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MINIREVIEW

# Oxygen activation by mononuclear nonheme iron dioxygenases involved in the degradation of aromatics

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**Abstract** Molecular oxygen is utilized in numerous metabolic pathways fundamental for life. Mononuclear nonheme iron-dependent oxygenase enzymes are well known for their involvement in some of these pathways, activating $O_2$ so that oxygen atoms can be incorporated into their primary substrates. These reactions often initiate pathways that allow organisms to use stable organic molecules as sources of carbon and energy for growth. From the myriad of reactions in which these enzymes are involved, this perspective recounts the general mechanisms of aromatic dihydroxylation and oxidative ring cleavage, both of which are ubiquitous chemical reactions found in life-sustaining processes. The organic substrate provides all four electrons required for oxygen activation and insertion in the reactions mediated by extradiol and intradiol ring-cleaving catechol dioxygenases. In contrast, two of the electrons are provided by NADH in the *cis*-dihydroxylation mechanism of Rieske dioxygenases. The catalytic nonheme Fe center, with the aid of active site residues, facilitates these electron transfers to $O_2$ as key elements of the activation processes. This review discusses some general questions for the catalytic strategies of oxygen activation and insertion into aromatic compounds employed by mononuclear nonheme iron-dependent dioxygenases. These include: (1) how oxygen is activated, (2) whether there are common intermediates before oxygen transfer to the aromatic substrate, and (3) are these key intermediates unique to mononuclear nonheme iron dioxygenases?

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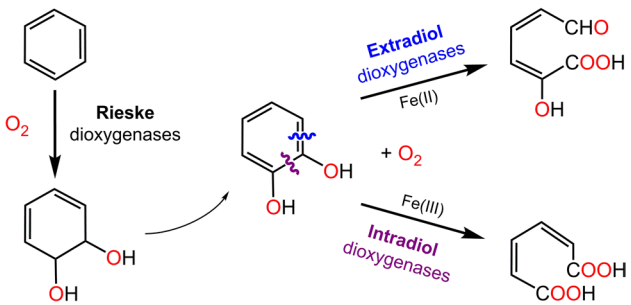
**Graphical Abstract**




**Keywords** Catalytic strategies · Crystal structure · High-valent iron species · Metabolism · Nonheme iron enzymes · Oxidative degradation · Reactive oxygen species · Ring-cleaving dioxygenase · Spectroscopy

## Introduction

### Discoveries of $O_2$ incorporation created the new field of oxygenases

Oxygen often serves as a direct oxidant in biodegradation pathways of functionally relevant organic metabolites. Molecular oxygen is a stable diradical molecule with two unpaired electrons, giving it a triplet ground state. In contrast, most organic molecules have singlet ground states with no unpaired electrons, so the reaction with triplet $O_2$ is a spin forbidden process. Oxygenase enzymes are utilized in nature to activate $O_2$ and sometimes also the organic molecule to allow them to react. The most common strategies employed by oxygenases are to utilize either a transition metal center or a flavin cofactor to activate $O_2$ in a controlled manner, so that it can react rapidly with the organic substrate bound nearby in the enzyme active site.

It is not unusual for the first discovered phenomenon to be thought of as prevalent or predominate, thereby creating an intangible barrier to acceptance of other alternative scenarios. Often times, the truth is not what was originally assumed, or only partially illuminated by the initial finding. For instance, before the 1950s it was thought that triplet dioxygen was too stable to be easily incorporated into organic substrates, making water the logical source for oxygen atoms found in products. For example, in carbohydrate metabolism, only oxygen from water is found in the $CO_2$ that results from the enzymatic oxidation of glucose via glycolysis, the pyruvate dehydrogenase complex, and the citric acid cycle. While it is true that $O_2$ ultimately plays a key role in the production of energy-rich molecules downstream of these processes, at that time it was believed only to serve as a terminal electron acceptor at the terminus of the oxidative phosphorylation pathway. This understanding was well established in the literature, so it was thought that the source of oxygen is water in most natural transformations. However, this concept was shown to be incorrect by the elegant and independent isotopic labeling studies performed by Hayaishi and Mason [1, 2], which demonstrated that oxygen from $^{18}O_2$ can be the direct source of oxygen incorporated during biological oxidations of organic substrates.

