and opening of heterocycles, oxidative scission of aromatic bonds, *etc*. The range of reactions of mononuclear non-haem Fe(II)-oxygenases appeared to be even wider than for cytochrome P450 and includes dehydrogenation, dealkylation, epimerisation and halogenation. Among great diversity of this class of enzymes, three families will be considered within the frame of this review.

The first family includes α-ketoglutarate-dependent enzymes. This is a versatile group of enzymes, which require the presence of an α-ketoacid as a cosubstrate (cofactor) of oxidation and perform functionalisation of non-activated C–H bonds in parallel with oxidative decarboxylation of α-keto carboxylate, usually α-ketoglutarate. Ketoglutarate is bound to iron as a bidentate ligand and provides two of four electrons required for reduction of O_2. In accordance with this, in hydroxylation reaction, one atom of dioxygen is included into the product while the other atom is included into the ketoacid residue, which transforms into a tricarboxylic acid residue and after elimination of CO_2, into succinate.[17,81]

The second family includes pterin-dependent hydroxylases, which use tetrahydrobiopterin (BH_4) as a cofactor donating two electrons. Phenylalanine-, tyrosine- and tryptophan-hydroxylases perform regioselective aromatic hydroxylation.[18,82]

The Rieske dioxygenases comprising the third family catalyse *cis*-dihydroxylation of a double bond in arenes, NADH being a source of two electrons; both atoms of dioxygen transfer to the substrate to form *cis*-diol.[83] These dioxygenases found in soil bacteria perform the first stage of degradation of aromatic hydrocarbons. The most comprehensively studied Rieske dioxygenase is naphthalene-1,2-dioxygenase (NDO), which dioxygenates naphthalene to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene.[84] The Rieske dioxygenases can also work as monooxygenases in reactions of hydroxylation and epoxidation where the second oxygen atom transforms into a water molecule.[85] The reactions catalysed by these enzymes proceed stereo- and enantioselectively; *cis*-dihydroxylation of arenes is a transformation unknown in synthetic organic chemistry.

In all these enzymes, the Fe(II) centre (Fig. 3) is coordinated to three amino acid residues: to two histidine and one aspartate or glutamate residues located in the apices of one of triangular faces of an octahedron. This configuration was called 2-his-1-carboxylate (*N*,*N*,*O*) facial triad.[86] While in cytochrome P450, there is only one coordination site at the

‡ See also review by P C A Bruijnincx, G van Koten, R J M Klein Gebbink *Chem. Soc. Rev.* **37** 2716 (2008).

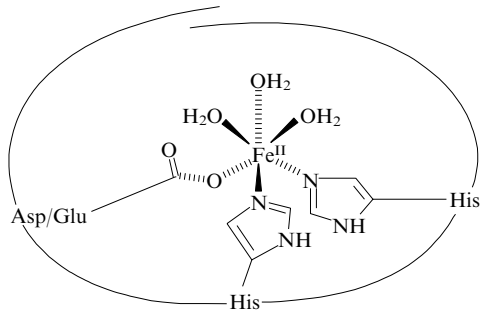


Figure 3. The active site of mononuclear non-haem oxygenases.

iron atom, which should be occupied by dioxygen or its reduced forms to effect catalysis, the metal centre of non-haem mononuclear oxygenases has three coordination sites on the opposite face of the octahedron accessible for binding exogenous ligands: substrate and/or cofactor and O_2. These labile coordination sites are usually occupied by water molecules or by other readily replaceable ligand. The *N,N,O* facial configuration allows activation of both the substrate and O_2 and enables their close arrangement for subsequent reaction. Unlike the haem iron atom, for which the high- and low-spin states are close in energy, the *N,N,O* triad stabilises the high-spin configuration of the iron atom.[87] Recently, first reports[88,89] have appeared concerning structural modelling of mononuclear non-haem centres with the *N,N,O* facial triad.

The generalised mechanism of catalysis by mononuclear non-haem enzymes[79] based on structural and spectroscopic data, studies of model systems and quantum chemical calculations is shown in Fig. 4. The enzyme active site is shown as a triangular pyramid with Fe(II) located at the vertex and the *N,N,O* triad forming the base.

In the 'resting' state of the enzyme (Fig. 4, structure **9**), the Fe(II) ion is six-coordinated and is not reactive with respect to O_2. As in the case of cytochrome P450, binding of the substrate and/or cofactor in the enzyme active site transforms the iron atom to the five-coordinated state **10**,[90] thus increasing its affinity to dioxygen. Both substrate and cofactor can be directly coordinated to the iron atom or can have binding sites in the proximity. In the case of the Rieske dioxygenases, the substrate and the cofactor are not bound directly to the Fe(II) atom, but the carboxylate group of the facial triad acts as a bidentate ligand. Coordinated O_2 (structure **11**) is reduced to peroxo intermediate **12** (Fig. 4); further activation of the intermediate generates high-valent ferryl intermediate **13** and initiates oxidation process special for each family of enzymes. Binding of O_2 is the limiting step of the overall process.[91] For this reason, the oxygen intermediate can be considered as a short-living intermediate of the catalytic cycle.[19] According to calculations,[92] it has the structure $Fe^{III}O_2^-$. Complete reduction of dioxygen includes two two-electron stages, which are peculiar for each group of enzymes. For ketoglutarate- and pterin-dependent enzymes, the first two-electron reduction of O_2 results in a bridged peroxo intermediate $Fe^{II}-OO-R$. The crystal structures of a similar intermediate and its precursor, η-$Fe^{II}O_2$ intermediate, have recently been established for 1,2-cathechol oxygenase performing oxidative opening of the aromatic ring.[93] Since in the case of the Rieske dioxygenases, one electron for the first two-electron reduction of O_2 is delivered from an external source and the second one from the Fe^{II} ion, terminal peroxide is formed bound to the Fe^{III} atom in the bidentate mode. The structure of such a peroxide for NDO is established from the X-ray data.[94]

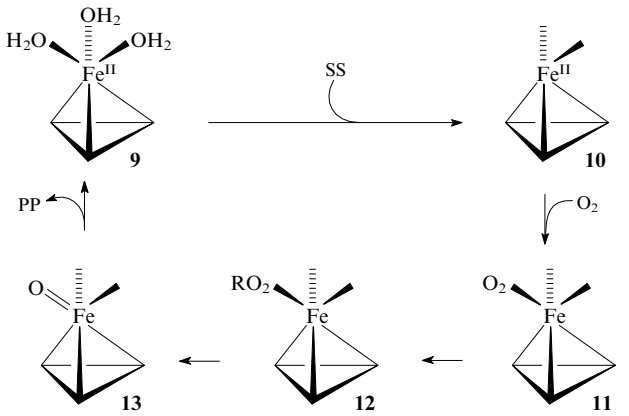


Figure 4. The generalised mechanism of catalysis by *N,N,O*-oxygenases.
The oxidation state of the iron atom in intermediates **11**–**13** depends on the type of oxygenase; SS are substrate and cofactor, PP are products, R = H or the cofactor residue.

Ketoglutarate- and pterin-dependent hydroxylases have both common and specific properties. According to calculations,[19] in the peroxide intermediate of BH_4-dependent hydroxylases, the water molecule coordinated to the iron atom participates in the heterolysis of the O–O bond donating a proton to the distal oxygen atom to form $HO-Fe^{IV}=O$. On the contrary, the Rieske dioxygenases resemble cytochrome P450 in many aspects. In particular, for completely oxidised enzyme, hydrogen peroxide can shunt the catalytic cycle.[95] Peroxo intermediate of the Rieske dioxygenase, $Fe^{III}-OO-H$, according to calculations,[19] is able to perform *cis*-dihydroxylation of the C=C bond; however, experimental data indicate that it transforms into high-valent oxoferryl intermediate, which is an actual hydroxylating agent:

$$Fe^{III}-OO-H \longrightarrow HO-Fe^{V}=O.$$

Appreciable incorporation of ^{18}O into the product upon addition of isotopically labelled water is in favour of this suggestion; the exchange is only possible in the case of the ferryl intermediate.[96] The formation of diol results in a Fe^{III} complex, which is reduced to the parent Fe^{II}-state under the action of the second external electron from NADH.[97] Evidence has been obtained[98] that monooxygenase reactions of NDO also proceed through the ferryl intermediate. Finally, structural and functional models of mononuclear non-haem oxygenases have recently been synthesised that well reproduce the *N,N,O* triad of NDO and performed selective epoxidation or *cis*-dihydroxylation of alkenes.[99] Incorporation of the labelled oxygen atom from the added $H_2{}^{18}O$ to the diol suggests that the ferryl intermediate is an active species in this case, too.

