DALTON
PERSPECTIVE

Principles of small molecule activation by metalloenzymes as exemplified by the soluble methane monooxygenase from *Methylococcus capsulatus* (Bath)*

Ann M. Valentine and Stephen J. Lippard

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

Many metalloenzymes activate small molecules in a manner that is unique to natural systems. In this Perspective we discuss the soluble methane monooxygenase protein system from *Methylococcus capsulatus* (Bath), which uses a mixed-function oxidase to convert methane selectively to methanol. Through a series of biophysical studies, theoretical calculations, synthetic model studies and mechanistic biochemical experiments, the respective roles of the carboxylate-bridged non-heme diiron center and the protein environment in controlling the enzyme mechanism have been delineated. These results are used to identify themes common among metalloenzymes that activate small molecules and to identify future directions for the study of this protein system.

Although the field of bioinorganic chemistry is young compared to other areas of the discipline, such as organic synthesis, its subject matter may be traced perhaps even to the origin of life itself. Nickel and iron sulfide surfaces have been implicated as catalysts responsible for the chemoautotrophic events which occurred during primordial metabolism.[1] In this scenario, chemical sources rather than sunlight provided energy for emerging organisms, and bioinorganic units were vital in harnessing this energy. During the subsequent evolution of higher life forms, the special properties of transition metals as catalysts were preserved in the form of metalloenzymes which could achieve difficult feats of activation such as occurs in CO dehydrogenase/acetyl CoA synthase.[2] As modern biochemistry evolved in the current century, metalloproteins attracted attention because of their bright colors and magnetic properties, the heme and iron–sulfur proteins providing especially good examples of these phenomena. Other, equally important, proteins which lacked these distinguishing features were sometimes overlooked. Urease, the first enzyme to be crystallized,[3] contains nickel, but this fact was revealed only decades following its isolation.[4] Today, catalysis by metal-containing enzymes is a topic of great interest, not only to provide an understanding of a specific biological function, but also because knowledge of these processes provides clues to fundamental principles of chemical reactivity which are of potential utility in the chemical industry.[5]

With the maturing of bioinorganic chemistry,[6] it became possible to formulate general principles which together created a body of knowledge by which new phenomena could be understood. Metalloenzymes have been a prominent focus of this work and will be the topic of most of the discussion in this Perspective. Apart from accelerating the rates of chemical reactions, however, metal ions can play key structural roles in biology by effecting the long distance transfer of electrons and controlling fundamental signalling mechanisms in the cell. Moreover, several active pharmaceuticals depend on metal chemistry, examples being bleomycin and cisplatin.[5a]

Metal ions are often key components of enzymes which manipulate or activate chemical units having only two to five atoms.[7] What might be the role of the metal in such a metalloenzyme? A classical view of enzyme mechanisms holds that binding energy provides a significant amount of the rate enhancement observed in biocatalysis.[8] By using binding energy, an enzyme increases the effective concentrations of reactants and holds them in the optimal position to interact with one another or with a protein component. When the substrate is a large molecule, such as a carbohydrate or another protein, binding energy is afforded by multiple weak, non-covalent forces such as hydrogen bonds or hydrophobic interactions. When the substrate is dioxygen or another small molecule (Fig. 1),[2,9–14] this form of rate enhancement is unavailable because molecules having so few atoms cannot provide a sufficient number of such weak interactions. Instead, advantage may be taken of co-ordination chemistry, the binding of small substrates as ligands to metal centres, thereby effecting kinetically difficult transformations. In addition, many of the metals employed in biological systems have redox potentials within the physiological range of ±800 mV. This characteristic, and the ability of these potentials to be tuned by the ligand environment, are exploited in the activation of several of the molecules in Fig. 1.[5a]

Redox-active metalloenzymes facilitate reactions of dioxygen because the metal can overcome the kinetic barrier imposed by the triplet ground state of O_2. Reactions of triplet oxygen with organic compounds are slow because they are spin-forbidden.[15] The barrier to reactions with photochemically generated singlet oxygen is 15 kcal mol^{-1} lower in energy than that with triplet oxygen. To react with a hydrocarbon by a non-photochemical route, triplet oxygen must be converted to singlet oxygen by a radical process or must be activated by co-ordination to a transition metal having an incompletely filled shell of d electrons. Because radical processes often leak toxic by-products capable of damaging organisms, many of the systems which require dioxygen employ metalloenzymes.

The discussion thus far has stressed the role of the metal in a metalloenzyme, but the amino acid side chains at the active site cannot be ignored. Proteins can use such cavities, as illustrated by the gray area in Fig. 2, to guide substrates to the active site, orient them with respect to metal-activated species, and increase the local concentration of substrates at the reactive center. An advantage of metalloproteins over simpler co-ordination compounds as catalysts is that a kinetically labile and reactive moiety can be stabilized by the protein environment. These stabilizing forces are represented in Fig. 2 by solid lines which tether metal ions to the active site cavity. Reactive

* Based on the presentation given at Dalton Discussion No. 2, 2nd–5th September 1997, University of East Anglia, UK.

Non-SI units employed: cal = 4.184 J, atm = 101 325 Pa, Da ≈ 1.66×10^{-27} kg.