In the classic dioxygen labeling experiment, Hayaishi showed in 1955 that both oxygen atoms from $^{18}O_2$ were incorporated into the ring-opened dicarboxylic acid product of catechol 1,2-dioxygenase (pyrocatechase) [1]. During the same period, Mason showed that $^{18}O_2$ is the source of the oxygen atom incorporated by tyrosinase during the formation of 3,4-dihydroxy-L-phenylalanine (DOPA) [2]. Two years later, Hayaishi showed that the first step of tryptophan degradation involves the incorporation of two oxygen atoms to form *N*-formylkynurenine. By mass spectrometric analysis of the isolated product, Hayaishi and coworkers determined that both atoms of oxygen in *N*-formylkynurenine were derived from $^{18}O_2$, rather than $H_2^{18}O$ [3, 4]. These exciting discoveries, along with subsequent findings by peers, established oxygenases as a new enzyme class. Hence, a new chapter of biological oxygen activation was started some 60 years ago.

## Nonheme iron-dependent oxygenases are prevalent in nature

The most common transition metal-containing oxygenases employ an iron cofactor for oxygen activation. After activation, one or both oxygen atoms are transferred to the organic substrate, consequently generating biological functionality. Oxygenases are broadly classified into two families, dioxygenases and monooxygenases. Dioxygenases incorporate both oxygen atoms into the substrate or a primary organic substrate plus a co-substrate. They either take all four electrons needed for the reaction from the substrate (or co-substrate) or two from the substrate and two from an external donor. In contrast, monooxygenases only incorporate one oxygen atom from $O_2$ into the product while reducing the second atom to water using electrons from an external donor. Both oxygenase classes differ from oxidase enzymes, which transfer two or four electrons from a donor (usually the substrate) to reduce $O_2$ to $H_2O_2$ or $H_2O$ without oxygen incorporation. Iron-dependent dioxygenases can employ either heme or nonheme cofactors in the active site. The heme-dependent dioxygenases are discussed in our recent review [5] and by Raven in this volume [6] and thus will not be included here.

In what follows, the mechanisms of three types of mononuclear nonheme iron-dependent dioxygenases will be discussed, namely extradiol dioxygenases, intradiol dioxygenases, and Rieske dioxygenases. Several representative enzymes from each class are shown in Table 1. We consider these three classes of mononuclear nonheme Fe dioxygenases to be able to promote "pure" dioxygenation reactions. The α-ketoglutarate-dependent dioxygenases and pterin-dependent aromatic amino acid hydroxylases are also prevalent and versatile in nature. However, in the latter two cases, only one atom of $O_2$ is incorporated into the primary organic substrate, while the other O-atom ends up on the organic co-substrate, which provides two of the four electrons needed for oxygen reduction. These latter types of mononuclear nonheme Fe dioxygenases are discussed elsewhere in this special issue [7, 8].

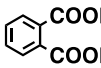
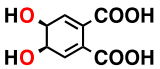
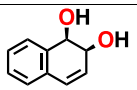
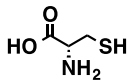
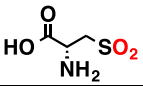
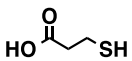
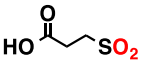
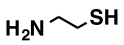
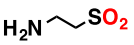
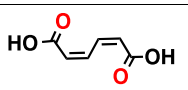
## Rieske dioxygenases require both $O_2$ and a reducing agent

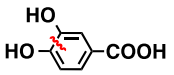
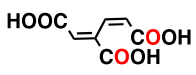
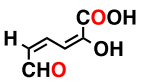
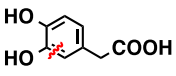
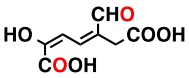
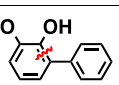
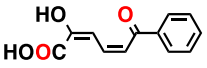
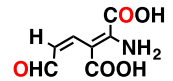
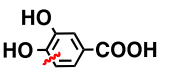
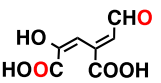
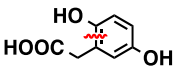
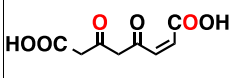
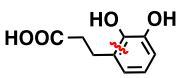
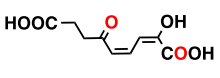
### A unique group of dioxygenases

Under aerobic conditions, Rieske-type iron-dependent dioxygenases transfer two oxygen atoms to arenes in a *cis*-dihydroxylation reaction to yield *cis*-dihydrodiol products with NADH and molecular oxygen as co-substrates (Table 1). Common examples are benzene dioxygenase, phthalate dioxygenase, toluene dioxygenase and naphthalene 1,2-dioxygenase. These enzymes carry out the first step in the degradation of aromatic compounds by soil bacteria, followed by formation of catechol and then ring cleavage by intra- or extradiol dioxygenases [9, 10]. Rieske dioxygenases exhibit stereo- and enantiospecificity in their reactions, which are difficult to be achieved in synthetic organic chemistry [11].