The ferryl intermediate of the ketoglutarate-dependent enzyme, taurin/ketoglutarate dioxygenase (TauD), has been well studied by different spectral methods.[20,100,101] High kinetic isotope effect (KIE) ($k_H/k_D = 50$) evidences for its involvement in hydroxylation reaction.[102] The high-spin

intermediate $Fe^{IV}=O$ (**J**) of TauD having UV absorption at ~318 nm has been first detected for the reaction of the enzyme–substrate complex with O_2 at 5 °C, along with an enzyme–product complex, by the combination of the SF and RFQ methods.[103] Direct evidence for the presence of the oxo group in the intermediate **J** was first obtained from the resonance Raman spectrum[104] where a typical band at 821 cm^{-1} was observed, which shifted to 787 cm^{-1} when $^{18}O_2$ was used. A comparison of the Mössbauer spectra for this intermediate with those for model ferryl complexes also confirmed its ferryl nature, which was later confirmed by the short Fe–O distance, according to the data from X-ray absorption spectroscopy.[105] Important information on the electronic structure and geometry of intermediate **J** was obtained from the comparison of parameters of experimental Mössbauer spectra with those calculated by the DFT method.[101,106] It is shown that intermediate **J** is the high-spin ($S=2$) Fe^{IV}-oxo complex located in the distorted-octahedral ligand environment of two histidine imidazole rings and two carboxylate groups (succinate and Asp101 residues) with the axial oxo ligand in the *trans* position to one and the *cis* position to the other imidazole ring. One carboxylate group is a monodentate ligand, while the other is a bidentate ligand. The calculated Fe–O distance (1.65 Å) for this intermediate is in agreement with the experimental value (1.62 Å). A rather high isomeric shift in the Mössbauer spectrum (0.3 mm s^{-1}) is due to a weak equatorial ligand field.

After the structure of the intermediate **J** of TauD has been established, high-spin ferryl intermediates were found in two other ketoglutarate-dependent oxygenases, *viz.*, prolyl-4-hydroxylase[107] and halogenase CytC3.[108]

Thus, in contrast to 'elusive' Cpd I of P450, intermediate **J** of TauD has been stidied in detail. Though both intermediates are iron oxo complexes, they have different structures. While Cpd I of P450 is a six-coordinated octahedral complex, in the intermediate **J** of TauD, five ligands compose a trigonal bipyramid or a distorted octahedron.[106] As a result, both systems have different sets of molecular orbitals and, therefore, different reactivity.[109] Unlike the haem iron atom, the highest spin state, which is allowed for a given amount of electrons, *i.e.* quintet is the ground electronic state of the ferryl intermediate in the N,N,O coordination environment having a weak ligand field. According to the experimental data and theoretical calculations, the high-spin $Fe^{IV}=O$ intermediate having formally four d-electrons at the iron atom, has the quintet ground state; after oxygenation it transforms into high-spin Fe^{II}, which also has the quintet ground state. Therefore, the reaction of oxygen transfer with the participation of this intermediate occurs adiabatically.[21]

Quantum chemical calculations for the reaction of intermediate **J** of TauD with propene showed that it is a strong oxidant capable of hydroxylating C–H bonds and epoxidating C=C bonds with a lower activation barrier than Cpd I of P450. Since in the case of intermediate **J** of TauD, the strength of the FeO–H bond formed[75] is 10 kcal mol^{-1} larger, the former is much more reactive as compared with Cpd I of P450.[21]

In conclusion of this chapter, α-ketoglutarate-dependent halogenases should be mentioned, which have recently been discovered;[80] they are structurally similar to ketoglutarate-dependent oxygenases, but have somewhat different ligand environment of the iron atom: in the active site, there is an exogenous halide ion (usually, chloride) instead of carboxylic group of the facial triad.[110] These enzymes perform halogenation of non-activated C–H bonds with the participation of the ferryl intermediate.[80]

IV. Methane monooxygenase: unprecedented activity and high selectivity

Among iron-containing non-haem oxygenases, multicomponent soluble methane monooxygenase (sMMO, further MMO) is of special interest;[22–25,90,111] it has been found, together with less studied copper-containing particulate membrane-bound methane monooxygenase (pMMO),[112] in methanotrophic microorganisms using methane as the only source of carbon and energy.§ The hydroxylase component of MMO works within the multienzyme complex together with reductase and a small, but extremely important for catalysis, coupling (regulatory) protein B.

Methane monooxygenase is a dinuclear biocomplex of iron and catalyses oxygenation of the simplest and least reactive saturated hydrocarbon, methane, under mild conditions:

$$CH_4 + O_2 + NADH + H^+ \longrightarrow MeOH + H_2O + NAD^+.$$

For the past two decades, this reaction unprecedented in chemistry has stirred up interest of many researchers. If a biomimetic industrial catalyst reproducing the reactivity of MMO was successfully constructed, large-scale transformation of methane to methanol would substantially increase its use as a cheap energy source and a material for a variety of industrial processes.

In addition to methane, MMO can oxygenate more than 50 different compounds, among them linear alkanes (up to n-octane) at the terminal methyl and adjacent methylene groups, cyclic and aromatic hydrocarbons, alkenes at the C–H bond (hydroxylation) or the C=C bond (epoxidation). However, MMO reacts with these hydrocarbons slower than with methane, despite the fact that the C–H bonds in them are weaker. In branched alkanes, the part of the molecule most remote from the branching point is oxidised. These facts demonstrate that the organisation of the active site rather than the strength of the bond that is attacked is the decisive factor in MMO-catalysed reactions.

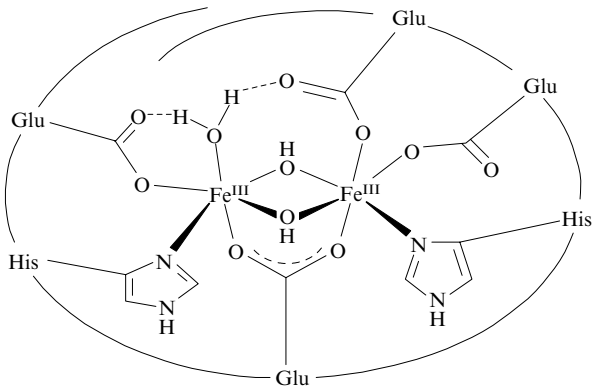


Figure 5. The active site of MMO.

In the active site of MMO[113] (Fig. 5), two iron atoms are bound to protein through four glutamine (Glu) and two histidine (His) residues. During the catalytic cycle, one of

§ See Appendix (1).

the carboxylate groups is conserved as a bridge between the iron atoms, which is important for their concerted functioning. In addition, several exogenous hydroxo and aqua ligands can be bound to the iron atoms, part of which, together with the carboxylate group, form three bridges maximum between the iron atoms. Unlike the haem iron atom in cytochrome P450 having one coordination site for the formation of oxygen intermediates, in the case of MMO, four internal coordination sites, which are occupied in the ferri-state by two bridging HO groups,[23, 114, 115] can participate in the catalytic process according to the mechanism of bridging activation of O_2 (see below).

The active site also contains a hydrophobic cavity with a volume of about 185 Å^3 binding the substrate; one side of the cavity is formed by the dinuclear iron complex, the other side is formed by hydrophobic amino acid residues of the protein.[113, 116] Binding of the substrate in this hydrophobic 'pocket' initiates rapid two-electron reduction of the Fe(III) atoms to Fe(II) in the cluster.[117]

By 2001, the structures of 34 biocomplexes have been established by X-ray studies of different forms of ferri- and ferro-MMOs; these data provided understanding of the detailed structure of the active site of MMO in the oxidised and reduced states.[22] Conformational changes in these structures can play an important role in different steps of catalytic process.

In the oxidised MMO (Fig. 5, its fragment is presented in Fig. 6, structure **14**), both iron atoms are located in the distorted octahedral environment and are non-equivalent. The Fe1 atom contains a terminal coordinated water molecule. The coordination environment at the Fe2 atom is more labile than that at the Fe1 atom. In addition, instead of bridging hydroxy group in the distal position to the histidine residues, other anionic or neutral oxygen donors can be arranged, among them methoxy group and ethanol. Spectral stidies indicate that high-spin Fe(III) ions are bound to one another in an antiferromagnetic mode to give the diamagnetic ground state.[26]

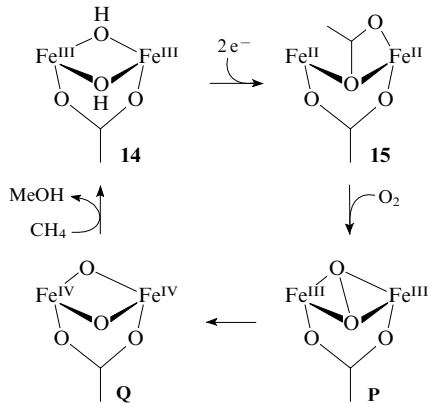


Figure 6. A simplified catalytic cycle of MMO.

According to X-ray, ESR and Mössbauer spectroscopy data, the reduced MMO (Fig. 6, structure **15**) contains two high-spin ($S=2$) Fe(II) atoms with different ligand environment bound in a ferromagnetic mode. The distance between the iron atoms is equal to 3.3 Å.[22, 111] In ferro-MMO, both iron atoms become five-coordinated after losing a weakly bound bridging water molecule located in the distal position to the histidine residues, which allows O_2 molecule to form a bridge between the iron atoms.[25] This side of the dinuclear complex adjoins the hydrophobic 'pocket' binding the substrate and is the scene for the overall catalytic process. Like cytochrome P450, the hydroxylase component of MMO contains a hydrogen bond network, which expands from the dinuclear core to the protein surface and acts as a channel for delivery of protons and, probably, electrons to the reaction zone. Studies of geometry and electronic structures of ferri- and ferro-MMOs by the DFT method showed that the protein environment markedly influences the energetics of the active site.[114, 118]

The hydroxylase component reduced by dithionite is able to perform stoichiometric oxidation of methane by dioxygen (one cycle) in the absence of reductase. In this case the addition of methane brings about only moderate influence on the rate of oxygen consumption.[23]

Catalytic cycles of MMO (Fig. 6), N,N,O-oxygenases (Fig. 4) and cytochrome P450 (Fig. 2) have both similarities and important differences. The reaction of ferro-MMO with O_2 has been studied by optical spectroscopy (SF mehtod).[119, 120] It was found that, in contrast to cytochrome P450, the key intermediates, compounds **P** and **Q** (Fig. 6) can be trapped in the native system of MMO and characterised. Despite the fact that they are accumulated in marked concentrations before reaction,[119, 120] their exact structure is still the matter of discussion. The first intermediate observed was diferriperoxo species **P**. There is indirect evidence for the formation of the precursor of intermediate **P**, whch can be a superoxo species.[121] The presence of the only quadrupole doublet in the Mössbauer spectrum of intermediate **P** indicates the symmetrical arrangement of the peroxo group between the iron atoms. Compound **P** spontaneously transforms into intermediate **Q**, the rate of the formation of the latter being independent of methane concentration, but the rate of disappearance being proportional to it. This has led to the conclusion that complex **Q** is a species reacting with methane. The disappearance of intermediate **Q** from the reaction with methane is distinctly registered by a decrease in intensities of characteristic bands in the absorption spectrum.