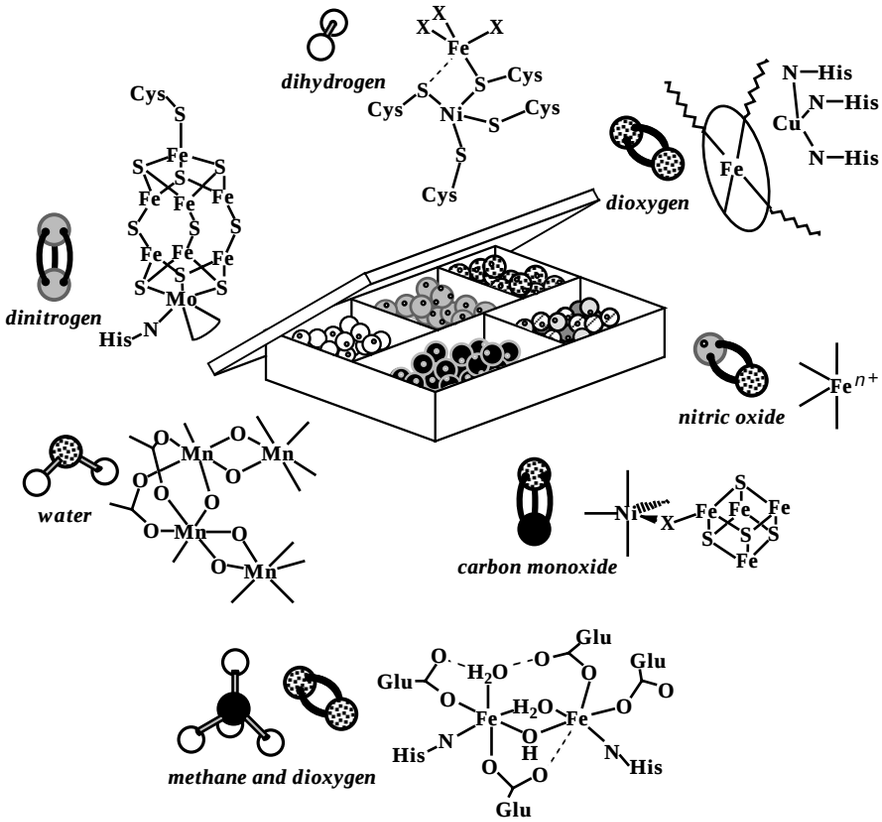

Fig. 1 Small molecules activated by metalloenzymes. Clockwise from top: H_2 and the active site of hydrogenase;[9] O_2 and the heme a_3-Cu_B site of cytochrome c oxidase;[10] NO and a model for the mononuclear iron active site of nitric oxide reductase;[11] CO and the nickel–iron–sulfur centre of carbon monoxide dehydrogenase/acetyl CoA synthase;[2] CH_4, O_2 and the non-heme diiron active site of methane monooxygenase;[12] H_2O and a proposed model for the oxygen-evolving complex of photosystem II;[13] and N_2 and the iron–molybdenum cofactor of nitrogenase[14]

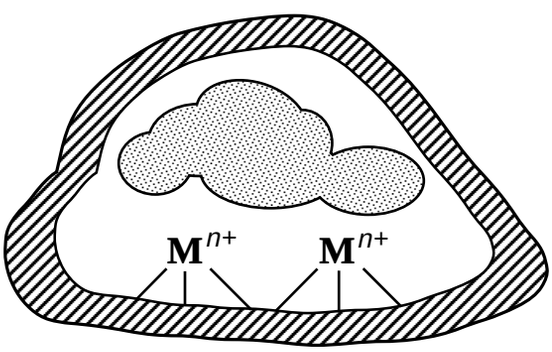

Fig. 2 Schematic representation of a metalloprotein active site. The protein construct stabilizes kinetically labile metals (forces represented by lines), sequesters active intermediates (barrier shown by striped bar), and provides a substrate binding site (depicted in gray)

intermediates which convert to non-productive products in free solution are sequestered within the protein environment, as symbolized in Fig. 2 by the striped enclosure. The presence of the protein scaffolding adds an element of control to the system by facilitating interactions with other protein molecules that modulate the activity of the catalyst. It can be disadvantageous for an enzyme to produce active species indiscriminately, and protein–protein interactions can regulate the formation and quantity of such species during catalysis.

Common Themes in Small Molecule Activation

Some of the reasons nature employs metalloenzymes to activate small molecules have been discussed. Beyond these rather basic concepts, however, additional common themes are emerging as more systems are examined. In the following sections we discuss these points and illustrate them with results of studies on the soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus* (Bath), carried out mainly in our laboratory. Certain of the conclusions have a generality which provide a common ground for comparing and contrasting the outcome of experiments performed on related systems.

Achievement of kinetically difficult transformations

Metalloenzymes can catalyze reactions which are difficult to duplicate outside of a natural system. Even when the active site structure can be modeled, its function is frequently not achieved. There are exceptions, of course, and some of the reactions catalyzed by metalloenzymes can be carried out by non-biomimetic systems, but mimicking bioinorganic reactions remains a significant challenge.

The sMMO system oxidizes methane to methanol using dioxygen, as indicated in equation (1).[16–18] In this mixed-

$$CH_4 + O_2 + NADH + H^+ \longrightarrow CH_3OH + H_2O + NAD^+ \quad (1)$$

function oxidation chemistry, one atom of dioxygen is incorporated into substrate and the other into water. The remarkable chemical reaction occurs at a carboxylate-bridged non-heme diiron active site housed in the hydroxylase enzyme, as illustrated in Fig. 3. The enzyme releases methanol before further oxidation can occur. For methane-requiring bacteria which employ sMMO, the reaction supplies both carbon and energy.[19] These methanotrophs and others like them play an important role in the global carbon cycle, preventing most of the methane produced by anaerobic microflora from reaching the atmosphere, where it contributes to the greenhouse effect, and recycling it back into the biosphere.[20] Methanotrophic bacteria have been used to remove oil spills from contaminated beaches and to degrade halogenoalkane contaminants in drinking water.[21]

The carboxylate-bridged diiron centres in sMMO activate

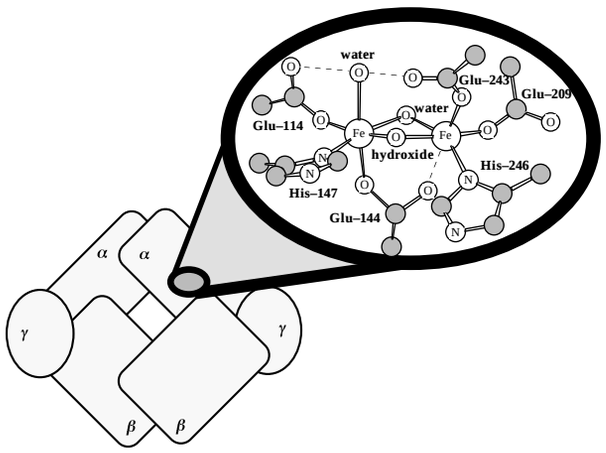

Fig. 3 View of the non-heme diiron active site housed in the α subunit of the sMMO hydroxylase enzyme. This diferric active site structure was determined at −160 °C.[12] An identical unit in the other α subunit is not shown

two small molecules, dioxygen and methane, to achieve a reaction which cannot be duplicated industrially except at high temperatures and pressures.[22] The conversion of methane to methanol is desirable because most of the abundant sources of natural gas are located in remote areas, and transport of a combustible gas poses significant problems. Conversion to methanol is economically competitive with shipping liquefied natural gas, even with the present inefficient conversion processes. The transformation of methane to methanol involves first its conversion to syngas ($CO + H_2$) by desulfurization followed by steam reforming [equation (2)], a process requiring

$$CH_4 + H_2O \longrightarrow CO + 3H_2 \qquad (2)$$

temperatures in excess of 800 °C. Syngas is then converted into methanol at 200–300 °C and 50–100 atm. Methods being developed for the direct oxidation of methane to methanol currently produce only very low yields of the desired product. If such methods could be improved, the liquefaction of methane would accrue significant economic benefits.