Two or three protein components comprise the majority of Rieske dioxygenases: a reductase containing flavin and ferredoxin cofactors, sometimes a ferredoxin-containing

**Table 1** Nonheme Fe dioxygenases discussed in this review

| Enzyme | Substrate | Product |
|---|---|---|
| **Rieske Dioxygenase** | | |
| Benzene 1, 2-dioxygenase |  |  |
| Phthalate 4,5-dioxygenase |  |  |
| Toluene dioxygenase |  |  |
| Naphthalene 1,2-dioxygenase |  |  |
| **Thiol Dioxygenase** | | |
| Cysteine dioxygenase |  |  |
| 3-Mercaptopropionic acid dioxygenase |  |  |
| Cysteamine dioxygenase |  |  |
| **Intradiol Dioxygenase** | | |
| Catechol 1,2-dioxygenase |  |  |

| Enzyme | Substrate | Product |
|---|---|---|
| **Intradiol Dioxygenase** | | |
| Protocatechuate 3,4-dioxygenase |  |  |
| **Extradiol Dioxygenase** | | |
| Catechol 2,3-dioxygenase |  |  |
| Homoprotocatechuate 2,3-dioxygenase |  |  |
| 2,3-dihydroxybiphenyl dioxygenase |  |  |
| 3-Hydroxyanthranilate 3,4-dioxygenase |  |  |
| Protocatechuate 4,5-dioxygenase |  |  |
| Homogentisate 1,2-dioxygenase |  |  |
| 2,3-dihydroxyphenyl propionate 1,2-dioxygenase |  |  |

The ring cleavage site is indicated by a jagged red line in the intradiol and extradiol dioxygenases

electron transfer protein, and a terminal oxygenase. The latter includes a Rieske [2Fe–2S] cluster, coordinated by two cysteines and two histidines, and a catalytic mononuclear iron center responsible for dioxygen activation and the *cis*-dihydroxylation of the aromatic ring.

The ~35 kDa reductase component acts to transfer electrons one at a time from the two-electron donor NADH, via the small ~15 kDa ferredoxin (when presents) to the Rieske [2Fe–2S] cluster found in the terminal oxygenase (MW = 150–220 kDa). The relatively large terminal oxygenase generally has a quaternary structure consisting of either an $\alpha_3$ or $(\alpha\beta)_3$ trimer [11]. Interestingly, crystal structures show that the catalytic iron center and the Rieske cluster within the same subunit are separated by 44 Å [12], beyond the limit for an anticipated efficient electron transfer. The longest separation distance in other systems is 30–35 Å found in ribonucleotide reductase [13, 14]. On the other hand, the head-to-tail trimeric structure of the oxygenase component places the catalytic Fe center only 12 Å from the Rieske cluster on the adjacent subunit, which would allow fast electron transfer. Indeed, fast electron transfer between the metal centers has been reported [12]. The pathway for inter-subunit electron transfer is postulated to proceed through a conserved Asp residue at the subunit–subunit interface that serves as a bridge between a histidine residue on the Rieske cluster and a histidine ligand on the iron center [15].

## Proposed mechanism of Rieske dioxygenase action

In the as-isolated enzyme, the nonheme iron center is typically found in the ferrous state coordinated by two water molecules, two histidines and a bidentate aspartate/glutamate residue [15, 16]. As the first step of the catalytic cycle, the arene substrate binds to the active site adjacent to the catalytic ferrous center (Fig. 1) and causes the dispatch of one of the water ligands to make the ferrous center 5-coordinate [17]. Reduction of the Rieske center causes allosteric changes in the geometry of the mononuclear iron center geometry in preparation for $O_2$ binding [18–20]. Oxygen is then proposed to coordinate to form a transient but yet unobserved Fe(III)-superoxo intermediate [21]. There are several possibilities for the next intermediate in the cycle. It has recently been proposed that the initially

