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Versatility of biological non-heme Fe(II) centers in oxygen activation reactions

Elena G Kovaleva and John D Lipscomb

Elena G. Kovaleva and John D. Lipscomb are in the Department of Biochemistry, Molecular Biology and Biophysics and the Center for Metals in Biocatalysis, University of Minnesota, 6-155 Jackson Hall, Minneapolis, Minnesota, 55455 USA.

Elena G Kovaleva: koval021@umn.edu; John D Lipscomb: lipsc001@umn.edu

Abstract

Oxidase and oxygenase enzymes allow the use of relatively unreactive O₂ in biochemical reactions. Many of the mechanistic strategies employed in nature for this key reaction are represented within the 2-His-1-carboxylate facial triad family of non-heme Fe(II) containing enzymes. The open face of the metal coordination sphere opposite the three endogenous ligands participates directly in the reaction chemistry. Here, data from several studies are presented showing that reductive O₂ activation within this family is initiated by substrate (and in some cases co-substrate or cofactor) binding, which then allows coordination of O₂ to the metal. From this starting point, both the O₂ activation process and the reactions with substrates diverge broadly. The reactive species formed in these reactions have been proposed to encompass four oxidation states of iron and all forms of reduced O₂ as well as several of the reactive oxygen species that derive from O–O bond cleavage.

Dioxygen serves at least three quite different roles that profoundly impact aerobic life. The most commonly appreciated role is to serve as the terminal electron acceptor in processes such as oxidative phosphorylation that yield the central energy-rich molecules used throughout metabolism. The second, no less important role is to serve as the source for many of the oxygen atoms found in the essential molecules of biological systems such as steroid hormones, aromatic amino acids, neurotransmitters, signalling molecules, and regulatory factors ¹. Also, processes operating on a global scale, such as the recovery of the enormous quantities of carbon sequestered as lignin in plant life or the oxidation of the billions of tons of methane generated by anaerobes before it can enter the atmosphere, also involve oxygen incorporation from O₂ ^{2,3}. On a more local scale, biodegradation of both aliphatic and aromatic toxic compounds often begins with the incorporation of oxygen ⁴. The third, and least appreciated role played by dioxygen in aerobic organisms involves neither energy conversion nor oxygen incorporation. Rather, some enzymes can convert dioxygen to alternative forms that are, in effect, highly specialized reagents that are used to catalyze the synthesis of important biomolecules. An excellent example of the latter role for O₂ is the biosynthesis of penicillin-type antibiotics ^{5,6}, which is discussed in more detail later in this review.

Dioxygen is an attractive reagent for use in a biological system because its high potential reactivity is held in check by its molecular structure. The triplet ground state of O₂ that results from the presence of two unpaired electrons in degenerate molecular orbitals makes the direct reaction with singlet molecules, the spin-paired state of most potential reaction partners, a forbidden process ⁷. The central question that has faced chemists and biochemists for the half

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century since the oxygen incorporation into a biological molecule from O₂ was first demonstrated⁸ is how O₂ can be made to react at ambient temperature and with high specificity. Of course, the answers to this question are of fundamental importance in understanding the myriad of interacting, O₂-dependent metabolic processes that make life possible while living in a sea of oxygen. However, an understanding of nature's oxygen activation strategies also has many direct applications including the design of new pharmaceuticals relevant to a wide variety of diseases, targeted biodegradation systems for the environment, catalysts for the conversion of abundant molecules for fuels and industrial synthons, and on the horizon, genetic alteration of oxygen activating enzymes to mitigate the effects of aberrant catalysis.

Dioxygen is activated from its abundant triplet ground state to reactive singlet or doublet (radical) species by oxidase and oxygenase enzymes. In many cases, these same enzymes generate species of even greater reactivity by cleaving the O–O bond. All of these reactions are tightly regulated both spatially and temporally so that specific chemistry occurs to yield biomolecules that are compatible with downstream metabolic pathways or function. When loss of regulation occurs, the well-characterized deleterious effects of reactive oxygen species ensue. The problem of specifically activating O₂ has been addressed and solved in a remarkably varied manner by nature. Generally, the solution involves either a transition metal, such as iron, copper, or manganese, an organic cofactor, such as flavin or pterin, or both, as is the case for heme. In all biological systems, the O₂ is reductively activated because simple inversion of an oxygen electron to yield the singlet state directly is highly endothermic. The source of the activating electron(s) is again quite varied; the list includes: the metal, the organic substrate itself, a co-substrate, a second redox active metal or cofactor, reduced pyridine nucleotide, and for some oxidases, ascorbate.