Kinetic data demonstrate the importance of the interaction of all three components of MMO having high affinity to one another and forming a multienzyme complex in which the Fe_2-cluster gains the capacity to two-electron reduction. Protein B (see above) plays the decisive role in performing the key steps: in its presence, O_2 binding occurs almost 1000 times faster, the rate of formation of intermediate **Q** is 40 times higher, the yield of products increases from 40% to 80%; the addition of reductase increases the yield to 100%. Protein B controls electron transfer from reductase to hydroxylase so that the process could occur only in the presence of substrates, it increases the reaction rate and can also alter the regioselectivity of alkane oxidation. Protein B is also supposed to control the substrate delivery to the active site of enzyme[122–125] opening or widening the hydrophobic channel, *i.e.*, it acts as a molecular sieve. Binding of protein B causes changes in some spectral characteristics of the reduced Fe_2-cluster, which indicate possible changes in the coordination environment of the iron atom, supposedly, Fe2.[123]

Efficient and substrate-specific transformation of methane to methanol requires the presence of a strong oxidant and simultaneously the control of the access of more

reactive substrates to it. Powerful oxidant of MMO, intermediate **Q**, is safely protected by the closed protein structure; fast delivery of methane and O_2 to it becomes possible only after a complex between protein B and oxygenase is formed. In this case, a physical channel is generated matching methane and O_2 molecules.[125]

In the absence of substrate in the system, intermediate **Q** can be accumulated in amounts sufficient for spectral investigations. The Mössbauer spectrum of frozen solutions of complex **Q** indicates that the iron atoms in this complex are in the high-spin Fe(IV) state and are bound in the antiferromagnetic mode (the parameter of the magnetic interaction $J < -30$ cm) to yield the symmetrical diamagnetic cluster $Fe^{IV}-O-Fe^{IV}$ (Ref. 126).

The bridging mechanism of O_2 activation and methane oxidation¶ (Fig. 6) takes into consideration the dinuclear structure of the active site of MMO. According to this mechanism, intermediate **Q** has the structure of $Fe(IV)_2(\mu\text{-}O)_2(\mu\text{-}COO)$ cluster; intermediate **P** has the similar structure of a bridging peroxide in which oxygen atoms are bound to one another and to both iron atoms. Activation of oxygen occurs through the homolytic O—O bond cleavage in the peroxide intermediate and does not require the involvement of a proton; methane is activated by the attack of the bridging oxygen atom (unlike the terminal oxygen atom of the ferryl intermediate **7**), probably, with the participation of the other μ-oxo bridge.

This mechanism is confirmed by experimental and theoretical studies.[126, 130] Thus, an analysis of the data from EXAFS spectroscopy (Extended X-ray Absorption Fine Structure) for intermediate **Q** revealed the presence of one short and one long Fe—O bonds and unusually short distance between the iron atoms.[126] The length of the short Fe—O (1.8 Å) bond appeared to be larger than those of the terminal Fe=O bonds in high-valent iron porphyrin complexes (1.67 Å)[51] and in other ferryl complexes, but comparable with those of the Fe—O bond in dinuclear μ-oxo complexes containing a Fe—O—Fe fragment. Model complexes with the $Fe_2(\mu\text{-}O_2)$ core have been synthesised;[131] this structure is confirmed by theoretical calculations.[132]

Some anomalies were found for the reaction of methane with complex **Q**, namely, unusually high kinetic isotope effect (>50) against virtually complete lack of the latter for reactions of other alkanes and the highest oxidation rate among alkanes, which is not consistent with the strongest C—H bond.[23] The dependence of the reaction rate on inverse temperature (the Arrhenius plot) for the interaction of intermediate **Q** with methane appeared to be non-linear, which points to the two-step mechanism of this process.[119, 120] The lack of the isotope effect and normal temperature dependence of the reaction rate with intermediate **Q** for other substrates indicate that the limining step of the process is not the C—H bond cleavage, but, probably, alkane binding to the active site. The limiting step of the reaction with methane is temperature-dependent: at low temperatures, it is substrate binding, while at high temperatures, the reaction at the C—H bond. Activation parameters for transformation of complex **P** to **Q** and for the reaction of intermediate **Q** with methane in the temperature range where the limiting step is C—H bond cleavage are in satisfactory agreement with those calculated by the DFT method.[132–134]

It appeared that it is possible to reproduce 'methane-like' kinetics for the reaction with ethane using a mutant protein B in which the amino acid residues in the region of contact with the hydroxylase component are smaller in size, which increases the diameter of the channel and facilitates binding of a larger ethane molecule to the active site.[125] In this case, the active site of MMO is equally accessible for both methane and ethane; the reaction rate constants for the interaction of these alkanes with intermediate **Q** differ by three orders of magnitude in accordance with the difference in the values of bond energies by 4 kcal mol^{-1}; the normal KIE is observed. It is also shown that the effect of proton quantum tunneling (under-barrier hydrogen transfer) suggested for the explanation of unusually large isotope effect is substantial only in the case of methane oxidation rather than that of ethane.

Recent quantum chemical calculations with the use of the hybrid DFT method and a large model (up to 100 atoms) revealed important details concerning the structures of intermediates **P** and **Q** and their reactions.[24, 27, 130, 132–134] The reliability of these methods was demonstrated by correct simulation of the X-ray data for the structures of ferro- and ferri-MMOs including the peripheral network of H-bonds.[133] A hydrogen bond network is necessary for the stabilisation of intermediates **P** and **Q**. It is transformations in H-bonding that control the distance between the iron atoms and conversion of one intermediate to the other. The water molecule coordinated to the Fe1 atom and involved into the network of hydrogen bonds appeared to play a key role in maintaining the structures of **P** and **Q**.[133] The calculated parameters of the Mössbauer spectra are in good agreement with the experimantal data; the calculations confirm the high-spin state of intermediate **Q**.[135] Calculations for the catalytic process of dioxygen activation show[24, 130] that in the O_2 binding to reduced MMO, electron transfer from one iron atom occurs first to form a superoxide complex, which gaining electrons from the other iron atom transforms to the $\mu\text{-}\eta^2,\eta^2$ peroxide. Experimental data indirectly confirm the presence of the superoxo intermediate as a precursor of intermediate **P**.[121]

The use of the large model for quantum chemical calculations gave the energies of transition states and rate constants for elementary reactions, which are in good agreement with the known experimental data, thus confirming the suggested oxidation mechanism. Strong influence of the protein environment on the energetics of reactive intermediates has been found.[136, 137] The protein helps to drive reactants toward products along the reaction path through a larger strain in the structure of intermediates formed at the beginning of the catalytic cycle, so that the strain relief at subsequent stages reduces their energy barriers.[137, 138] The electronic structure of the dinuclear centre of intermediate **Q** appeared to lie between those of Fe(IV)—Fe(IV) and Fe(III)—Fe(V).[132, 137] In the reaction with methane, asymmetry in the geometry of complex **Q** is increased; this structural distortion plays an important role in methane activation, because it increases significantly the electrophilicity of the bridging O atom giving it a radical-like character.[24, 139] The limiting step for methane hydroxylation is the abstraction of an H atom from methane to form a radical-like intermediate (proton transfer to the O

¶ This mechanism, which was first suggested more than 10 years ago,[127–129] was termed in this whay in order to differentiate it from the terminal mechanism of O_2 activation in the mononuclear active site of cytochrome P450.

atom and withdrawal of an electron from the C–H bond by Fe2). After the first transition state is passed, the process follows two routes with different mechanisms,[115,132] radical-bound and concerted. Both routes have similar second transition states giving the methanol complex after rotation of the OH group.[140] According to the second route, the rearrangement step occurs with the retention of the tight binding of the methyl fragment to the hydroxy group. In this case, the configuration is retained in the hydroxylation of a chiral alkane, whereas according to the first route, partial racemisation takes place the degree of which depends on the lifetime of the radical intermediate. The contribution of these routes depends on the substrate nature; the actual process is a superposition of two extreme cases. Thus the retention of the configuration in the hydroxylation of ethane containing H, D and T atoms in each methyl group, according to the calculations,[141] should be 69%–84%, which is in good agreement with the experimental value of 72% obtained for *R*-enantiomer of labelled ethane.[142]