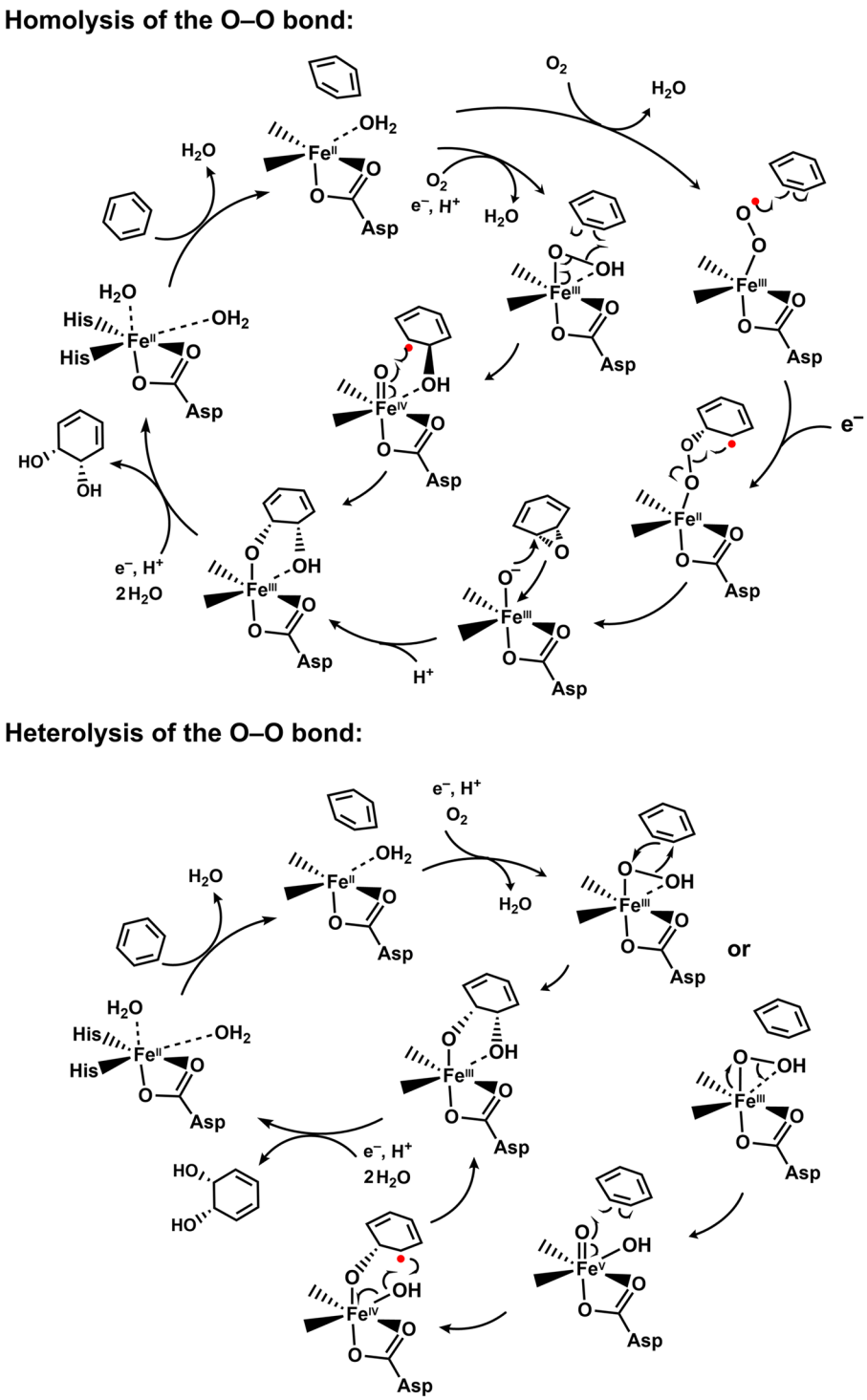



**Fig. 1** Proposed catalytic routes of Rieske dioxygenases through O–O bond homolysis (*top*) or heterolysis (*bottom*)

formed Fe(III)-superoxo intermediate attacks the aromatic substrate to form an Fe(III)-peroxo-aryl radical intermediate, which is then reduced by electron transfer [21] from the Rieske cluster to form the Fe(II) analog. This proposal is based on the observation that the substrate type and the electron-donating capacity of aromatic ring substituents affect the rate constant for electron transfer from the Rieske center, so that electron transfer cannot precede attack on the substrate. However, side-on bound Fe(III)-hydroperoxo intermediates have been observed in crystals of Rieske dioxygenases [11, 22] and trapped and spectroscopically characterized in samples taken during "peroxide shunt" turnover in which hydrogen peroxide serves as the source of oxygen atoms [23].