The understanding of biological oxygen activation mechanisms has been advanced in recent years through the coordinated use of chemical, structural, spectroscopic, and computational approaches^{9–12}. A surprisingly comprehensive overview of the lessons learned from these studies can be acquired by focusing on specific representative members of the large family of non-heme iron containing oxygenases and oxidases that bind the metal using weakly charge donating ligands. Most of the members of this class utilize two histidine and one glutamate or aspartate endogenous ligands arranged on one face of the iron coordination sphere (2-His+Asp/Glu facial triad motif)¹³. The opposite face is usually occupied by solvents that can be directly or indirectly displaced as substrates or co-substrates and then O₂ bind to the enzyme^{14,15}. This allows the iron to participate in the oxygen activation and substrate oxidation process in a variety of ways. Here, five representative types of non-heme oxygenases and related oxidases that have adopted different solutions to the oxygen activation process will be considered.

Extradiol catecholic dioxygenases

Roughly 30 % of the mass of woody plant material on earth is comprised of aromatic compounds^{2,16}. The inherent stability of these compounds would make them a natural sink for a significant fraction of the bio-available carbon were it not for the action of dioxygenase enzymes that cleave the aromatic ring and initiate pathways leading to central metabolites^{17–20}. Invariably, the aromatic compound is first functionalized, generally by the introduction of two or more hydroxyl or amino substituents on the aromatic ring to increase its reactivity. The aromatic ring is then cleaved by one of several types of dioxygenase enzymes. The extradiol dioxygenases cleave the aromatic ring of catechol analogs adjacent to the vicinal hydroxyl substituents by inserting both atoms of oxygen from O₂ to yield muconic semialdehyde adducts. Structural studies show that these enzymes bind Fe(II) in the 2-His+Asp/Glu facial triad coordination environment^{21,22}. To date, there is no evidence that the iron changes redox state during the catalytic cycle. Moreover, no external reductant is needed for catalysis, showing that the electrons required to activate O₂ derive from the substrate. The

structure and utility of the 2-His+Asp/Glu facial triad were first recognized in the extradiol dioxygenases^{13–15}, and elements of their catalytic mechanism are carried through most other members of the broad family. Thus, it is instructive to first examine the extradiol dioxygenases in some detail.

The extradiol dioxygenases form an active site Fe(II)-chelate complex with the catecholic substrate and then bind O₂ in an adjacent metal ligand site^{14,20,23–25}. As illustrated in Fig. 1, this allows a concerted activation of substrate and O₂ by transfer of an electron from the former to the latter via the iron. Recombination of the resulting radicals in a position enforced by the geometry of the metal complex gives a specific Fe(II)-bound alkylperoxo intermediate. This intermediate was originally proposed to break down by a concerted Criegee rearrangement to yield a lactone intermediate^{14,23,26}. The heterolytic O–O bond cleavage during this reaction would be facilitated by protonation of the oxygen atom bound to the iron by an active site acid catalyst. Finally, the open ring product would result from hydrolysis of the lactone by the second oxygen atom from O₂, which remains bound to the iron at the level of water. Recent computational studies are in accord with this mechanism except that they predict formation of the lactone by a multistep process involving an intermediate unstable epoxide^{27,28}.

Direct evidence for several aspects of this mechanistic proposal has come from our recent study in which reaction intermediates of the extradiol dioxygenase homoprotocatechuate 2,3-dioxygenase (HPCD) were trapped in a single crystal²⁹. In this study, crystals of HPCD were allowed to react with the slow alternative substrate 4-nitrocatechol (4NC, **2**) in the presence of a low concentration of O₂ prior to cryofreezing and structure solution. Three intermediates of the reaction cycle were found in different subunits of the homotetrameric enzyme, of which two are shown in Fig. 1. One subunit contained the chelated substrate-Fe(II)-O₂ complex. The oxygen in this complex is bound in the side-on configuration, thereby aligning it for reaction with the correct ring carbon of 4NC. Also, the ring of the 4NC is not planar, suggesting that it gives up an electron to become a semiquinone radical. The long Fe–O (2.4 Å) bond lengths are consistent with the iron remaining in the Fe(II) state, suggesting that the electron from the 4NC is transferred to the oxygen to form a bound superoxide.