Other non-haem oxygenases are known, which, like to MMO, contain a dinuclear iron complex in the active site and catalyse oxidation of non-activated C–H bonds:[28] alkane monooxygenase (AlkB), toluene monooxygenase, (TMO), xylene monooxygenase, alkene monooxygenase, *etc*. These enzymes are able to oxygenate different hydrocarbons: alkanes (except for methane), alkenes and arenes.[143] Among these oxygenases, a TMO family, which hydroxylates regioselectively toluene at the *para*-, *meta*- or *ortho*-position relative to the methyl group is best studied,[144,145] as well as a non-haem family AlkB. The results of structural and spectral studies show that the dinuclear core of the active site of TMO is very much similar to that of the active site of MMO. However, in contrast to the chain of small hydrophobic cavities leading to the active site of MMO,[146] a large hydrophobic 'pocket' makes the dinuclear centre of TMO more accessible for the substrate.[147] Spectral studies at low temperatures found peroxo intermediate of TMO, which differs in the structure from intermediate **P** of MMO.[148]

Chemists are most interested in alkane monooxygenase from *Pseudomonas oleovorans* called sometimes ω-hydroxylase,which has a dinuclear active site with the carboxylate bridge and catalyses hydroxylation of n-alkanes (C_5–C_{12}) at the terminal methyl group.[149] Butane monooxygenase described recently hydroxylates butane at the terminal methyl group with the regioselectivity exceeding 80%.[150] Since the reactions catalysed by ω-hydroxylase, cytochrome P450 and MMO have equal stoichiometry, it is supposed that the active intermediate of the former is also the high-valent iron-oxo complex.[151] An important difference in the structures of ω-hydroxylase and MMO consists in the predominance of donor nitrogen atoms in the coordination sphere of AlkB and the presence of the oxo-bridge between the iron atoms.[149,152,153] Nevertheless, it is shown[154,155] that the mechanisms of alkane hydroxylation by TMO and by ω-hydroxylase have very much in common with the mechanism of action of MMO. All these oxygenases require for efficient catalysis the participation of the regulatory protein B, which increases their activity 30–150-fold.[28] It is supposed that protein B facilitates the access of O_2 and methane to the active site[125] and/or the addition of O_2 according to the bridging mechanism, due to either dissociation of water molecule bound to the iron atom[28] or change in the geometry of the dinuclear centre.[123]

Thus, in recent years, substantial progress has been achieved in understanding of the nature of the strongest oxidant in biology, active intermediate of MMO, which possesses the exceptional ability to perform selective oxidation of such an inert molecule as methane under mild conditions. An important difference between haem and non-haem oxygenases is that in the former, the iron atom exists in a low-spin state, while in the latter, it is always in the high-spin state. This, as will be shown below, leads to larger reactivity of non-haem oxygenases. In spite of substantial differences in the structures of active sites, haem- and non-haem oxygenases have much in common in the mechanisms of O_2 activation and alkane oxidation.

V. Strategy of Nature in oxidation of non-activated C–H and C=C bonds

As was already mentioned, different oxygenases have similarities and differences. All these enzymes use the same scheme of dioxygen activation on iron complexes to form essentially the same reactive oxidant and similar interaction of the latter with hydrocarbons. The metal centre retains reactive oxygen intermediates in its coordination sphere and provides a possibility to avoid their uncontrolled radical reactions. An outstanding role of iron in metabolism of dioxygen can be explained, on the one hand, by its abundance in the crust and, on the other hand, by large affinity to oxygen donors and favourable redox characteristics. For this reason, in the course of evolution, the oxygenase route of functionalisation of the most inert non-activated C–H and C=C bonds was naturally involved in the common metabolism of O_2 with involvement of iron compounds. That is why the structures of active sites of iron-containing oxygenases are somewhat similar to those of the corresponding oxidases, catalases, peroxidases, *etc*.

In oxygenase reactions, the process of dioxygen reduction includes transfer of four electrons to the O_2 molecule. This process can be divided into two two-electron stages: (i) activation of O_2 to form the oxidising agent, high-valent iron-oxo complex (oxoferryl or ferryl reagent) and (ii) substrate activation accompanied by the O atom transfer from the oxidising agent to the substrate. The mechanism of O_2 activation includes binding to the Fe(II) atom, formation of a peroxo intermediate followed by homolytic or heterolytic O–O bond cleavage to form the ferryl intermediate that performs hydrocarbon oxygenation:

$$Fe + O_2 \longrightarrow FeO_2 \longrightarrow$$

$$\longrightarrow Fe{-}OOH \text{ (or } Fe{-}OOR) \longrightarrow Fe{=}O,$$

R is the cofactor residue.

Ferryl intermediates are electrophylic agents. Their initial interaction with C–H and C=C bonds occurs according to the mechanism that is similar for different oxygenases, namely, abstraction of an H atom from alkanes or addition to the unsaturated system to form, in both cases, a radical intermediate [FeOH·R]. This process results in hydrocarbon activation. At the next stage, the recombination of the radical with HO$^{\bullet}$ occurs to give final products. The major characteristics of some oxygenases and the structures of their intermediates are presented in Table 2.

The structures of active sites of oxygenases, as well as of their active intermediates are different. The activities of oxygenases are also different: only MMO is able to oxidise

Table 2. Characteristics of main type oxygenases.

Characteristic	Cytochrome P450	The Rieske oxygenase	Hydroxylase	MMO
Fe state in 'resting' enzyme	Fe^{III}	Fe^{II}	Fe^{II}	$(Fe^{III})_2$
Stationary ligands [a]	$N_4(S\cdots H)$	N_2O_2	N_2O	$2\ NO_3$
The number of labile ligands [b]	1	2	3	4
The number of electrons in redox process	1	2	2	2
Source of electrons	NAD(P)H	NADH	RH_2	NADH
Configuration of O atoms in a peroxide intermediate	η^1-OOH	η^2-OO(H)	η^1-OOR	μ-η^2:$\eta^2-O_2^{2-}$
The type of O−O bond cleavage	heterolytic	heterolytic	homolytic	homolytic
Ferryl intermediate	$P^{+\cdot}Fe^{IV}=O$ ($PFe^{V}=O$)	$Fe^{V}=O(OH)$	$Fe^{IV}=O$	$Fe^{IV}-(\mu\text{-}O)_2-Fe^{IV}$ ($O=Fe^{V}-O-Fe^{III}$)

[a] Atoms coordinated to metal are shown, the subscript shows their number; [b] also means the number of coordination sites accessible for catalysis.

methane. Binding of O_2 requires Fe(II) atom, which is present only in the 'resting' state of mononuclear non-haem oxygenases. Since cytochrome P450 and MMO in the 'resting' state contain Fe(III), the process for these oxygenases starts from electron transfer. An important distinction is the source and the character of electron transfer to the metal-coordinated O_2 molecule (Table 2). Two of four electrons required for complete reduction of O_2 are usually supplied by the substrate (all four in the case of dioxygenases), and two others are supplied by a biological reductant (or a cofactor). In the case of cytochrome P450, MMO and the Rieske dioxygenases, the electrons are delivered step by step from NAD(P)H to form Fe(III)-peroxo and Fe(V)-oxo intermediates. In the case of a series of mononuclear non-haem oxygenases requiring a cofactor (for instance, 2-ketoacid or tetrahydrobiopterin), two electrons are simultaneously delivered from the cofactor to form Fe(II)-peroxo and Fe(IV)-oxo intermediates.

An analysis of literature data on oxygenases shows that, despite a number of structural and functional differences, they have much in common. It is most substantial that high-valent iron-oxo complexes are the major and versatile oxidants for various reactions catalysed by different oxygenases.[13]

The concept on the ferryl intermediate has come to biology from chemistry in the course of long discussion on the nature of the reactive oxidant, Fenton's reagent ($Fe^{II}+H_2O_2$).[156] This system exhibits strong oxidising ability and activates sufficiently strong C−H bonds in different compounds. The generally adopted concept is that it is associated with the participation of the radical $HO^{\bullet}$, the reactivity of which is well-known. However, high regio- and stereoselectivity of cyclohexanol oxidation by Fenton's reagent in acetonitrile to form *cis*-cyclohexane-1,3-diol suggests the participation of the ferryl intermediate in oxidation, because such selectivity is in principle impossible for the freely diffusing radical $HO^{\bullet}$ (Ref. 70).†

The reactivity of ferryl species originates from their electronic structures, which is usually calculated by the methods of quantum chemistry or determined from spectral characteristics of these short-living intermediates. A ferryl species can be simple (mononuclear) or complex (dinuclear as in MMO or bound to the radical centre as in cytochrome P450) and contain iron atom in different oxidation states (IV, V, VI) and in several spin states. An analysis of experimental and theoretical data shows that the strength and the distances of Fe−O bonds are approximately the same for all mononuclear ferryl complexes independent of the ligand nature (haem or non-haem), oxidation state and spin state. This can be explained by a marked similarity of $d_\pi(Fe)-p_\pi(O)$ orbital interactions leading to a rather strong covalent Fe−O bond for all these species.[157, 158] The qualitative picture of binding in $[Fe=O]^{2+}$ ion ($S=1$) is presented in Fig. 7.[159] In symmetrical octahedral field, five degenerate 3d orbitals of the Fe atom form two groups: $t_{2g}(d_{xy}, d_{xz}, d_{yz})$ and $e_g(d_{x^2-y^2}, d_{z^2})$. Low-energy t_{2g} orbitals interact with an O atom to give π-bonds, while the high-energy orbitals are involved into the formation of σ-bonds. The interaction of d_{z^2} orbital of the Fe atom with one of 2p orbitals of the oxygen atom gives bonding and antibonding σ-orbitals of the ferryl group. Two other 2p orbitals of the O atom are combined with two $t_{2g}(d_{xz}, d_{yz})$ orbitals to form two bonding and two antibonding π-orbitals of the ferryl group. Thus, a [Fe=O] species contains one σ- and two half-π-bonds with an Fe−O bond order equal to 2. Strong covalent π-bond in all ferryl complexes results in a high density of unpaired electron at the oxygen atom. The orbitals d_{xy} and $d_{x^2-y^2}$ are antibonding, because they do not participate in the formation of the Fe−O bond. Ten electrons occupy, correspondingly, low-energy orbitals to form the $(d_{xy})^2(d_{xz},d_{yz})^2$ electronic configuration of the ferryl complex with the $[Fe=O]^{2+}$ ion ($S=1$).