Some possible mechanistic routes to *cis*-diol formation in Rieske dioxygenases are presented in Fig. 1. If the intermediate in Rieske dioxygenases is the proposed Fe(II)-peroxo-aryl radical, it might evolve through homolytic cleavage of the O–O bond, leading to formation of a substrate epoxide and Fe(III)–OH. Opening of the epoxide would yield a cation on the adjacent carbon which could abstract the hydroxide from the iron to complete the formation of the non-aromatic *cis*-diol product [21]. If the Fe(III)-bound hydroperoxo species is the reactive intermediate, it could function as the direct oxidant, transferring both oxygen atoms simultaneously to the aromatic substrate, perhaps via a dioxetane intermediate [11]. Two other plausible reaction routes have been proposed based on how the O–O bond is cleaved [24]. Homolytic cleavage followed by substrate oxidation would yield a substrate phenol radical and an enzyme-based Fe(IV)=O species, which could transfer the second oxygen to the substrate ring in a subsequent step. The Fe(IV)=O species is chemically equivalent to compound II in most P450 catalysis [25, 26] and heme-dependent tryptophan 2,3-dioxygenase [27, 28]. Alternatively, heterolytic O–O bond cleavage would generate an HO–Fe(V)=O complex, analogous to compound I in heme enzymes, to react with the substrate to give rise to an Fe(IV)-substrate radical complex [23]. The final intermediate in all proposed mechanisms is a ferric *cis*-diol product complex. The product is released only after an external electron is transferred to the mononuclear iron via the Rieske cluster to regenerate the resting state.

Studies of biomimetic catalysts have provided insight into how a water ligand might facilitate the reaction of an Fe(III)–OOH intermediate. Based on isotopic labeling experiments, the Que group has proposed a water-assisted mechanism in which that $H_2^{18}O$ binds to the ferric center *cis* to the hydroperoxo ligand to incorporate an $^{18}O$ into an $H^{18}O$–Fe(V)=O oxidant that is generated via heterolytic cleavage of the O–O bond [29–31]. This mechanism was more recently supported by kinetic studies showing the decay of an observed Fe(III)–OOH intermediate to be accelerated as a function of water concentration [31]. An observed $H_2O/D_2O$ kinetic isotope effect of 2.5 further demonstrated the key role of a water proton in facilitating the proposed heterolytic O–O bond cleavage step. In strong support, Costas and coworkers have reported convincing spectroscopic evidence for the existence of the $[Fe^{V}(O)(OH)(L)]^{2+}$ ion by cryospray mass spectrometry [32, 33]. More mechanistic details are discussed in a review on bioinspired nonheme iron catalysis in this issue [34]. Thus, the notion of an $Fe^{V}(O)$ oxidant presented in Fig. 1 for the Rieske oxygenase mechanism has a plausible basis.

## Oxygen insertion into catechol: a tale of two dioxygenases

### Extradiol and intradiol ring-cleaving dioxygenases

It is estimated that roughly 30% of the mass of woody plant material on earth comprises cross-linked aromatic compounds in the form of lignin. Nature has devised a variety of strategies to break down the lignin into individual aromatic molecules, which are much more stable to degradation than aliphatic hydrocarbons [35, 36]. One strategy devised by nature to overcome the stability of aromatic compounds is to utilize oxygenases to couple ring cleavage with oxygen insertion and form ring-opened products that can re-enter the Krebs cycle.

Hashimoto and Hayaishi isolated the first intradiol ring-cleaving dioxygenase, pyrocatechase, from *Pseudomonas* (Fig. 2). This enzyme breaks the catechol aromatic ring between the hydroxyl-bearing carbons with incorporation of both atoms of oxygen from $O_2$ to form *cis*, *cis*-muconate [1, 37, 38]. Protocatechuate-3,4-dioxygenase (3,4-PCD), first studied by Stanier and Ingraham [39], and later by Hayaishi [40], converts 3,4-dihydroxybenzoate to 3-carboxy-*cis,cis*-muconate in a reaction analogous to that of catechol 1,2-dioxygenase.

Protocatechuate 4,5-dioxygenase and catechol 2,3-dioxygenase (metapyrocatechase) were the first enzymes shown to insert both atoms of oxygen from $O_2$ into enzyme-bound catecholic substrates at positions adjacent to the two phenolic hydroxyl groups, leading to the production of 2-hydroxymuconate semialdehydes [38, 41, 42]. Senoh and his coworkers found another enzyme, 3,4-dihydroxyphenylacetate-2,3-dioxygenase (also known as homoprotocatechuate 2,3-dioxygenase, 2,3-HPCD) [43], that performs a similar reaction oxidizing 3,4-dihydroxyphenylacetate to 2-hydroxy-5-carboxymethylmuconate semialdehyde (Table 1). Due to the position of ring cleavage, these enzymes were termed extradiol ring-cleaving dioxygenases (Fig. 2).