Two of the other subunits of HPCD in the crystal contained the Fe(II)-alkylperoxo intermediate with continuous electron density from the iron through both oxygen atoms to the C2 of 4NC, which becomes fully tetrahedral. Finally, the fourth subunit of HPCD contained the Fe(II)-product complex, showing that the enzyme is capable of turnover in the crystal.

The structural studies indicate that two residues in the second sphere of the metal coordination sphere play important roles in the catalytic process. The first is Tyr257, which is within hydrogen bonding distance of the deprotonated 4NC hydroxyl substituent at C2. This distance becomes shorter (2.4 Å) in the observed intermediates, suggesting that formation of a strong hydrogen bond by Tyr257 draws the reaction toward the intermediates. The second residue is His200, which is positioned to donate a proton to the alkylperoxo intermediate to promote heterolytic O–O bond fission. Alteration of the reaction kinetics and specificity caused by mutation of His200 to residues that cannot act as acid catalysts supports its essential function^{30,31}. Without a histidine at position 200, an activated oxygen species is stabilized in solution, greatly slowing the reaction cycle³⁰. Thus, O₂ activation and subsequent reaction occur by a collaboration of electron donation from the substrate and acid catalysis by an active site histidine.

Rieske *cis*-diol forming dioxygenases

One way in which the catecholic substrates for the extradiol dioxygenases are formed in nature is through the direct addition of both atoms of oxygen from O₂ to adjacent carbons of an unactivated aromatic ring in the reaction catalyzed by *cis*-dihydrodiol-forming dioxygenases

^{32,33}. Re-aromatization of the *cis*-dihydrodiol product by the next enzyme in the pathway yields the catechol, which, in turn, becomes a substrate for downstream ring-cleaving dioxygenases. In the *cis*-diol forming reaction, one more bond is formed than is broken in the substrate and O₂, so two external electrons must be supplied. This also serves a mechanistic purpose because it allows activation of O₂ to a more potent form than occurs in the extradiol dioxygenase mechanism, a necessity when the goal is to attack an unactivated aromatic ring.

The Rieske *cis*-diol dioxygenases fall into 4 subfamilies with differing numbers and types of protein components and subunits, but a similar active site region is retained⁴. Crystal structures show that each enzyme has a substrate-binding site located in the oxygenase component that contains a mononuclear iron bound by the 2-His+Asp/Glu motif in which the carboxylate ligand is often bidentate^{34–38}. Spectroscopic studies show that, in solution, the iron is 6-coordinate in the absence of substrate, indicating that additional solvent molecules or weak protein-derived ligands are bound^{39,40}. Approximately 12 Å away from the mononuclear iron, and across a subunit boundary, is a Rieske-type 2Fe–2S cluster that acts to store and supply an additional electron for the reaction. A separate reductase component, sometimes acting through electron transfer proteins, couples NADH oxidation to Rieske cluster reduction.

As has been found to be the case for each of the O₂ activating enzymes discussed here, studying the enzyme reaction under single turnover conditions provided important clues to the mechanism of the Rieske *cis*-diol forming enzymes^{41–44}. Exposure of the fully reduced oxygenase component (in which each metal center is one-electron reduced) to substrate and O₂ resulted in one turnover to form a nearly stoichiometric yield of *cis*-dihydrodiol product in the case of naphthalene dioxygenase (NDO). At the end of the single cycle, both metal centers were found to be oxidized, suggesting that the O₂ is activated by a two-electron reduction process. As illustrated in Fig. 2, this led to the hypothesis that the reactive form of oxygen is a peroxy or hydroperoxy species bound to Fe(III). In heme containing oxygenases and methane monooxygenase (MMO), the equivalent species undergoes O–O bond cleavage upon protonation to yield a species at the oxidation level of Fe(V)=O, which is the true hydroxylating reagent^{25,45,46}. In each of the latter species, there is more than one oxidized center in close proximity, so the highest localized valence for iron is Fe(IV). A similar formal Fe(V)=O species can be proposed for the Rieske dioxygenases, although in this case there is no obvious candidate for the second nearby oxidized center, so the oxidizing potential may not be delocalized. Also, a mechanism to retain both atoms of oxygen for incorporation into the substrate must be proposed. One solution to both of these issues is to retain both atoms of oxygen in the metal coordination sphere to form an Fe(V)–oxo–hydroxo moiety in which the hydroxo substituent would partially stabilize the high valent metal–oxo⁴¹.