The electronic configurations for different mononuclear ferryl complexes including only the key orbitals are given in

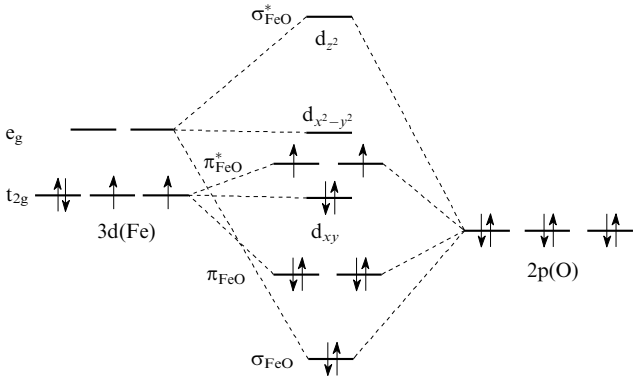


Figure 7. The electronic structure and binding in $Fe^{IV}=O$ complexes ($S=1$).

† See Appendix (2).

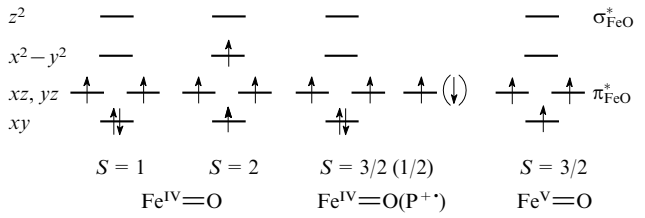


Figure 8. The qualitative orbital diagrams of the electronic structure of ferryl complexes.

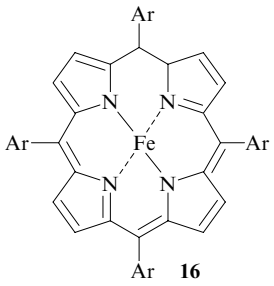
Fig. 8. For complexes $Fe^{IV}=O$, the spin ground state is determined by the strength of the ligand field in the equatorial plane (orthogonal to the Fe–O bond), because equatorial donor atoms change relative energy of the orbital $d_{x^2-y^2}$. Strong σ-donor equatorial ligands, for example, amines, favour the low-spin ground state. Weak-field equatorial ligands such as oxygen donors, as well as elongation of the Fe–N bond, favour the high-spin state.[158]

It is worthwhile to start the consideration of peculiarities of the electronic structure of ferryl complexes from mononuclear complexes $Fe^{IV}=O$, which are the most thoroughly studied experimentally and theoretically. Although active sites of non-haem oxygenases contain iron atom in the high-spin state, almost all ferryl complexes synthesised are low-spin complexes. The only exclusion is the aqua complex $[FeO(H_2O)_5]^{2+}$, which was obtained by treatment of an aqueous solution of Fe(II) salt with ozone[160] and is the only synthetic high-spin iron-oxo complex fully characterised by chemical and spectroscopic methods.[161] It is stable for 10 s in acidic aqueous solution at room temperature and behaves as a strong oxidant abstracting H atom from alkanes and oxidising alcohols to ketones. Theoretical interest in this complex was first of all associated with the discussion on its role in the Fenton system.[162] The electronic structure[161, 163, 164] of the cation $[FeO(H_2O)_5]^{2+}$ is similar to that of the non-solvated ion $[Fe=O]^{2+}$ due to weak donor properties of a water molecule. The ground state of the species $[FeO(H_2O)_5]^{2+}$ is high-spin ($S=2$): four orbitals contain each one electron (the quintet ground state, Fig. 8). 3σ*-Orbital is the lowest unoccupied orbital and an important boundary orbital for interactions with hydrocarbons. The high-spin state should be more reactive due to lowering of the energy level of the lowest unoccupied σ*-orbital as a result of strong exchange stabilisation of unpaired electrons in this state. These conclusions are in accordance with high catalytic activity of non-haem oxygenases, where oxygen donors dominate in their coordination environment and ferryl intermediates are in the high-spin ground state.

As was mentioned above, the group $Fe^{IV}=O$ ($S=1$) is incorporared in Cpd I of P450 and together with porphyrin radical cation ($S=1/2$) forms the almost degenerate ground state from two closely located spin states, a quartet ($S=3/2$) and a doublet ($S=1/2$) (Fig. 8). Moreover, in contrast to non-haem systems, a large set of porphyrin orbitals determines a dense set of excited states in Cpd I of P450. The calculations indicate that the elctron character of Cpd I of P450 is determined by the degree of hydrogen bonding and by polarity of the environment of the thiolate ligand.[11] In the gas phase, Cpd I has predominantly the S-radical character due to a significant admixture of p-orbital of the sulfur atom to the porphyrin a_{2u}-orbital, whereas, taking into account hydrogen bonding and polar environment of the thiolate ligand, this mixing decreases and the unpaired electron is mainly localised on porphyrin. These changes influence the reactivity of intermediate Cpd I and relative selectivity of hydroxylation and epoxidation of propene. It was found that external electrostatic field along the S–Fe–O bond axis results in changes in the calculated spin distribution between thiolate and porphyrin and simultaneously in the regioselectivity of oxidaton. Intermediate Cpd I of P450 was called a 'chemical chameleon' for this ability to change its electronic structure depending on protein environment.[165]

Only the most stable $Fe^{IV}O$-complexes with haem- and non-haem ligands and the iron atom in the low-spin state ($S=1$) have been relatively well studied.[29] A comparison of experimental and calculated characteristics of these complexes allows calibration of the results of the corresponding quantum chemical calculations and extrapolation of these results to high-spin intermediates of natural oxygenases.[166, 167] Theoretical study of spectral characteristics of short-living active intermediates can facilitate their detection.[166, 168]

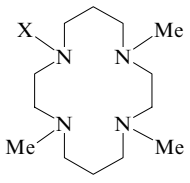
Model six-coordinated ferryl complexes $P^{+\bullet}Fe^{IV}=O$ with tetraarylporphyrin ligand **16** have been obtained at low temperatures; the structures were confirmed by different spectral methods including ESR and ^1H NMR, resonance Raman, Mössbauer and X-ray absorption spectroscopic methods.[169, 170]



16

Ar = Ph (TPP), *etc.*

Mononuclear non-haem $[Fe^{IV}=O]$-complex was obtained by the reaction of ozone with the iron complex containing the cyclame-acetate ligand (**17**, $X=CH_2CO_2^-$) at −80 °C and characterised in solution by Mössbauer spectroscopy.[171]



17

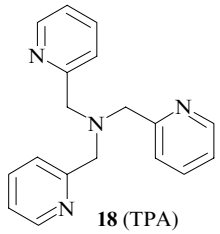
X = $CH_2CO_2^-$, Me (TMC), $CH_2CH_2S^-$ (TMCS), CH_2Py.

Similar complex having the TMC ligand (**17**, X = Me) was successfully isolated in the crystal state and its X-ray structure was established.[172] Recently, a series of biomimetic six-coordinated $[Fe^{IV}=O]$ complexes with non-haem ligands has been obtained; many of these complexes were characterised by structural and spectral methods.[30, 31, 173] Although attempts to obtain a model haem $Fe^{IV}O$ complex with the thiolate ligand failed, the non-haem analogue of Cpd I of P450, complex $[Fe^{IV}=O(TMCS)]^+$ (TMCS is ligand **17**, $X=CH_2CH_2S^-$) has been synthesised and char-

acterised by spectral methods.[174] The thiolate group in this complex is stabilised due to a covalent attachment to trimethylcyclame in the pentadentate ligand TMCS.

In spite of enormous efforts, up to date all attempt to trap and characterise spectroscopically highly active short-living ferryl intermediates of some oxygenases containing a group with the iron atom in higher oxidation state, [Fe^{V}=O] (Fig. 8) were unsuccessful. To a certain extent, it also refers to intermediate Cpd I of P450, which is formally compound [Fe^{V}=O]. In experiments with laser flash photolysis of iron corrole complex (analogue of porphyrin complex) and CYP119, which have been recently carried out,[32] the spectra observed (Table 1) were assigned to short-living [Fe^{V}=O] intermediates. However, the QM/MM calculations (Quantum Mechanical/Molecular Mechanical Calculations) for camphor cytochrome $P450_{cam}$ did not confirm involvement of the intermediate [Fe^{V}=O] in the reaction, though a possibility for participation of other excited states with a lower barrier to abstraction of H atom could not be ruled out.[175] Since these intermediates can be detected only by spectral methods, reliable prediction of the spectra of these intermediates from quantum chemical calculations is extremely important.[168] Unfortunately, only a small number of calculations of intermediates [Fe^{V}=O] has been published.[168, 176]

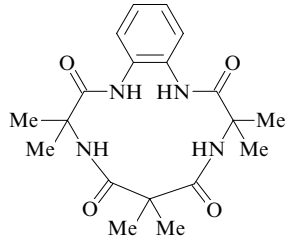
Theoretical calculations were performed for intermediate [HO(TPA)Fe^{V}=O] [TPA is tris(2-pyridylmethyl)amine, ligand **18**].