Apart from its industrial potential, the selective activation of unactivated carbon–hydrogen bonds in homogeneous solution was recently identified as one of the 'Holy Grails' of modern chemistry.[23] Attempts to achieve this transformation have typically failed by virtue of their inability to mimic one or more of the steps in the biological oxidation of methane by sMMO. Some reactions are not catalytic, others require *tert*-butyl hydroperoxide or another active oxygen source other than O_2, and still others demand high temperatures (>100 °C) and pressures (>50 atm). Many simply cannot oxidize methane or they oxidize it beyond the level of methanol. Recently, methane was oxidized to methanol by using dioxygen at 40 °C.[24,25] The best catalytic system, a $EuCl_3$–Zn–CF_3CO_2H–TiO_2 system, converted methane into methanol with a turnover number of about 10 h^{-1}.[25] The sMMO enzyme from *M. capsulatus* (Bath) performs the reaction with a turnover number of $\geqslant 0.19\ s^{-1}$.[26]

Multi-protein enzyme systems

Like many bioinorganic systems, sMMO is a multi-protein construct. Our group focuses on sMMO from *Methylococcus capsulatus* (Bath), a strain native to the thermal springs at Bath, UK, and kindly provided by Professor H. Dalton.[27] Other laboratories investigate material isolated from a different bacterial strain, *Methylosinus trichosporium* OB3b.[17,18] The sMMO from *M. capsulatus* (Bath) has been resolved into three constituent proteins.[27] The hydroxylase is a dimeric 251 kDa enzyme (MMOH) having $\alpha_2\beta_2\gamma_2$ stoichiometry with one non-heme diiron active site in each α subunit.[28] Its crystal structure[12,29,30] revealed the active site and overall protein structure shown schematically in Fig. 3. A second component protein is a 38.5 kDa reductase (MMOR) which contains an Fe_2S_2 cluster and an FAD moiety which accepts electrons in the form of hydride ion from NADH.[27,31] A 16 kDa coupling protein, designated protein B (MMOB),[32] couples consumption of electrons by the reductase with reduction of the diiron centers in the hydroxylase, preparing them for reaction with dioxygen and, ultimately, productive hydroxylation of the substrate. Binding of protein B to the hydroxylase affects the active site geometry, the regioselectivity of oxidation, and the lifetime of intermediates observed spectroscopically in single turnover reactions (see below).[33]

The compartmentalization of functions among the three proteins regulates the system at several levels. If the hydroxylase active sites were to react directly with NADH, this reducing agent might be consumed in the absence of substrate. Without MMOB, the hydroxylase undergoes non-productive futile reaction cycles with dioxygen to make water.[33*b*] Early evidence that component protein interactions regulate the system was the discovery that the resting state $Fe^{III}Fe^{III}$ hydroxylase could be titrated with electrons to yield the mixed valent $Fe^{II}Fe^{III}$ and fully reduced $Fe^{II}Fe^{II}$ forms,[34] but that, in a reconstituted system containing MMOH, MMOR, MMOB, and substrate, the potential for transfer of the second electron became more positive than for the first.[35] As a consequence, the diiron site was reduced by two electrons from the diferric directly to the diferrous state, forming little or none of the non-physiologically active mixed valent $Fe^{II}Fe^{III}$ form. Recently, the reductase was identified as the component responsible for this behaviour, and remarkably, it could exert the effect when present at only 10% of the concentration of active sites.[36] These and other experiments indicate that the MMOB and MMOR components help maintain exquisite control of the catalytic activity of the dinuclear active sites in MMOH.

Electron transfer over a distance

A significant property of metal ions is their ability to undergo reversible electron-transfer reactions at physiologically relevant potentials. In bioinorganic systems in which redox catalysis is employed, electrons are often transported over long distances (>10–20 Å) such that sites of reduction and oxidation can be widely separated. The donor and acceptor sites can even be in different proteins in a multi-component system, as discussed in the previous section, requiring long range electron transfer between protein molecules. The pathway of electron transfer from donor to acceptor through and between protein molecules has also been the subject of intense experimental and theoretical scrutiny, with attention focused on the question of whether through-bond or through-space movements are more likely to be involved.[37]

As mentioned earlier, the initial electron acceptor from the NADH molecule in sMMO is MMOR. This protein in turn reduces the hydroxylase, poising it to react with dioxygen. Exactly how MMOH and MMOR interact and how electrons access the diiron active sites remain unresolved. As of this writing, no crystal structures of complexes between sMMO components are available, although chemical cross-linking studies have indicated that MMOR binds to the β subunit of MMOH.[38] To address this question further, the MMOH crystal structures were systematically examined to identify putative sites of protein–protein interactions.[39] In this analysis, it was hypothesized that extended helix–helix contacts between two individual hydroxylase molecules in a crystal lattice might mimic contacts between MMOH and either of its partner proteins, MMOB and MMOR. Interacting regions having more than three helical turns were analyzed to determine whether they shared homology with sequences in MMOB and MMOR. One eleven-amino-acid sequence in domain 2 of the α subunit

Fig. 4 The α subunit of sMMO hydroxylase showing the hydrophobic cavities leading from the active site (cavity 1) to the protein surface (cavity 3). Iron atoms are depicted as striped spheres

of MMOH[29] displayed homology to a region of MMOR. This peptide was synthesized and added to a kinetic assay of MMO activity which it inhibited with an IC_{50} corresponding to a K_d value of <100 μM. By using the structure of a homologous reductase, a binding model was developed for the interaction between MMOH and MMOR, which if correct would help to identify the point of initiation of electron transfer into the hydroxylase.