An alternative mechanistic hypothesis has received support from studies of phthalate dioxygenase which gives only ~50 % yield during single turnover⁴⁷. It was suggested that after achieving the formal Fe(V)=O state, the mononuclear iron accepts another electron from a Rieske cluster located in a remote subunit of the multimeric enzyme. Thus, the true reactive species would be either an Fe(II)–(hydro)peroxy or an Fe(IV)–oxo–hydroxo, which would presumably be more readily stabilized in a biological system.

Both mechanistic scenarios have received support from computational studies^{48,49}. One direct approach to resolving this controversy has been to show that hydrogen peroxide added to the substrate complex of naphthalene or benzoate dioxygenase (BZDO) in the resting state with the Rieske cluster oxidized and the mononuclear iron reduced yields nearly stoichiometric amounts of product (a peroxide shunt)^{50,51}. In this case, ¹⁸O-labeling studies showed the peroxide is the source of the oxygen in the product, and because it can supply only two electrons, the two-electron activated oxygen proposal is favored. In the case of BZDO, a form

with both the Rieske and mononuclear iron sites fully oxidized was prepared, and this state was also active in the peroxide shunt despite the lack of any source for a third electron ⁵¹.

It has thus far not proven possible to distinguish between an Fe(III)–(hydro)peroxo and an Fe(V)–oxo–hydroxo reactive species. Computational studies favor the former for enzyme reactions, but show that the latter is a possible reactive species in small molecule model compounds where the low spin state of the metal can be accessed ^{48,49}. The crystal structure of the side-on bound hydroperoxo complex of NDO has been solved after exposing the substrate-bound crystal of NDO to O₂ as shown in Fig. 252. Also, an intermediate with EPR and Mössbauer parameters similar to a side-on bound peroxo adduct of model complexes has been found after adding H₂O₂ to fully oxidized BZDO in solution ⁵¹. Results favoring the (perhaps subsequent) formation of an intermediate Fe(V)–oxo–hydroxo species have derived from the use of probe molecules that are monooxygenase substrates for NDO ⁵³. Both norcarane and bicyclohexane form high percentage yields of products that reveal the intermediate formation of long-lived substrate radicals with no evidence for cation intermediates. Studies of a variety of oxygenases in recent years have shown that Fe(III)–hydroperoxo intermediates are unlikely to yield radical intermediates, while this is the expected route for formal Fe(V)=O species ⁴⁶.

Dioxygenases using a 2-oxo-acid as a co-substrate

Many mammalian and bacterial hydroxylating dioxygenases reductively activate O₂ by utilizing a mechanism that is substantially different from those considered above for extradiol and Rieske *cis*-diol dioxygenases ⁵⁴. X-ray crystallographic studies have shown that the 2-oxo-acid linked dioxygenase family utilizes a mononuclear Fe(II) in a 2-His+Asp/Glu ligand motif that chelates a 2-oxo-acid (generally α -ketoglutarate (α KG, ³)) co-substrate in ligand sites adjacent to the probable O₂ binding site on the iron (Fig. 3) ^{55–58}. This is analogous to substrate binding to extradiol dioxygenases, and similarly, the bound O₂ attacks the oxo-acid. However, the reactions diverge in several respects at this point, as illustrated in Fig. 3. First, the 2-oxo-acid is much less electron donating than the catecholate substrate of the extradiol dioxygenases, and thus the reactive oxygen species is likely to be an Fe(III)–O₂^{•–} rather than the Fe(II)–O₂^{•–}. Second, the role of these dioxygenases is to hydroxylate a substrate, similar to the roles of Rieske dioxygenases, cytochrome P450, and MMO, and thus, a high valent iron-oxo species of some sort is likely to be required. This mandates the input of electrons to break the O–O bond. In the case of the α -ketoglutarate dioxygenases, these electrons are derived not from reduced pyridine nucleotide, but rather from bond cleavage of the co-substrate to yield the intermediate products CO₂ and succinate, the latter containing one atom of O from O₂. Finally, the Fe(IV)=O species also generated in this reaction is used to incorporate one oxygen atom into the substrate to form a hydroxylated product. This means that the dioxygenase stoichiometry is satisfied by addition of the two atoms from O₂ to different molecules.