18 (TPA)

The formation of the latter in model systems was suggested on the basis of incorporation of a labelled oxygen atom from water into the oxidation product due to a possibility for fast exchange of the ferryl oxygen atom with water.[177] According to the calculation, a model species has the quartet ground state ($S=3/2$) with rather short Fe–O bond(1.64 Å) and elongated Fe–OH bond (1.77 Å).[178, 179] The Fe–O bond distance and spin density distribution in the ground state are close to those calculated for Cpd I of P450 and related model complexes. However, in contrast to Cpd I of P450, the TPA ligand is not oxidised; both electrons required for the formation of Fe^{V}=O are delivered by the iron atom.

Recently, a first model [Fe^{V}=O]-complex with the tetraamide macrocyclic ligand TAML (**19**), which is stable against oxidation, has been synthesised and characterised by spectral methods.[180]



19 (TAML)

The group Fe^{V}=O in the relatively long-living complex [(TAML)Fe^{V}=O]$^{-}$ (several hours at $-60\,^{\circ}C$) can be stabilised due to the presence of four expremely strong donors, amide nitrogen atoms in the deprotonated form of the ligand. Spectroscopic data and DFT calculations indicate that this complex is a low-spin one ($S=1/2$). Its lowest excited state is the Fe^{IV}–radical cation TAML ($S=3/2$), as is the case for the corresponding state for Cpd I of P450. This compex is able to oxygenate non-activated C–H and C=C bonds, similar to Cpd I of P450.

The reactivity of the ferryl group is determined by the lowest (unoccupied or semi-occupied) boundary MOs, which are $3\sigma^{*}$- and $2\pi^{*}_{x,y}$-orbitals. Relative contribution of these acceptor orbitals into the interaction with the substrate donor orbital is determined by both their relative energies and the degree of overlap with the substrate donor orbital, overlapping being dependent on geometrical factors.[181] The difference in activities of high-spin and low-spin ferryl species originates from relative contribution of these orbitals into the interaction with a substrate.[181]

The calculation of O atom transfer from $[FeO(H_2O)_5]^{2+}$ to the methane molecule to form methanol indicated [182] that the reaction follows the oxygen rebound mechanism. The first step including hydrogen transfer from methane to the oxo ligand and formation of a complex $Fe^{III}OH$ with the bound radical CH_3 proceeds rather easily, because cleavage of the C–H bond, occurs simultaneously with the O–H bond formation. The radical CH_3 is bound to the complex by a three-centred two-electron bond with the strength intermediate between that of hydrogen bond and simple covalent bond. Elongation of the C–H bond strongly increases its polarity thus stabilising the reaction transition state. The second stage leading to the formation of methanol bound to the complex also has a low activation barrier and is strongly exothermal. The theory of valence bonds [183] gives a reasonable explanation of the oxygen rebound mechanism and allows a quite reliable prediction of activation barriers for the stage of the abstraction of H atom from different alkanes.[184]

It is worth considering the problem of possible relationship between the activity of ferryl complexes and involvement of the oxyl excited states $Fe^{III}-O^{\bullet}$ and $Fe^{IV}-O^{\bullet}$ in their electronic structures (Refs 185 and 186).‡ Participation of these forms is considered important in the trasition state of reactions of ferryl intermediates.[151, 181] The oxyl structure is suggested for ferryl species in iron-containing zeolites capable of oxidising methane at room temperature.[187] In contrast to well-separated low-lying states of O_2 molecule, for the ion $[Fe{=}O]^{2+}$ the energy of the excited state $^{5}B_2$ is sufficiently close to that of the ground state.[188] Malykhin *et al.*[188] believe that the radical properties of ion $[Fe{=}O]^{2+}$ in this state enhance, because the oxygen atom becomes a σ-radical, which predetermines the activity of the ferryl intermediate with respect to H abstraction. However, the majority of researchers consider high spin population on the oxo ligand as a consequence of covalent delocalisation of π-bonds of the ferryl fragment.[158]

The observation that reactions of cytochrome P450 proceed, as if several oxidants participated in these reactions, has led to the development of a concept on two-state and multi-state reactivity(MSR),[33] which originates from studies of gas phase reactions of $Fe{=}O^{+}$ with H_2.[189] The

‡ See Appendix (3).

point of this concept applied to high-valent metal-oxo complexes is that the process can simultaneously proceed through several routes (potential energy surfaces, PES) corresponding to different spin states. Different states jointly give a set of different intermediates and products. In the case of cytochrome P450, the oxo complex in the doublet state reacts according to the concerted mechanism (barrierless oxygenation), whereas in the quartet state, it reacts according to the step wise mechanism (with a high oxygenation barrier). Since in the region of intersection of PESs, the energy values of different spin states are close, the transition from one surface to another is possible (spin crossing). Such transition to the barrierless surface leads to a significant acceleration of the process and to high stereoselectivity.

Hydroxylation of methane and propane by intermediate $[HO(TPA)Fe^{V}{=}O]$, according to calculations, occurs on the PES of the quartet state.[178] However, since the lifetime of the radical intermediate is very short, this process appeared to be efficiently concerted, which explains essentially stereospecific hydroxylation of alkanes observed experimentally.[177] The calculation of the effect of substituents in pyridine rings of the ligand[190] demonstrated that electron-withdrawing groups reduce the activation barriers to hydroxylation, whereas electron-donating groups increase it. The introduction of six NO_2 groups into ligand **18** decreases the activation energy to 12 kcal mol^{-1} (Ref. 190).

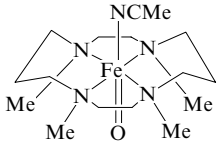
The reactivity of haem and non-haem synthetic ferryl complexes is significantly affected by *cis*-equatorial and *trans*-axial ligands.[34] The calculations demonstrated[191] that axial ligands control the activity of high-spin ferryl complexes in substrate activation changing the energy level of the acceptor $3\sigma^*$-orbital. The activity increases in the series: $NH_3 < MeCN < H_2O \approx H_2S < BF_3 < 0$ (the digit 0 implies the lack of an axial ligand). In the latter case, the interaction with methane would proceed without activation barrier.

Theoretical calculation suggested a probable explanation of the influence of axial ligands on the reactivity of haem ferryl complexes: weakening of the Fe–O bond in the transition state by donor ligands[192] and change in the spin state of these reactive species.[193] It was found for non-haem iron-oxo complexes[194] that, as well as for porphyrin complexes,[195] electron-withdrawing substituents in the *cis*-position increase electrophilicity of the $Fe^{IV}{=}O$ centre. The effect of axial ligands in these complexes appeared to be stronger and strictly inversed: electron-donating substituents increase oxidising activity. Unlike haem oxygenases having a relatively rigid porphyrin-thiolate environment, in the case of non-haem oxygenases, different ligands can occupy both *cis*- and *trans* positions with respect to the oxo group, which provides a wider possibility to vary oxidising properties of native oxygenases.

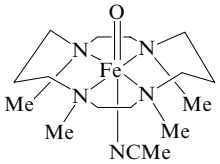
The TSR concept can be also applied to non-haem low-spin complexes.[196] Experimental and theoretical studies of reactivity of non-haem model complexes $[Fe^{IV}{=}O(TMC)L]^{2+(1+)}$, where L = MeCN, $CF_3CO_2^-$, N_3^-, RS^-, provide understanding of the nature of high reactivity of cytochrome P450. While, as expected, upon O atom transfer to phosphine, its activity is increased with an increase in electrophilicity of the ferryl complex, the order of the increase in activity upon H abstraction from C–H bonds unexpectedly becomes opposite: $MeCN < CF_3CO_2^- < N_3^- < RS^-$, *i.e.* a donor ligand enhances oxidant activity.[197,198] This result was rationalised within the TSR concept.[199] According to this concept, all reactions include two adjacent spin states of the fragment $Fe^{IV}{=}O$: the triplet ground state having a high barrier and the quintet excited state with a lower barrier. On each PES, barriers decrease with an increase in electrophilicity of the ferryl complex. The rationale for this unusual activity enhancement upon H atom abstraction consists in the fact that the PES for the quintet state 'crosses' the barrier of the triplet state through the spin inversion from the triplet to the quintet state, thus ensuring the lowest-energy route for the reaction. An increase in electron-donating character of the axial ligand decreases the energy difference between the triplet and quintet states, facilitates inversion and, therefore, increases the contribution of the more reactive quintet state to the reaction. This explains the enhancement of activity of Cpd I of P450 by the thiolate ligand in the reaction of H abstraction from alkanes. Thus, in the case of higher organisms, Nature uses a less aggressive reagent, which, due to the presence of the axial cysteine residue, ensures the most efficient low-energy reaction route.