Access to the MMOH active sites

The three-dimensional structure of a metalloenzyme, determined by X-ray crystallographic or NMR spectroscopic methods, reveals the position and geometry of the active site. When the metal core is buried within the protein matrix, it is interesting to inquire how the substrates reach the active site and by what pathway products depart.

The sMMO hydroxylase can oxidize a broad range of substrates, including alkanes, alkenes, ethers, amines and halogenocarbons.[40–45] The binding of these different substrates in the vicinity of the diiron centers in the α subunits was considered following the determination of the MMOH crystal structure.[29,39] Hydrophobic pockets were identified in a chain leading from the very hydrophobic cavity right at the active site, cavity 1, and extending outward through domain 1 of the α subunit toward the protein surface (Fig. 4).[29] The cavities were not connected into a channel, however. For example, in the first structure analyzed the cavity adjacent to the active site, cavity 2 in Fig. 4, was partitioned from cavity 1 by side chains of a leucine, a phenylalanine and a threonine residue. Similar side chain contacts separated other pairs of adjacent cavities from one another. In more recent experiments, xenon gas was used as a probe for putative methane binding pockets in MMOH. Xenon has the same size as methane but, owing to its high electron density, is much more readily identified by X-ray crystallography. When MMOH crystals were pressurized with Xe gas, a well ordered xenon atom was found in cavity 2.[39] In other forms of the crystal, including one grown from solutions containing MMOB, the leucine residue blocking access to cavity 1 from cavity 2 had an altered side chain conformation such as to open a connection between the two cavities.[39,46] This so-called 'leucine gate' was also in the open position in crystals where the diiron centre had been chemically reduced to the $Fe^{II}Fe^{II}$ state. The leucine gate thus appears to regulate substrate access to the active site, and may represent one way in which protein–protein interactions might control the chemistry at the catalytic iron center. For example, dioxygen or methane might be excluded from the active site until the gate opens, a process which could be triggered by binding of one of the other protein components and/or reduction of the hydroxylase diiron centres.

Theoretical calculations have also been used to identify possible sites of substrate binding to MMOH. One study[47] used a docking methodology and energy minimization which combined Monte Carlo and molecular dynamics techniques. Several putative binding sites were identified, with the favored ones depending mostly on the size of the substrate. A site corresponding to cavity 1 (Fig. 4) was favored by small substrates such as methane. Two other sites displayed favorable interactions with larger substrates and were located in pockets at or near the protein surface, somewhat removed from the active site. None of the sites was the one occupied by Xe in the crystal structure experiment.[39] Studies of this kind do not address the dynamics of substrate binding to the active site, and it is not obvious how substrates would make their way to the catalytic center without movement of protein side chains.

Substrate selectivity and diagnostic probes

Bioinorganic catalysts act upon a selected set of substrates, accelerating the rate of the desired chemical transformation. The attendant selectivity and rate enhancement may reflect the steric requirements of a substrate binding pocket and the power of the active species generated in the catalytic reaction cycle. The wide range of substrates oxidized by the soluble MMO systems,[41] for example, contrasts with the relatively narrow set of molecules which can be hydroxylated by the copper-containing particulate MMO (pMMO) hydroxylase.[48] Both will oxidize methane, which has a C–H bond energy of 104 kcal mol^{-1}, and release the product without further oxidation, despite the fact that the C–H bond strength of methanol is only 94 kcal mol^{-1}.[49] Mechanistic proposals must account for these properties. Since hydroxylating species in both sMMO and pMMO must be sufficiently powerful to be able to oxidize methane, one might therefore surmise that the active site of the latter is smaller to account for its more stringent substrate specificity. Both must be sufficiently hydrophobic such that methanol is extruded before further oxidation can occur. The hydrophobic character of the active site of sMMOH has already been established from the crystal-structure determination.[29]

The ability of sMMO to oxidize a wide range of substrates has enabled the use of diagnostic substrate probes to delineate aspects of the enzyme mechanism. Such probes were used extensively in mechanistic studies of cytochrome P-450, the heme iron analog of sMMO.[50] Radical clock substrate probes are molecules that contain strained rings which open if radical intermediates are formed during the course of an oxidation reaction [Fig. 5(*a*)]. When the rate constant for the ring opening step is known, the lifetime of the putative radical intermediates can be estimated from the ratio of acyclic to cyclic products obtained in the reaction. The oxidation of such probes by the cytochrome P-450 enzyme system has been used as evidence to support the widely accepted radical rebound mechanism,[50] although recent work has questioned this interpretation.[51] Radical clock substrate probes applied in the sMMO system revealed no or only very small amounts of ring opened products with *M. trichosporium* sMMO[43,44] and none for the *M. capsulatus* sMMO enzyme.[44,45] These results suggested either formation of a radical species with an exceedingly short lifetime ($\tau < 10^{13}$ s) or no substrate radical at all during the course of the hydroxylation reaction.[44]

Both sMMO systems have been probed mechanistically by using alkanes made chiral through the use of the three isotopes of hydrogen.[52,53] If a substrate radical were to form upon activation of $[^1H][^2H][^3H]C–CH_3$, for example, rotation about the

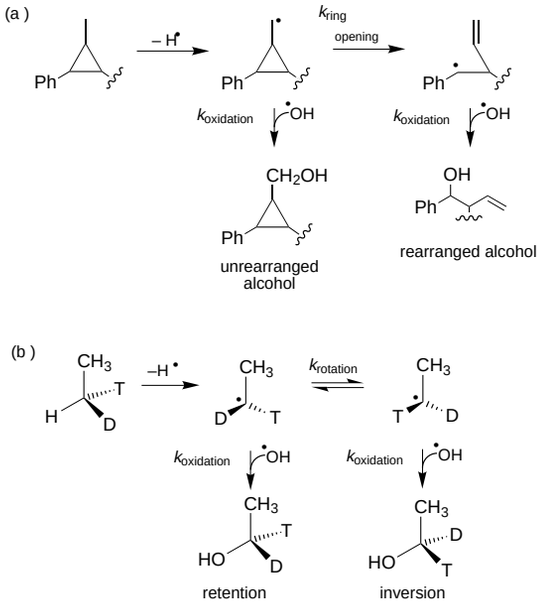

Fig. 5 (*a*) Ring opening of a radical clock substrate probe. If hydrogen-atom abstraction occurs, hydroxylation competes with ring opening, giving rise to cyclic and acyclic products. (*b*) Oxidation of isotopically substituted chiral ethane. If a radical is formed, rotation about the C–C bond results in products displaying retention or inversion of configuration