The use of transient enzyme kinetic techniques in combination with spectroscopy has allowed very significant progress in understanding the mechanism of 2-oxo-acid dioxygenases in recent years ^{59,60}. Oxygen binding in this class is triggered by the binding of both substrate and 2-oxo-acid co-substrate. In studies of the enzyme taurine- α KG dioxygenase (TauD), an enzyme that hydroxylates 2-amino-1-ethane sulfonic acid (taurine, **4**) as part of a pathway that allows bacteria to release sulfite as a sulfur source for growth, it was shown that rapid mixing of the TauD- α KG-taurine complex with O₂-containing buffer resulted in the formation of at least 2 intermediates. One intermediate, termed J, was trapped by rapid freezing and shown by Mössbauer spectroscopy to have parameters similar to those of known Fe(IV) containing intermediates, in particular intermediate X of ribonucleotide reductase and Q of MMO ^{60–62}. Formation of J required α KG, and the rate of its decay exhibited a very large KIE upon deuteration of the C1 of taurine ^{63,64}. Resonance Raman spectra of the intermediate showed a

stretching mode characteristic of Fe(IV)=O ⁶⁵ and EXAFS spectra showed the appropriate Fe–O distance⁶⁶. Together these approaches revealed the first mononuclear non-heme Fe(IV)=O intermediate and offered strong support for the proposed mechanism shown in Fig. 3 that invokes a high-valent iron-oxo species to attack the substrate. The large KIE suggests that the mode of this attack is hydrogen atom abstraction from the substrate followed by rebound of hydroxyl radical to yield the hydroxylated product.

It is useful to note that the 2-oxo-acid dioxygenases, in effect, activate oxygen twice using different strategies that favor the specific chemistry that follows activation. In the first activation step, an $\text{Fe(III)-O}_2^{\bullet-}$ species is generated to carry out the relatively facile attack on the 2-oxo-acid. This leads to the second activation step in which the much more potent Fe(IV)=O species is generated by O–O bond cleavage. Because the iron begins and ends its cycle in the Fe(II) state, the even more reactive formal Fe(V)=O state characteristic of the reactive species of Rieske *cis*-diol dioxygenases, cytochrome P450 and MMO is not generated and is apparently not required.

Enzymes similar to the 2-oxo-acid dioxygenases

The number of recognized 2-oxo-acid dioxygenase enzymes is large and growing rapidly as modern proteomic techniques are employed. However, two other types of enzymes appear to use analogous O_2 activation mechanisms. The first includes enzymes such as 4-hydroxyphenyl-pyruvate dioxygenase (HPPD), which is part of the tyrosine metabolic pathway⁶⁷. In this case, the 2-oxo-acid is the pyruvate substituent of the substrate, which binds directly to the iron to allow O_2 binding and activation. Following $\text{Fe(III)-O}_2^{\bullet-}$ attack and release of CO_2 , the presumed Fe(IV)=O intermediate carries out aromatic ring hydroxylation. The reaction differs from the mechanism shown in Fig. 3 in that it is proposed to occur by direct attack of the Fe(IV)=O species on the aromatic ring to yield an intermediate Fe(II)-O -substrate arenium cation. The presence of the cation is thought to allow ketonization and substituent migration prior formation of homogenisate product.

The second related enzyme class catalyzes halogenation of amino acids and related molecules bound to carrier proteins in preparation for non-ribosomal peptide formation in a variety of natural products⁶⁸. These enzymes use Fe(II) , O_2 , and αKG like the traditional 2-oxo-acid dioxygenases, but they also require a halide, and the normal carboxylic acid from the 2-His + Asp/Glu Fe(II) binding motif is absent. A crystal structure of syringomycin halogenase shows that the place in the ligand sphere normally occupied by the carboxylic acid residue is occupied by the halogen that will be transferred⁶⁹. It is proposed that the normal oxygen activation scheme for a 2-oxo-acid dioxygenase shown in Fig. 3 proceeds through the substrate hydrogen atom abstraction step. Then, instead of $\text{OH}^\bullet$ rebound, the halogen reacts with the substrate radical to complete the reaction. In accord with this mechanistic proposal, a transient Fe(IV)=O species has now been trapped and spectroscopically characterized^{70,71}.