Electrophilicity of the ferryl group in intermediate Cpd I is decreased due to pronounced donor properties of porphyrinate and thiolate It was found by experimental and theoretical methods that the thiolate ligand increases the basicity of the ferryl complex.[46,76] High basicity is supposed to be the stategy of Nature ensuring H atom removal from alkanes at a lower redox potential, which protects the protein environment from oxidative destruction.[77]

High potential to influence the ferryl centre by changing its nearest environment has recently been demonstrated using ferryl complex with the cyclam ligand as a model.[200] It was shown that even insignificant changes in the coordination sphere of the iron atom can strongly affect the reactivity of the ferryl complex. For example, changes in the arrangement of axial ligands, oxo group and acetonitrile, markedly increase oxidising ability of complex **A** as compared to that of complex **B**.§

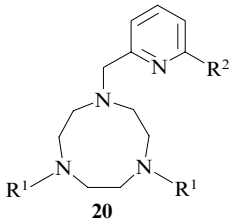


A



B

Similarly, small variations of R^1 and R^2 in ligand **20** strongly affect the selectivity and stereospecificity of cyclohexane oxidation catalysed by the corresponding iron complex.[201]



20

R^1 = Me, Pr^i; R^2 = H, Me.

§ See Appendix (2).

QM/MM calculations and numerous experimental observations demonstrate that the protein environment strongly influences the rate and selectivity of reactions of ferryl intermediates.[202, 203] The enzyme compensates a large entropy contribution into free activation energy for H abstraction from the substrate associated with a loss of translational and rotational degrees of freedom, at the stage of substrate binding through hydrophobic interactions. Due to this, for example, free activition energy for camphor hydroxylation with $P450_{cam}$ is decreased by about 10 kcal mol^{-1} (Ref. 204). Changes in the position and dynamics of adjacent amino acid residues of protein molecules near the active site decrease the activation barrier not only through stabilisation of the transition state, but also increasing the contribution of the tunnel effect,[205] which plays a significant role in the interaction of ferryl complex with compounds containing C–H bonds.[206]

Recently, chemical evolution of some forms of cytochrome P450 has been achieved using directed mutations in order to 'tune' it for the oxidation of simple hydrocarbons, which are not their natural substrates.[207] Preparation of novel, much more efficient, forms simulates the process of natural evolution of this oxygenase and demonstrates that 'tuning' for the substrate consists of the search for the optimum configuration of the enzyme–substrate complex.¶

In spite of the fact that mononuclear oxygenases, including cytochrome P450, are able to oxygenate strong C–H bonds, Nature has selected the dinuclear centre for methane oxidation. The active species is supposed to be an intermediate containing the $Fe^{V}-O-Fe^{III}$ fragment;[132, 208] the role of the second Lewis centre consists in the enhancement of electrophilicity of the ferryl complex [191] in order to attack a very strong C–H bond of methane. However, the nature of extremely high activity of MMO is yet to be established.

The majority of oxygenases represent multicomponent enzymes; the interaction between protein components is often decisive in 'tuning' the activity. This is especially true for the so-called 'effectory' proteins. Though nowadays there is a number of hypotheses, structural background for effectory function of these proteins has not yet been finally established.[28, 209]

Thus, Nature employs the most diverse tactics in the general strategy for oxidative activation of C–H and C=C bonds based on ferryl intermediates. High activity and extreme selectivity of oxygenases is achieved at different levels of their hierarchal organisation, starting from metal ion and its proximal coordination environment up to multicomponent interactions in the enzyme system. To solve the problem of creation of a catalyst for oxidative functionalisation of the most inert bonds including C–H bonds in methane, it is necessary to meet two mutually exclusive requirements: to create an exclusively reactive agent and to ensure a possibility to operate with it selectively and without damaging the molecules comprising the background of living systems. Nature succeeded in this task. Study of different oxygenases, creation and investigation of their chemical models and quantum chemical calculations of processes involving enzymes and models allow undertanding of the strategy of Nature in oxidation of non-activated C–H and C=C bonds and the use of its experience in human practice.

¶ See Appendix (4).

VI. Prospects for bio-inspired oxidation

Inertness of hydrocarbons substantially limits their use. At the same time, the lack of efficient methods for selective oxidation of non-activated C–H and C=C bonds in complex multifunctional molecules is a serious problem for fine organic synthesis. Both problems have been successfully solved for billion years of evolution by the creation of efficient and selective natural catalysts, iron-containing oxygenases. Tthis altogether encourages chemists for development of analogous catalysts, for example, on the base of high-valent metal-oxo complexes similar to reactive intermediates of oxygenases. High reactivity and selectivity of these systems are achieved through the design of the first and the second coordination sphere of metal complexes based on biomimetic ligands fixed on a relatively rigid scaffold, because the organisation of the active site is especially important. Advantages of biological and chemical systems can be combined in biomimetic catalysts. Chemists are not limited in the choice of material and can use other transition metals (Mn, Ru, Co, Cu, Ni, *etc.*) and any ligands. The problem is to achieve the final goal at minimum design, namely, to create chemical catalysts, which would be equal to oxygenases in their efficiecy and selectivity. Since these catalysts are important for both synthetic chemistry and chemical industry, biomimetic oxidation with the use of synthetic complexes has attracted great interest.[6, 7, 35, 210]

The first simple models of cytochrome P450, which allowed oxygen atom transfer to double bonds of alkenes and C–H bonds of alkanes, were iron tetraarylporphyrin complexes **16**, Fe(TPP)Cl and others applied as catalysts for alkane hydroxylation and alkene epoxidation by iodosobenzene.[211, 212]

Later, these models were substantially improved by the introduction of electron-withdrawing substituents into the phenyl groups of porphyrin, then by complete halogenation of the porphyrin macrocycle, which decreased oxidative destruction of the ligand and enhanced reactivity of the intermediate.[36, 213] On the basis of these electron-deficient porphyrins, rather efficient catalysts have been created ensuring high yields of products at high oxidation rates of hydrocarbons including n-alkanes.[214, 215] Mainly the models using oxygen donors such as H_2O_2, PhIO, NaOCl, *etc.* have been developed, whereas the creation of models based on O_2 was hindered by the difficulties concerning the competition between substrate and reductant for the reactive oxygen species.

Bio-inspired oxidation maintains many distinguished features of biocatalytic oxidation. Selectivity of the formation of the major product is usually above 80%; in most cases, it reaches 90%–100%, while the oxidation rate can be very high. Thus the rate of selective hydroxylation of the tertiary C–H bond in adamantane at 65 °C is 1000 turnovers of catalyst per minute.[216] The process is highly regio- and stereoselective.[37, 170, 177] In most cases, the efficiency of the oxidant use is higher than 60%. Application of H_2O_2 as a cheap and environmentally safe oxidant is especially attractive for biomimetic oxidation of hydrocarbons.[34] Recently, it has been found that biomimetic oxidation of alkanes by hydrogen peroxide can be performed with satisfactory efficiency using non-haem complexes [37, 177] or, in the case of models of cytochrome P450, complexes with electron-deficient ligands.[214, 217, 218]

Modification of the nearest surrounding of the metal-bound O atom through, for example, the introduction of bulky or chiral groups into the *ortho*-position of the phenyl rings of TPP provides a possibility to influence regio- and enantionselectivity of oxidation. Catalytic asymmetrical monooxygenation of alkanes, alkenes and alkyl sulfides was performed using Fe and Mn complexes of binaphthyl-substituted porphyrin.[219] The introduction of bulky substituents into porphyrin changes the regioselectivity of n-alkane oxidation toward an increase in the proportion of oxidation products at the terminal (ω) methyl group and/or adjacent methylene (ω−1) group,[220] selectivity of formation of primary alcohols achieving 26%–30%.[221,222] The possibility of obtaining primary alcohols from n-alkanes makes these systems similar to ω-hydroxylase.

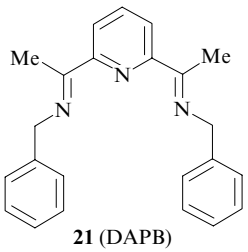
Quite perfect models of cytochrome P450 have been created on going from simple sterically hindered metalloporphyrins to systems with hydrophobic cavities constructed either through covalent 'attachment' of molecules with cavities (cyclodextrins, cyclophanes) or through self-assembling into organised molecular assemblies through non-covalent interactions.[38] However, the majority of these models are designed for alkene epoxidation, which is a simpler task than alkane hydroxylation.

A great number of non-haem biomimetic complexes capable of catalysing oxidation of non-activated C−H and C=C bonds by hydrogen peroxide has been synthesised.[37,177,223–225] A series of systems based on non-haem complexes of iron and other transition metals has been suggested for selective alkene oxygenation.[224–227] In contrast to porphyrin complexes, these compounds appeared to be able to catalyse *cis*-dihydroxylation of alkenes by hydrogen peroxide, similar to non-haem Rieske oxygenases.† With these complexes, the diol : epoxide ratio is determined by the nature of the ligand environment and varies within the limit 0.1–14; the efficiency of the oxidant used achieved 85%.[226] The corresponding design of the ligand provides a possibility to perform asymmetric *cis*-dihydroxylation with 97% enantiomeric excess.[228]

In the presence of iron complexes with ligand **20**, cyclohexane is oxidised by hydrogen peroxide with turnover number (*N*) of up to 64 and up to 74% use of the reagent. Oxidation of olefins to form a mixture of epoxide and diol proceeds with *N* of up to 252.[201]

Manganese complex with ligand **20** epoxidises selectively *cis*-olefins with peracetic acid, *N* being above 1000 with a conversion > 98% and selectivity > 99%.[229] Mn complex with a tridentate nitrogen ligand (triazacyclononane) and chiral carboxylate catalyses enantioselective *cis*-dihydroxylation of olefins by hydrogen peroxide with $N > 2000$.[230]