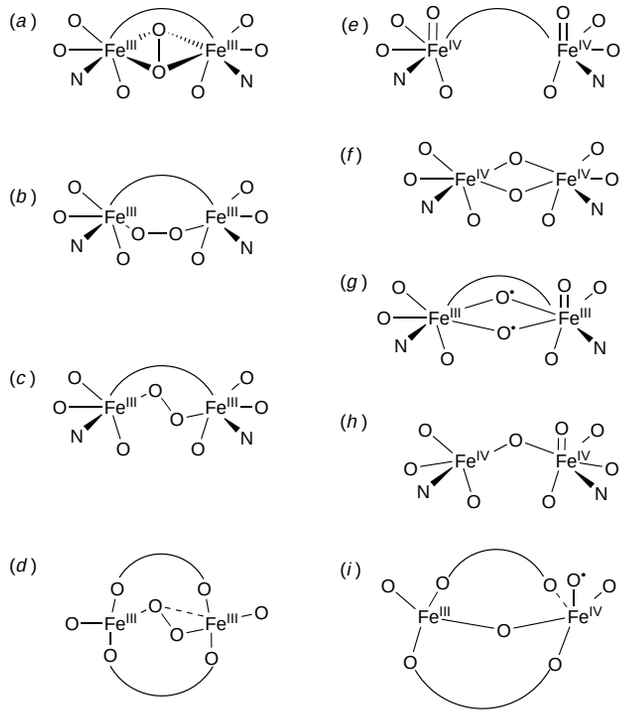

Fig. 6 Candidates for the structures of intermediates in the sMMO catalytic cycle. (*a*)–(*d*) Possible structures of the diiron(III) peroxo intermediate. (*a*) An η^2,η^2-peroxo species. The Fe_2O_2 unit could be either planar or folded about the O–O bond. (*b*) An η^1,η^1 species with a dihedral Fe–O–O–Fe angle of 0°. (*c*) Another version of the η^1,η^1-peroxo conformation with a significant dihedral angle. (*d*) The lowest energy configuration of a diiron(III) peroxo species as determined by density functional theory.[58] (*e*)–(*i*) Possible structures of intermediate Q. (*e*) A diiron(IV) dioxo, or diferryl, configuration. (*f*) A di(μ-oxo)diiron(IV) species. (*g*) A di(μ-oxyl)radical diiron(III) species. (*h*) A species with one bridging and one terminal oxo group. (*i*) A configuration determined by density functional calculations, featuring an oxo-bridged structure with a terminal oxygen radical[58]

C–C bond in the resulting ethyl radical before recombination would give rise to inverted products [Fig. 5(*b*)]. The rate of this rotation can be calculated at a given temperature from transition-state theory and the free energy for the process. As in the case of the radical clock substrate probes, the product distribution can be used to compute a rate constant for a putative radical rebound step. If a very long-lived radical develops, racemization will occur, whereas fully concerted processes will give rise either to complete retention or complete inversion. The sMMO systems from both *M. capsulatus* (Bath) and *M. trichosporium* OB3b yielded ≈30% inverted products when chiral ethane was employed as the substrate, and even less when the butane analog was used. In the case of *M. trichosporium* OB3b, the presence of inverted products was interpreted as evidence for a very short-lived alkyl radical intermediate.[52] For the *M. capsulatus* (Bath) enzyme, the calculated radical lifetime of less than 100 femtoseconds was deemed too short to support a radical intermediate, and a non-synchronous concerted mechanism was invoked instead.[53] The question of whether or not discrete substrate radicals are formed during the course of sMMO oxidations remains a topic of debate.

Detailed steps in the catalytic mechanism

When a catalytic reaction can be synchronized so that all of the active sites undergo the same transformations simultaneously, transient kinetics can be employed to identify possible intermediate species. In this manner, the discrete steps between the initiation of a reaction and return to the enzyme resting state can be defined. Metalloenzymes are often amenable to such studies because spectroscopy can be applied to probe the existence of intermediates as well as stable states.[7] Charge transfer or d–d electronic transitions enable the use of UV/VIS spectroscopy, and when excitation of one of these electronic bands enhances a vibrational mode, resonance-Raman spectra can be obtained. Unpaired electrons at the metal center allow the use of electron paramagnetic (EPR) and other more advanced magnetic resonance techniques. Mössbauer spectroscopy provides valuable insight into the oxidation state and electronic environment of iron proteins, in particular. X-Ray absorption spectroscopy can provide information about oxidation state and geometry of the metal. Information about metastable intermediates is helpful in constructing the detailed reaction cycle of a metalloenzyme and guides the synthesis of spectroscopic and functional models.

For sMMO, the hydroxylase can be chemically reduced in an anaerobic environment. When MMOB is added and the resulting solution mixed rapidly with dioxygen in the absence of substrate, several intermediate species are observed.[33a] The single turnover reaction can be monitored continuously, by using stopped-flow spectroscopy, or discontinuously, with freeze–quench methodology. Experiments of this kind led to the postulated existence of an early intermediate in the reaction cycle of MMOH from *M. trichosporium* OB3b,[54] and this species was directly observed in subsequent studies of the *M. capsulatus* (Bath) proteins. In a series of freeze–quench Mössbauer experiments, the intermediate was determined to attain its maximum concentration 156 ms after mixing.[55] The isomer shift ($\delta = 0.66$ mm s^{-1}) suggested a diferric site and later stopped-flow UV/VIS spectroscopic experiments accumulated additional evidence that the species was a (μ-1,2-peroxo)diiron(III) complex, designated H_{peroxo} or P.[56,57] In particular, it had an optical absorption band with $\lambda_{max} \approx 725$ nm. Several possible symmetric diferric peroxo structures for H_{peroxo} are depicted in Fig. 6(*a*)–6(*c*). Shortly after the peroxo intermediate was identified in MMOH, three model compounds featuring (μ-1,2-peroxo)diiron(III) cores were crystallographically characterized,[59–61] all having spectral features similar to those of the protein intermediate. In two of the three complexes,[60,61] the Fe–O–O–Fe dihedral angles were approximately 0°, resembling the structure in Fig. 6(*b*). In the third, however, the conformation of the peroxo unit more closely resembled bridging mode

(*c*) in Fig. 6, with a dihedral angle of 53°.[59] The single quadrupole doublet in its Mössbauer spectrum and isomer shift were nearly identical to the corresponding values in intermediate H_{peroxo}. Because of the close match in Mössbauer parameters, this bridging mode is the one currently favored as that most likely to occur in H_{peroxo}.