Tetrahydropterin-containing oxygenases

The neurotransmitters DOPA (4) and serotonin (5) and the amino acid tyrosine are synthesized by mammals in reactions catalyzed by hydroxylases containing an Fe(II) bound in a 2-His+Glu motif⁷². In addition, crystal structures show that a tetrahydropterin cofactor binds near the Fe(II) (Fig. 4)⁷³. The reactions of these enzymes require an activated oxygen such as the high valent Fe(IV)=O species found in the 2-oxo-acid dioxygenases. As shown in Fig. 4, the binding of substrate near, but not to, the Fe(II) facilitates O_2 binding. Two electrons are required to break the O–O bond to generate the high valent iron oxo species. It is postulated that the intermediate in this case contains a peroxo bridge between the Fe(II) and the tetrahydropterin⁷⁴. Thus, an intermediate iron-superoxo or peroxo species is not formally required, although one is likely to transiently form. The pterin is believed to supply the two electrons required to

cleave the O–O bond in much the same way that a flavin stabilizes the bound O₂ in the peroxy state in flavo-monooxygenases. Heterolytic cleavage of the O–O bond would yield a 4a-hydroxy pterin and an Fe(IV)=O species prepared for hydroxylation chemistry. Recently, the Fe(IV)=O species has been directly detected and trapped⁷⁵. The mechanism of hydroxylation remains under investigation. It is possible that electrophilic attack of the Fe(IV)=O species on the aromatic ring of the substrate results in a two-electron oxidation to yield an Fe(II)–O–substrate arenium cation in one step⁷². Alternatively, the Fe(IV)=O species may abstract one electron to yield an intermediate ring radical and an Fe(III)–oxo species. Subsequent reaction of the oxo species with the substrate radical would yield the same arenium cation as the first mechanism⁷⁶. The second mechanism is more in line with reactions of high valent iron-oxo in other systems, but the final intermediate in either case would collapse to yield the hydroxylated product and leave the Fe(II) resting state of the enzyme.

In the tetrahydropterin-containing oxygenases, elements of the O₂ activation mechanism described above are combined with the novel application of a reduced cofactor that participates directly in the reaction to yield the reactive oxygen species. The initial peroxo complex is similar to that formed by the extradiol dioxygenases in that iron remains formally Fe(II) and an O–O bridge is formed with a molecule that can supply reducing equivalents. However, the stability of the 4a-hydroxy pterin causes the heterolytic O–O bond cleavage to occur in the opposite direction such that the high valent Fe(IV)=O species is formed rather than the Fe(II)-hydroxy intermediate of the extradiol dioxygenases. Similar to the Rieske *cis*-diol dioxygenases, a second electron donating cofactor is required to activate oxygen. However, the reduced pterin can supply both electrons while the Rieske cluster of the Rieske *cis*-diol dioxygenases can supply only one, thereby requiring the Fe(II) to supply the second electron for bond cleavage, becoming Fe(III). The formation of these distinct intermediate states dictates that the product of heterolytic cleavage is at the level of a formal Fe(V)=O in the case of the Rieske *cis*-diol dioxygenases and Fe(IV)=O for the pterin-linked hydroxylases.

Oxidase enzymes in the 2-His+Asp/Glu family

Although the most familiar oxidase enzymes serve roles as terminal electron acceptors in energy storage pathways, there are many oxidases that make use of the oxidizing potential of O₂ to promote biosynthetic reactions. Through the application of the analytical techniques developed in the study of the oxygenase enzymes described here, and the advent of X-ray crystal structures of several members of the biosynthetic oxidase family, it is now known that many of these enzymes belong to the 2-His+Asp/Glu family^{77,78}. The mechanisms of O₂ activation proposed for this subgroup are very diverse, but they contain elements of the mechanisms discussed here. The general strategy appears to be to bind both the substrate and O₂ to the iron as in the extradiol and 2-oxo-acid dioxygenases to promote electron transfer away from the substrate and, in most cases, the iron. The reduced oxygen then undergoes O–O bond cleavage to yield a molecule of water and leave an Fe(IV)=O species as in the 2-oxo-acid dioxygenases or tetrahydropterin-linked hydroxylases. Finally, the high valent iron species is used as a reagent to complete a second part of the reaction, and in doing so, accepts two more electrons to form the second molecule of water.