Both intradiol and extradiol dioxygenases operate by utilizing a nonheme Fe center to allow triplet $O_2$ to react rapidly with singlet substrates, but they use different mechanistic strategies. Both mechanisms progress from an initial chelate complex of the catecholic substrates with the iron. Both classes have a third iron ligand site adjacent to the substrate ligand sites that can be occupied by oxygen or an activated oxygen intermediate at some stage of the reaction cycle. The mechanism for Fe(II)-dependent extradiol dioxygenases involves nucleophilic attack by an iron-bound oxygen on the organic substrate to yield an alkyl peroxo

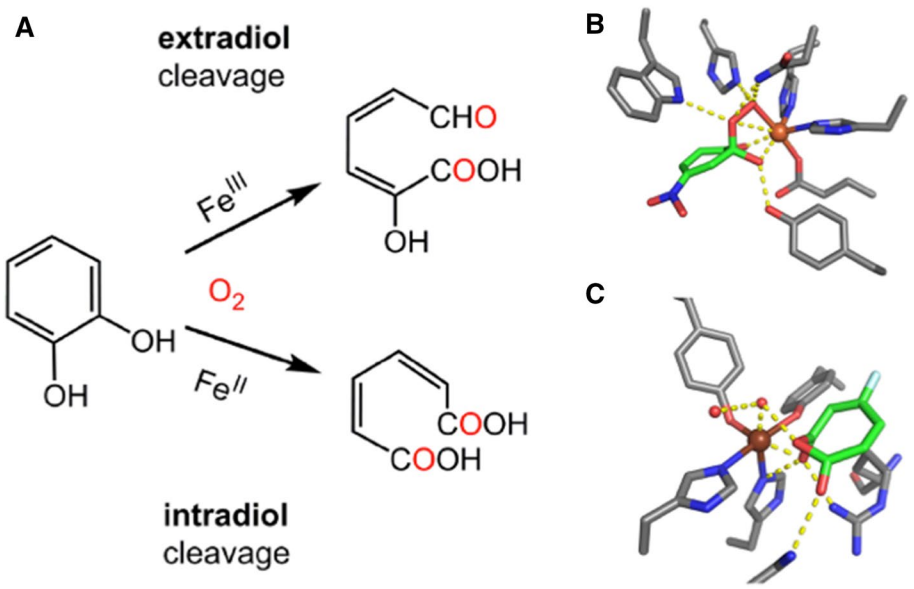



**Fig. 2** Distinct natural catechol dioxygenase activities. **a** Extradiol and intradiol cleavages, **b** the alkylperoxo intermediate structurally characterized in the extradiol dioxygenase 2,3-HPCD (from 2IGA.pdb), and **c** the structure of the anhydride intermediate in the intradiol dioxygenase 3,4-PCD (from 4WHR.pdb)

intermediate. In the case of the Fe(III)-dependent intradiol dioxygenases, the iron serves to activate substrate to allow formation of a similar alkylperoxo intermediate via a different strategy. No additional electron source is required in either of these reactions. The catalytic Fe center, with aid from active site residues, is sufficient to promote electron transfer from the organic substrate to $O_2$.

Extradiol ring-cleaving dioxygenase genes are more commonly found in the NCBI protein database (https://www.ncbi.nlm.nih.gov/protein) than their intradiol counterparts, with 126,278 entries versus 9994 (both with redundancies) as of November 1st, 2016, respectively. Thus, the extradiol ring-cleaving enzymes predominate, and they are essential biological catalysts for carbon and nitrogen cycles in the aerobic world.

## Proposed mechanism of extradiol ring-cleaving dioxygenases

Extradiol dioxygenases cleave a wide variety of substrates [44–51]. There are three subfamilies of extradiol dioxygenases that differ in their protein structures and evolutionary relationships [52, 53]. Type I and II enzymes share partial sequence similarity. Type II enzymes are believed to be evolved from a type I enzyme through gene duplication [52]. As a result, the type II enzymes are composed of two domains with approximately the same folding pattern as type I [54, 55]. The well-studied catechol 2,3-dioxygenase and 2,3-HPCD belong to type I enzymes, while 2,3-dihydroxybiphenyl dioxygenase typifies type II enzymes. Type III enzymes belong to the functionally diverse Cupin superfamily [56–59]. These enzymes are far removed evolutionarily from the other groups of extradiol dioxygenases, and they are distinct in sequences as well as structures. 3-Hydroxyanthranilate-3,4-dioxygenase (HAO, also known as HAD) and protocatechuate 4,5-dioxygenase (4,5-PCD) are representative members of this group [60, 61]. However, type III enzymes do share most of the active site characteristics and mechanistic properties of the other two types of enzymes [59, 61, 62].