The nature of intermediates in the MMOH reaction cycle has been probed by various theoretical methods. An approximate extended-Hückel calculation interrogated structures for the peroxo intermediate,[62,63] the one of lowest energy being the μ-1,2-peroxo binding mode of Fig. 6(*c*). Methane did not interact favorably with the peroxo intermediate in any of the binding modes investigated. Density functional theory (DFT) has also been employed to investigate the sMMO oxidation mechanism.[58] The lowest energy structure for the peroxo intermediate was similar to that favored by the extended-Hückel calculations, but with one iron atom experiencing η^2 character with respect to peroxide ligand [Fig. 6(*d*)].

Despite the availability of several structural and spectroscopic models for the diiron(III) peroxo intermediate of sMMO, none exhibits properties that mimic its functional chemistry. Such a species should, like the enzyme active site, spontaneously convert to intermediates observed later in the catalytic cycle and/or selectively hydroxylate alkanes. Instead, without the protective scaffolding afforded by the protein, the peroxo complexes are metastable and decay mainly by nonproductive bimolecular pathways.[64,65] The formation of an active hydroxylating species from a diferric peroxo intermediate remains a major goal of this field.

In the protein system, H_{peroxo} spontaneously converts to a bright yellow intermediate known as Q, which has been extensively studied by Mössbauer ($\delta = 0.21$ mm s^{-1}, $\Delta E_Q = 0.68$ mm s^{-1} and $\delta = 0.14$ mm s^{-1}, $\Delta E_Q = 0.55$ mm s^{-1}) and optical spectroscopy ($\lambda_{max} = 350$ and 420 nm).[33a,54,66] These spectral properties suggest a diamagnetic, high valent iron(IV) oxo species but offer no unique interpretation. A recent extended X-ray absorption fine structure (EXAFS) experiment provided evidence for a 2.45 Å Fe···Fe distance in Q from *M. trichosporium* OB3b, together with two 1.77 Å Fe–O bonds.[66] The former distance is short and suggests multiple single atom bridges linking the iron atoms. The Fe–O bond lengths are consistent with either Fe^{III} or Fe^{IV} oxidation levels. The EXAFS results were interpreted as evidence for a di(μ-oxo)diiron(IV) rhombus like the one illustrated in Fig. 6(*f*), but other structures are equally likely.

The nature of intermediate Q has also been investigated by extended-Hückel theory.[62,63] A di(μ-oxo) structure like the one just discussed[66] interacted repulsively with methane and therefore was excluded as the active hydroxylating species. Instead, a five-co-ordinate ferryl intermediate was preferred that interacted with methane, distorting it into a geometry having C_{3v} symmetry and an Fe–C bond. In the DFT calculations,[58] the di(μ-oxo)diiron(IV) structure was invoked as being on the pathway, but was not considered to be the likely activator of substrate. Instead, this configuration was proposed to rearrange into an Fe^{III}–O–Fe^{IV}–O$^{\bullet}$ species [Fig. 6(*i*)], which would react with methane by hydrogen-atom abstraction and Fe–C bond formation. These issues must be resolved because, if there is to be any hope of producing functional models of enzymatic systems, we must understand the transient intermediates, what causes them to form, and what chemistry they can achieve.

Intermediate Q is widely held to be the one which reacts with substrate to form product. Its decay is accelerated by addition of substrate,[33a] and several intermediates following Q in the reaction cycle have been postulated.[18] A product-bound intermediate has been observed by optical spectroscopy for the reaction of nitrobenzene with *M. trichosporium* OB3b hydroxylase.[54] Once Q delivers its oxidizing equivalents to substrate, the diiron center returns to the $Fe^{III}Fe^{III}$resting state. In the absence of additional reducing equivalents in the form of NADH/MMOR or another reductant, the single turnover experiment

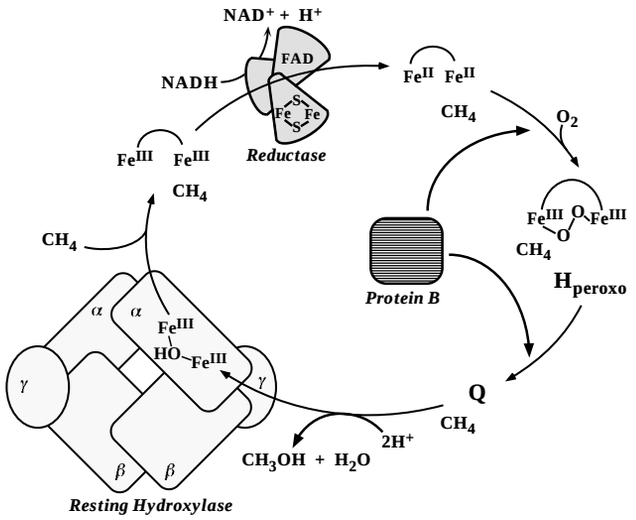

Fig. 7 Catalytic cycle for sMMO from *M. capsulatus* (Bath) including all observed intermediates

is concluded. The *M. capsulatus* (Bath) catalytic cycle showing the intermediates thus far identified is depicted schematically in Fig. 7.