Some well-studied examples of 2-His+Asp/Glu oxidases are isopenicillin N-synthase (IPNS)^{79–81}, fosfomycin synthase (FOS)^{82,83}, and 1-aminocyclopropane-1-carboxylate oxidase (ACCO), which catalyzes the formation of ethylene, a hormone in plants^{84–86}. These enzymes activate O₂ and ultimately form water using either 4 electrons from substrate (IPNS), two from substrate and two from NADH (FOS), or two from substrate and 2 from ascorbate (ACCO). The proposed mechanism of IPNS, of which two key steps are shown in Fig. 5, will serve as an example of the mechanistic strategy.

Spectroscopic and crystallographic studies show that substrate, δ -(L- α -aminoadipoyl)-L-cysteiny-D-valine (ACV, **6**) binds to the iron via its cysteinyl sulfur^{80,87}. The O₂ analog NO can be bound in an adjacent site suggesting that oxygen and substrate bind at the same time to the iron⁸⁸. Recent coordinated spectroscopic studies of the substrate-IPNS-nitrosyl complex have been extended computationally to the oxy complex⁸¹. These show that electron donation from the S-Fe bond would stabilize a Fe(III)-O₂^{•-} in an orientation that would favor abstraction of a hydrogen atom from the β -carbon of the ACV cysteinyl moiety. This prevents formation of a peroxo bridge, which would lead to oxygenase chemistry as in the cases of extradiol and 2-oxo-acid dioxygenases. Instead, a second hydrogen atom is transferred to the activated O₂ to give O-O bond cleavage coincident with formation of the β -lactam ring of penicillin N (7). The Fe(IV)=O species resulting from the O-O bond cleavage then becomes a reagent for the second ring-closing reaction. It is postulated that it abstracts a hydrogen atom from the β -carbon of the valinyl moiety of ACV^{79,87}. This radical intermediate rebounds to the original cysteinyl sulfur ligand rather than the hydroxyl ligand to form the thiazolidine ring of penicillin N and provide the final electron required to form the second water from the oxidase reaction. The rebound reaction to an atom other than oxygen is reminiscent of the halogenase reaction described above and prevents oxygenase chemistry from occurring during the second half of the reaction.

Conclusion

The broad application of the 2-His+Asp/Glu Fe(II) binding motif illustrated in Fig. 6 demonstrates the ability of nature to adapt a functional structural building block to catalyze a remarkable variety of oxygen activation chemistries. The key to this flexibility appears to be the open face of the iron coordination sphere, which can be used to bind O₂ and in some cases substrate or co-substrate to carry out a determining first step in the catalytic process. The organization of the second sphere environment of this open face can also be tuned by the strategic placement of acid/base catalysts, hydrogen-bonding partners, non-bonding substrates, electron supplying metal clusters, and cofactors. We have illustrated here that the combination of these elements allows the enzymes of the 2-His+Asp/Glu class to activate O₂ as: a) Fe(II)-O₂^{•-}, b) Fe(II)-alkylperoxo c) Fe(III)-O₂^{•-}, d) Fe(III)-(hydro)peroxo, e) Fe(IV)=O, and possibly, f) Fe(V)=O-hydroxo. All of these activation mechanisms share the common theme that the O₂ is reduced as part of the activation process and the O-O bond is broken during the reaction. However, by controlling the structural elements of the 2-His+Asp/Glu Fe(II) center and its local environment, the point at which the O-O bond breaks during the reaction cycle can be regulated. Moreover, it is possible to direct the distribution of electrons in the immediate product of the O-O bond cleavage reaction. The result is exquisite control over the O₂ activation process and the specific oxygenated and oxidized products that result.

The past decade has witnessed a great expansion in our appreciation of the detailed mechanisms used by non-heme Fe(II) containing oxidase and oxygenases. This was due to the application of new kinetic techniques that allowed intermediates in the reaction cycles to be recognized in combination with structural and spectroscopic techniques that allowed them to be characterized. As a result, several of the key oxo-, peroxo- and high-valent metal complexes described here have quickly moved from the hypothetical to the anchors in the mechanistic proposals. The search for additional intermediates in the reaction cycles in the future can now be much more focused, but at the same time, it is likely that these intermediates will be less stable and more difficult to detect and trap. While the route to success is unknown in this endeavor, it is likely to build on our ability to rationally control the progress of reactions through changes in the local metal environments, as well as to modulate the dynamics of the global enzyme structures by carrying out the reactions in atypical media such as crystals. It is also likely that computational studies will play a significant role by forecasting the properties of

intermediates so that experiments can be designed to optimize their stability and hence our ability to trap them for characterization.