Iron complexes with tetradentate nitrogen ligands **17**–**20**, *etc.* are of interest, for they allow stereospecific (up to 100%) alkane oxidation with hydrogen peroxide.[177] Efficient and selective alkane hydroxylation with *m*-chloroperoxybenzoic acid catalysed by Ni(II)(TPA) and related complexes has been reported.[231] At room temperature, the catalyst's turnover number is about 600 to give exclusively alcohol (the ratio alcohol : ketone ≈ 40). Oxidation of cyclohexane with hydrogen peroxide in an argon atmosphere at 50 °C in the presence of the catalytic system $Fe(ClO_4)_3$−DAPB−MeCN (DAPB is ligand **21**)[232] gave 93% overall yield of ketone and alcohol (calculated per hydrocarbon) with 100% selectivity. Efficient catalytic alkane oxidation by different peroxide compounds with *N* up to 7500 was achieved using a catalyst based on the system Os^{VI} – Lewis acid.[233]



21 (DAPB)

Specially prepared iron-containing zeolites appeared to be efficient biomimetic catalysts for direct oxidation of benzene to phenol by nitrogen(I) oxide (N_2O) with selectivity close to 100% at 50% benzene conversion.[234]

Recently a strategy of molecular recognition has been successfully used[235–237] for directed catalytic oxidation of definite C−H bonds in a complex molecule in the presence of more reactive bonds. For example, highly selective hydroxylation of steroid molecules into position 9 was successfully performed in aqueous medium using a biomimetic Mn-porphyrin catalyst with several cyclodextrin molecules linked to it. The latter should be responsible for hydrophobic substrate binding and proper orientation of the C−H bond at the C(9) atom with respect to the attacking reactive oxygen species. Like cytochrome P450, this catalyst manifests good substrate selectivity, excellent regio- and stereoselectivity with turnover number of up to 3000.[235] Selective hydroxylation of steroids at position 9 is of considerable practical interest for pharmaceutical chemistry.

Dinuclear manganese complex with two tridentate nitrogen ligands containing rigidly fixed COOH groups has been reported.[236] Each of these groups can interact through hydrogen bonds with the carboxylate group of the substrate, such as ibuprophen, providing its orientation in the way when only one C−H bond is accessible for oxygenation. This catalyst allowed oxidation of the substrates that can adopt different conformations with *N* of up to 700 and very high (>98%) regio- and stereoselectivity.[236,237]

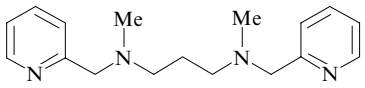
First steps have been undertaken in biomimetic methane oxidation. First of all highly selective (100%) stoichiometric oxidation of methane to methanol by nitrogen(I) oxide at room temperature using a dinuclear iron complex on the surface of zeolite FeZSM-5 should be mentioned.[238,239] A study by Mössbauer spectroscopy indicated the involvement of two long-living intermediates similar to intermediates **P** and **Q** of MMO.[240] The peroxo intermediate was characterised by electronic absorption and Raman spectra.[241] It was found that upon treatment with benzene, the absorption bands of the intermediate disappear, but the spectrum of phenol coordinated to the iron atom appears. Similar Cu-containing zeolites catalyse selective oxidation of methane and other lower alkanes with dioxygen at 125 °C to give alcohols.[242] Spectral evidence for the involvement of copper-bis-μ-oxo complexes, which are similar to the putative active oxidant of MMO, has been obtained.[243]

Finally, catalytic methane oxidation with hydrogen peroxide in water to give MeOH, CH_2O and HCOOH at 25–60 °C with a dinuclear iron phthalocyanine complex containing the fragment $Fe^{III}-N=Fe^{IV}$ has recently been reported.[244] Maximum turnover number achieved in this

† See Appendix (5).

reaction was 438, the product yield calculated per peroxide was 30% – 50%. Spectral and isotopic studies demonstrate that peroxide and/or ferryl ($O{=}Fe^{V}-N{=}Fe^{IV}$) intermediates participate in this process.[244]

Creation of biomimetic systems for hydrocarbon oxidation with dioxygen [245] is a more complicated task, because it requires a high degree of organisation of a catalyst, in particular, to suppress the competition between substrate and reductant for the reactive oxygen species. Nevertheless, certain hope for the creation of these systems has appeared recently. For example, pyridylamine complex of Fe(III), $[L^3{}_4Fe^{III}(Cl)(\mu\text{-}O)Fe^{III}Cl_3]$ (L^3_4 is ligand **22**) catalyses selective hydroxylation of n-hexane in the system O_2 – reductant – proton donor with *N* achieving 50.[246] The authors used trimethylhydroquinone as a reductant; ferryl intermediate is supposed to participate in oxidation.



22

Catalytic oxidation with dioxygen and formation of a ferryl complex stabilised by cyclam was conducted in the presence of a source of electrons and protons, alcohols,[247] ascorbic acid or the system $BPh_4^- - HClO_4$.[248] Kim *et al.*[247] suggested the mechanism of O_2 activation with the participation of two iron atoms, as is the case for MMO. However, Thibon *et al.*[248] provided serious evidence that a source of protons and electrons is required for this process. This is in accordance with the transient formation of a peroxo intermediate, the precursor of the ferryl complex, and simulates the mechanism of O_2 activation by cytochrome P450 and the Rieske dioxygenase.

In recent years, more and more publications have appeared devoted to preparative biomimetic oxidation of hydrocarbons. Iron non-haem complexes perform 90% conversion of an alkene to the epoxide using only 1.5 equiv. of hydrogen peroxide.[249] Accessible and cheap iron phenanthroline complex appeared to be a more efficient catalyst for preparative alkene epoxidation with the use of peracetic acid as an oxidant.[250] Finally, almost 100% conversion of an alkene to the epoxide was achieved with the use of H_2O_2 and 0.5 mol % of an iron complex with the tetradentate nitrogen ligand (TPA) in a mixture of acetonitrile and acetic acid,[251] which inhibits diol formation.

Investigations in the field of biomimetic oxidation have also laid the foundation for the creation of preparative methods for selective oxidation of non-activated C – H and C = C bonds in complex multifunctional molecules. In addition to above-mentioned examples of directed oxidation of steroids [235] and ibuprophen,[236] efficient one-step oxidation of one of C – H bonds of multifunctional artemisinine molecule without introduction of protective or activating groups is worth noting.[252] Under the action of hydrogen peroxide, stereochemically pure product (yield 45%) maintaining labile functional groups is formed from artemisinine.

Thus, joint efforts of chemists resulted in the design of ligands and preparation of active biomimetic catalysts for oxygenation of the strongest bonds with the efficiency (turnover number) of up to hundreds and thousands. The possibility has been demonstrated to achieve high chemo-, regio-, stereo- and enantioselectivity with a sufficiently high degree of substrate conversion and economic oxidant consumption. These studies are extremely important for the development of 'green chemistry'. Although application of biomimetic catalysts for large-scale functionalisation of hydrocarbons with the aim of more rational utilisation of oil and gas raw materials is still a long-term prospect, such catalyst can be demanded now for the creation of innovation oxidative processes for fine organic synthesis and pharmaceutical chemistry. In accordance with the lessons of Nature and using a great arsenal of tools of chemical science, new interesting results are expected on the way of further design and synthesis of more organised ‡ and oxidatively robust [253] bio-inspired catalysts.

VII. Appendix

Newer data relevant to the review have appeared since submission of the manuscript to the journal; these are given below.

1. Although most researchers are prone to support a Cu-active centre in pMMO [S I Chan, S S-F Yu *Acc. Chem. Res.* **41** 969 (2008); R Balasubramanian, A C Rosenzweig *Acc. Chem. Res.* **40** 573 (2007)], some data {M Martinho, D W Choi, A A DiSpirito, W E Antholine, J D Semrau, E Münck *J. Am. Chem. Soc.* **129** 15783 (2007); L V Tumanova, I A Tukhvatulin, D Sh Burbaev, R I Gvozdev, K K Andersson *Bioorg. Khim.* **34** 194 (2008) [*Russ. J. Bioorg. Chem.* **34** 177 (2008)]} attest to an Fe-active centre as in sMMO.

2. Recently, it has been shown [F Shi, M K Tse, Z Li, M Beller *Chem. – Eur. J.* **14** 8793 (2008)] that the medium pH can control the oxidation processes involving hydrogen peroxide and iron salts by switching the mechanism from non-selective radical ($HO^{\bullet}$-inintiated) to selective reactions involving a ferryl intermediate.

3. See also recent article: F Banse, J-J Girerd, V Robert *Eur. J. Inrg. Chem.* 4786 (2008). Higher reactivity of the oxyl complexes was postulated in C – H bond oxygenation by oxo complexes of high-vallent maganese [D Balcells, C Raynand, R H Crabtree, O Eisenstein *Inorg. Chem.* **47** 10090 (2008)].

4. Of special note is the regulatory role of the secondary coordination sphere including H-bonding interactions [R L Shook, A S Borovik *Chem. Commun.* 6095 (2008)] in the active sites of metallooxygenases.

5. Recently, it has been shown [Y Feng, C Ke, G Xue, L Que Jr *Chem. Commun.* 50 (2009)] that the complex $[Fe(TPA)(MeCN)_2]^{+}_2$ can catalyse cis-dihydroxylation of the aromatic double bond thus modelling the natural functions of NDO.

‡ S J Lee, S-H Cho, K L Mulfort, D M Tiede, J T Hupp, S B T Nguyen *J. Am. Chem. Soc.* **130** 16828 (2008).

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