The identification of intermediates on the catalytic pathway is the key to understanding all metalloenzymes. Because many small molecule substrates have strong or multiple bonds, very reactive species may be required for their activation. In order for a spectroscopically identified species to qualify as an intermediate on the catalytic pathway, it must be both catalytically and chemically competent. Catalytic competence requires that the observed transient appear and convert on a time-scale fast enough for it to account for product accumulation. This criterion requires study of the system under steady-state conditions. In the present example, the turnover number for methane has been reported to be 0.19 s^{-1},[26] and a more recent determination suggests a value of 0.6 s^{-1}.[33b] No step can occur more slowly than this limiting value, and postulated mechanisms which include slower steps can be discounted. The second criterion, that of chemical competence, means that the observed intermediate can effect the transformation attributed to it. Comparisons with known model chemistry can help to assure chemical competence, as can well executed theoretical calculations.

Conclusion

Significant progress has been made in understanding how the soluble methane monooxygenase system achieves the hydroxylation of methane under physiological conditions. Through application of a wide range of methodologies from both biochemistry and inorganic chemistry, we now know the three-dimensional structure of the hydroxylase enzyme, the nature of key intermediates in the reaction cycle, and aspects of how the three protein components interact and regulate the system. Several issues remain unresolved, however. We should like to understand more fully the mechanisms which control the selectivity of hydrocarbon oxidation, determine the structures of the MMOB and MMOR components and complexes between all three proteins, uncover how substrates and products traverse the protein matrix into and out of the active site, and probe the mechanism by which different substrates react. The nature of the existing intermediates is not fully understood and, especially, the key C–H bond-cleavage and C–O bond-forming steps are unknown. Finally, we should also like to be able to mimic the alkane hydroxylation chemistry with small molecule synthetic analogs.

What features of the sMMO system are likely to be shared by other metalloproteins that activate small molecules? Co-

ordinative unsaturation and unusual oxidation states observed in the reduced hydroxylase and intermediate Q are two properties that are likely to be of more general consequence. The control of the system by multi-protein interactions, and the separation of electron entry from substrate activation units on different proteins, should also find common ground among the systems that achieve some of the other functions illustrated in Fig. 1. Formation of reactive intermediates, and protection of those intermediates by the protein environment, may also be recurring features of these systems. Specific mechanisms that recruit small substrates to the active sites will be better defined with additional macromolecular structure determinations.

Enough work has now been done in the area of bioinorganic enzymology to define it as a distinct field, to fill textbooks and special issues of journals, and to begin to identify common themes. Still, many key problems in the field are far from being solved. The perspective in five years time should look quite different.

Acknowledgements

The work discussed herein was supported by grants from the National Institute of General Medical Science, the National Science Foundation, and Shell. We thank our many collaborators whose work is cited in this Perspective for fruitful interactions, and D. A. Whittington for help in preparing this manuscript.