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Figure 1.

Oxygen activation by extradiol dioxygenases. This type of activation involves simultaneous activation of O_2 and substrate, once both are bound to the active site Fe(II). It proceeds through formation of an alkylperoxo intermediate in which activated dioxygen attacks the substrate before O–O bond cleavage. The R-groups of the substrates considered here are $-CH_2-COO^-$ (HPCA, **1**) or $-NO_2$ (4NC, **2**). The structures shown are the *Brevibacterium fuscum* HPCD 4NC-semiquinone–Fe(II)– $O_2^{\bullet-}$ (top) and Fe(II)- alkylperoxo (bottom) intermediates (PDB 2IGA)²⁹ Atom color code: gray, carbon (enzyme residues); yellow, carbon (substrate); blue, nitrogen; red, oxygen; cyan, iron. Red and grey dashed lines show hydrogen bonds and potential bonds to iron, respectively.

Figure 2.

Oxygen activation by Rieske *cis*-diol dioxygenases. This type of reaction requires formation of a more reactive species that is found in the extradiol dioxygenase pathway of Fig. 1. One electron from the Rieske cluster and one from the Fe(II) are used to form Fe(III)–OOH which may then form Fe(V)=O–OH as the reactive species. The structure shown is of the hydroperoxo intermediate of *Pseudomonas* sp. strain NCIB 9816-4 NDO with the substrate analog indole bound nearby (PDB 1O7N)⁵² Atom color code: gray, carbon (enzyme residues); yellow, carbon (substrate); blue, nitrogen; red, oxygen; cyan, iron.

Figure 3.

Oxygen activation by 2-oxo-acid dioxygenases. An initial Fe(III)-O₂^{•-} species attacks the Fe-bound αKG co-substrate to yield an Fe(IV)=O reactive species. This species, in turn, attacks the substrate by hydrogen atom abstraction. The structure shown is of the initial reactive complex of *Escherichia coli* TauD with αKG bound to the iron and taurine bound nearby (PDB 1GQW)⁵⁵ Atom color code: gray, carbon (enzyme residues); yellow, carbon (substrate); bronze, carbon (co-substrate); blue, nitrogen; red, oxygen; green, sulfur; cyan, iron.

Figure 4.

Oxygen activation by tetrahydropterin-dependent hydroxylases. A peroxo bridge is formed between Fe(II) and an electron donating tetrahydropterin cofactor in this O_2 activation mechanism. The stability of the 4a-peroxypterin adduct allows the heterolytic $O-O$ bond cleavage to yield an $Fe(IV)=O$ species that reacts, in turn, with the aromatic substrate by electrophilic aromatic substitution. The structure shown is of human phenylalanine hydroxylase complexed with a substrate analog and tetrahydropterin (PDB 1MMK) ⁷³. Atom color code: gray, carbon (enzyme residues); yellow, carbon (substrate); bronze, carbon (co-substrate); blue, nitrogen; red, oxygen; green, sulfur; cyan, iron.

Figure 5.

Oxygen activation in the mechanism of formation of isopenicillin N by IPNS. Binding of O₂ to the active site Fe(II) yields an ACV-bound Fe(III)-O₂^{•-} reactive intermediate. Hydrogens (orange) and electrons derived from the closure of the β-lactam ring are then used to break the O-O bond and generate an Fe(IV)=O intermediate that is proposed to serve as a reagent to effect the thiazolidine ring closure reaction. The structure shown is of *Aspergillus nidulans* IPNS complexed with ACV (PDB 1BK0)⁸⁰ Atom color code: gray, carbon (enzyme residues); yellow, carbon (substrate); blue, nitrogen; red, oxygen; green, sulfur; cyan, iron.

Figure 6.

Oxygen activation by the flexible 2-His+Asp/Glu facial triad motif. All reactions begin by binding a substrate or cofactor to or near the iron resulting in release of solvents to open an O_2 binding site on the iron. A myriad of downstream chemistries are possible as determined by the nature of the exogenous molecules and active site residues near the catalytic surface of the iron. The three ligand sites shown to be occupied by H_2O in the central structure can be vacant, occupied by OH^- , or occupied by a weak protein ligand in different enzymes from the 2-His+Asp/Glu family.