Each type of extradiol dioxygenase has been well studied, especially the Type III 4,5-PCD and there is considerable evidence with which to build a consensus chemical mechanism as illustrated in Fig. 3 [24, 44–46, 48–51, 63]. In particular, Fe-bound superoxo, alkylperoxo, gem-diol intermediates and product-bound enzyme portrayed in this reaction cycle have all been captured from 2,3-HPCD and characterized by X-ray crystallography in the Lipscomb laboratory [64, 65]. Notably, this work is fully supported by spectroscopic data [66–70]. The catalytic iron center of extradiol dioxygenases employs the 2-His-1-carboxylate facial triad structural platform [71]. The ferrous ion is coordinated by two histidines and one glutamate with labile water-derived ligands occupying other empty positions [54, 72]. The glutamate ligand in some enzymes binds in a bidentate mode to the catalytic iron center. In an ordered binding sequence (Fig. 3), the organic substrate first binds to the ferrous center and replaces water ligand(s). A proton from only one of the two hydroxyl groups on the substrate is abstracted by an identified active site base, causing asymmetric binding of the catecholic hydroxyl groups to the iron center. Thus, the binding of the organic substrate reorganizes the iron center and promotes dioxygen binding and subsequent reactions [24, 47, 49, 59]. The dioxygen binds to the ferrous ion and may form a transient Fe(III)-bound superoxo radical. The Fe(III) ion then activates the catechol

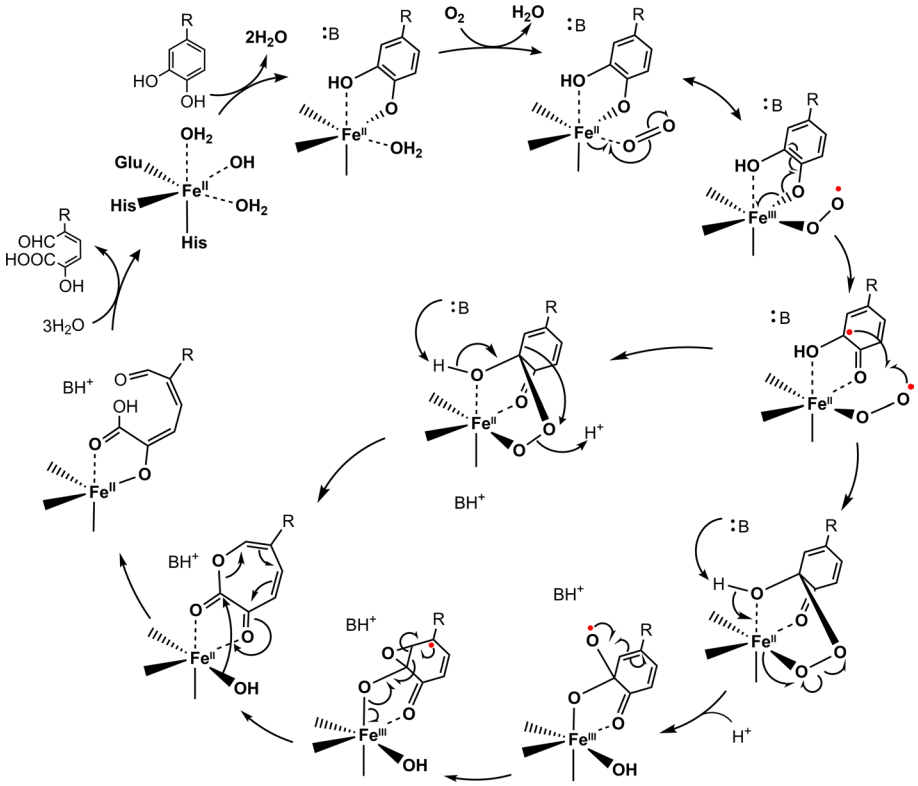


**Fig. 3** The catalytic mechanism of extradiol ring-cleaving dioxygenases

substrate by accepting an electron to generate the substrate semiquinone radical-Fe(II)-superoxo species. The two activated substrates undergo a radical–radical recombination reaction, forming an Fe(II)-bound alkylperoxo intermediate (Fig. 3) [73]. In 2,3-HPCD, His200 acts as an active site base to promote formation of the alkylperoxo intermediate [67].