References

1 C. Huber and G. Wächtershäuser, *Science*, 1997, **276**, 245.
2 S. W. Ragsdale and M. Kumar, *Chem. Rev*., 1996, **96**, 2515.
3 J. B. Sumner, *J. Biol. Chem*., 1926, **69**, 435.
4 N. E. Dixon, C. Gazzola, R. L. Blakeley and B. Zerner, *J. Am. Chem. Soc*., 1975, **97**, 4131.
5 (*a*) S. J. Lippard and J. M. Berg, *Principles of Bioinorganic Chemistry*, University Science Books, Mill Valley, CA, 1994; (*b*) J. J. R. Fraústo da Silva and R. J. P. Williams, *The Biological Chemistry of the Elements: The Inorganic Chemistry of Life*, Clarendon Press, Oxford, 1991.
6 S. J. Lippard, *Science*, 1993, **261**, 699.
7 R. H. Holm, P. Kennepohl and E. I. Solomon, *Chem. Rev*., 1996, **96**, 2239.
8 W. P. Jencks, *Adv. Enzymol*., 1975, **43**, 219.
9 R. K. Thauer, A. R. Klein and G. C. Hartmann, *Chem. Rev*., 1996, **96**, 3031.
10 S. Ferguson-Miller and G. T. Babcock, *Chem. Rev*., 1996, **96**, 2889.
11 B. A. Averill, *Chem. Rev*., 1996, **96**, 2951.
12 A. C. Rosenzweig, P. Nordlund, P. M. Takahara, C. A. Frederick and S. J. Lippard, *Chem. Biol*., 1995, **2**, 409.
13 V. K. Yachandra, K. Sauer and M. P. Klein, *Chem. Rev*., 1996, **96**, 2927.
14 J. B. Howard and D. C. Rees, *Chem. Rev*., 1996, **96**, 2965.
15 T. J. Kappock and J. P. Caradonna, *Chem. Rev*., 1996, **96**, 2659.
16 K. E. Liu and S. J. Lippard, in *Advances in Inorganic Chemistry*, ed. A. G. Sykes, Academic Press, San Diego, CA, 1995, vol. 42, p. 263.
17 J. D. Lipscomb, *Annu. Rev. Microbiol*., 1994, **48**, 371.
18 B. J. Wallar and J. D. Lipscomb, *Chem. Rev*., 1996, **96**, 2625.
19 C. Anthony, *The Biochemistry of Methylotrophs*, Academic Press, New York, 1982, p. 296.
20 I. J. Higgins, D. J. Best and R. C. Hammond, *Nature* (*London*), 1980, **286**, 561.
21 J. R. Bragg, R. C. Prince, E. J. Harner and R. M. Atlas, *Nature* (*London*), 1994, **368**, 413.
22 *Methanol Production and Use*, eds. W.-H. Cheng and H. H. Kung, Marcel Dekker, New York, 1994.
23 B. A. Arndtsen, R. G. Bergman, T. A. Mobley and T. H. Peterson, *Acc. Chem. Res*., 1995, **28**, 154.
24 I. Yamanaka, M. Soma and K. Otsuka, *J. Chem. Soc*., *Chem. Commun*., 1995, **95**, 2235.
25 I. Yamanaka, M. Soma and K. Otsuka, *Chem. Lett*., 1996, 565.
26 J. Green and H. Dalton, *Biochem. J*., 1986, **236**, 155.
27 J. Colby and H. Dalton, *Biochem. J*., 1978, **171**, 461.
28 M. P. Woodland and H. Dalton, *J. Biol. Chem*., 1984, **259**, 53.
29 A. C. Rosenzweig, C. A. Frederick, S. J. Lippard and P. Nordlund, *Nature* (*London*), 1993, **366**, 537.
30 A. C. Rosenzweig and S. J. Lippard, *Acc. Chem. Res*., 1994, **27**, 229.
31 J. Colby and H. Dalton, *Biochem. J*., 1979, **177**, 903.
32 J. Green and H. Dalton, *J. Biol. Chem*., 1985, **260**, 15 795.
33 (*a*) K. E. Liu, A. M. Valentine, D. Wang, B. H. Huynh, D. E. Edmondson, A. Salifoglou and S. J. Lippard, *J. Am. Chem. Soc*., 1995, **117**, 10 174; (*b*) G. T. Gassner and S. J. Lippard, unpublished work.
34 M. P. Woodland, D. S. Patil, R. Cammack and H. Dalton, *Biochim. Biophys. Acta*, 1986, **873**, 237.
35 K. E. Liu and S. J. Lippard, *J. Biol. Chem*., 1991, **266**, 12 836.
36 Y. Liu, J. C. Nesheim, K. E. Paulsen, M. T. Stankovich and J. D. Lipscomb, *Biochemistry*, 1997, **36**, 5223.
37 H. B. Gray and J. R. Winkler, *Annu. Rev. Biochem*., 1996, **65**, 537.
38 B. G. Fox, Y. Liu, J. E. Dege and J. D. Lipscomb, *J. Biol. Chem*., 1991, **266**, 540.
39 A. C. Rosenzweig, H. Brandstetter, D. A. Whittington, P. Nordlund, S. J. Lippard and C. A. Frederick, *Proteins*, 1997, in the press.
40 J. Colby, D. I. Stirling and H. Dalton, *Biochem. J*., 1977, **165**, 395.
41 J. Green and H. Dalton, *J. Biol. Chem*., 1989, **264**, 17 698.
42 B. G. Fox, J. G. Borneman, L. P. Wackett and J. D. Lipscomb, *Biochemistry*, 1990, **29**, 6419.
43 F. Ruzicka, D.-S. Huang, M. I. Donnelly and P. A. Frey, *Biochemistry*, 1990, **29**, 1696.
44 K. E. Liu, C. C. Johnson, M. Newcomb and S. J. Lippard, *J. Am. Chem. Soc*., 1993, **115**, 939.
45 S.-Y. Choi, P. E. Eaton, P. F. Hollenberg, K. E. Liu, S. J. Lippard, M. Newcomb, D. A. Putt, S. P. Upadhyaya and Y. Xiong, *J. Am. Chem. Soc*., 1996, **118**, 6547.
46 D. A. Whittington and S. J. Lippard, unpublished work.
47 A. R. George, P. C. Wilkins and H. Dalton, *J. Mol. Catal. B: Enzym*., 1996, **2**, 103.
48 D. D. S. Smith and H. Dalton, *Eur. J. Biochem*., 1989, **182**, 667.
49 *CRC Handbook of Chemistry and Physics*, ed. R. C. Weast, CRC Press, Inc., Boca Raton, FL, 64th edn., 1983.
50 P. R. Ortiz de Montellano, in *Cytochrome P450: Structure, Mechanism, and Biochemistry*, ed. P. R. Ortiz de Montellano, Plenum Press, New York, 2nd edn., 1995, p. 245.
51 (*a*) M. Newcomb, M.-H. Le Tadic-Biadatti, D. L. Chestney, E. S. Roberts and P. F. Hollenberg, *J. Am. Chem. Soc*., 1995, **117**, 12 085; (*b*) A. F. Shestakov and A. E. Shilov, *J. Mol. Catal. A: Chem*., 1996, **105**, 1.
52 N. D. Priestley, H. G. Floss, W. A. Froland, J. D. Lipscomb, P. G. Williams and H. Morimoto, *J. Am. Chem. Soc*., 1992, **114**, 7561.
53 A. M. Valentine, B. Wilkinson, K. E. Liu, S. Komar-Panicucci, N. D. Priestley, P. G. Williams, H. Morimoto, H. G. Floss and S. J. Lippard, *J. Am. Chem. Soc*., 1997, **119**, 1818.
54 S.-K. Lee, J. C. Nesheim and J. D. Lipscomb, *J. Biol. Chem*., 1993, **268**, 21 569.
55 K. E. Liu, D. Wang, B. H. Huynh, D. E. Edmondson, A. Salifoglou and S. J. Lippard, *J. Am. Chem. Soc*., 1994, **116**, 7465.
56 K. E. Liu, A. M. Valentine, D. Qiu, D. E. Edmondson, E. H. Appelman, T. G. Spiro and S. J. Lippard, *J. Am. Chem. Soc*., 1995, **117**, 4997.
57 A. M. Valentine and S. J. Lippard, unpublished work.
58 P. E. M. Siegbahn and R. H. Crabtree, *J. Am. Chem. Soc*., 1997, **119**, 3103.
59 K. Kim and S. J. Lippard, *J. Am. Chem. Soc*., 1996, **118**, 4914.
60 T. Ookubo, H. Sugimoto, T. Nagayama, H. Masuda, T. Sato, K. Tanaka, Y. Maeda, H. Okawa, Y. Hayashi, A. Uehara and M. Suzuki, *J. Am. Chem. Soc*., 1996, **118**, 701.
61 Y. Dong, S. Yan, V. G. Young, jun. and L. Que, jun., *Angew. Chem., Int. Ed. Engl*., 1996, **35**, 618.
62 K. Yoshizawa and R. Hoffmann, *Inorg. Chem*., 1996, **35**, 2409.
63 K. Yoshizawa, T. Yamabe and R. Hoffmann, *New J. Chem*., 1997, **21**, 151.
64 A. L. Feig, M. Becker, S. Schindler, R. v. Eldik and S. J. Lippard, *Inorg. Chem*., 1996, **35**, 2590.
65 A. L. Feig, A. Masschelein, A. Bakac and S. J. Lippard, *J. Am. Chem. Soc*., 1997, **119**, 334.
66 L. Shu, J. C. Nesheim, K. Kauffmann, E. Münck, J. D. Lipscomb and L. Que, jun., *Science*, 1997, **275**, 515.

Received 17*th July* 1997; *Paper* 7/05116F