In support of this mechanism, an Fe(III)-superoxo radical has been trapped and spectroscopically characterized in a variant of 2,3-HPCD in which a key active site acid/base catalyst has been removed to slow the reaction chemistry [66]. The actual Fe(III)-superoxo species has been structurally characterized through *in crystallo* chemical reactions in both 2,3-HPCD and homogentisate 1,2-dioxygenase (HGDO), along with the alkylperoxo intermediate [64, 74]. The structures are similar, but the $O_2$ is bound *trans* to the oxygen of glutamate in 2,3-HPCD in the alkylperoxo intermediate, whereas $O_2$ is observed *trans* to a His ligand in HGDO [74]. This variance appears to be due to the difference in the active site geometry of the ternary complex in the two enzymes.

Computational studies suggest that O–O bond cleavage of the alkylperoxo intermediate results in formation of a short-lived gem-diol radical at a ring carbon and then an epoxide at the position of ring cleavage [75–78]. The epoxide undergoes a rearrangement to form a seven-membered ε-lactone intermediate, which is hydrolyzed by the Fe(II)-hydroxide to produce the extradiol product. The product-bound intermediate has been structurally defined in both 2,3-HPCD and HGDO. The gem-diol intermediate is structurally characterized in a crystal the E323L variant of 2,3-HPCD containing the alternative substrate 4-sulfonylcatechol [65]. The lactone intermediate has also been proposed based on the $^{18}O$-labeling experiment of 2,3-dihydroxyphenylpropionate 1,2-dioxygenase (MhpB) [79]. The hydrolysis of a lactone by the iron(II)-bound hydroxide is supported experimentally by the MhpB-catalyzed hydrolysis of a saturated lactone analog [79]. Another possibility for the O–O bond cleavage is via 1,2-alkyl migration, which was supported by using mechanistic probes [80] and also hybrid DFT studies [81]. This mechanism suggested that Criegee rearrangement involves concerted acid catalysis by a base residue, which deprotonates the C-3 hydroxyl group to promote a ring expansion, forming the seven-membered ε-lactone intermediate by 1,2-alkyl migration [82].

## Proposed mechanism of intradiol ring-cleaving dioxygenases

The mononuclear Fe(III) cofactor of the intradiol catechol dioxygenases is coordinated by two histidine and two tyrosinate residues with a fifth hydroxide ligand in a trigonal bipyramidal structure [83] (Fig. 4). When the organic substrate binds to the enzyme, the axial tyrosinate ligand and equatorial hydroxide ligand of the Fe(III) center are

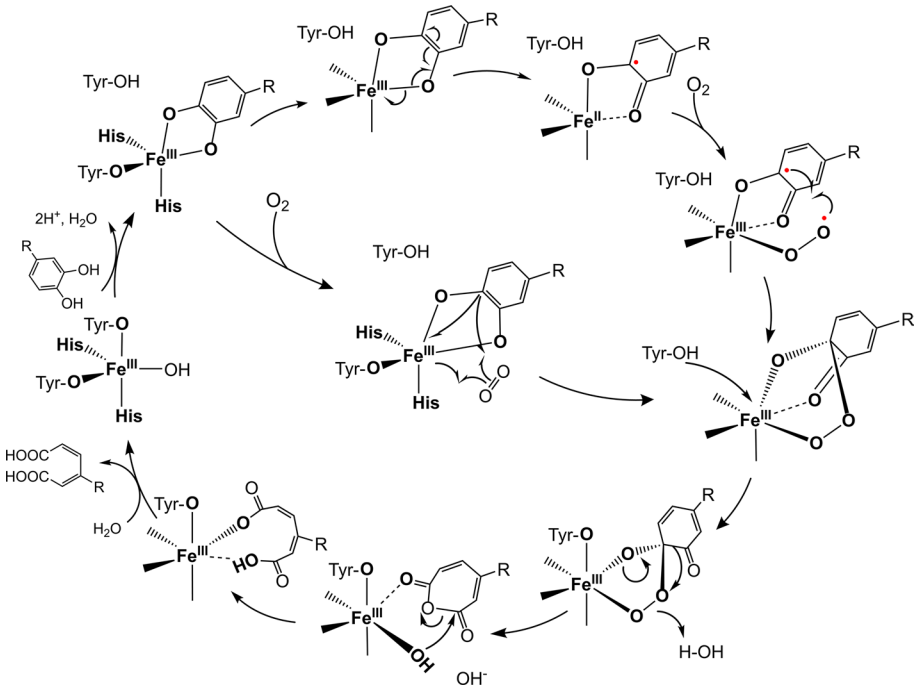



**Fig. 4** The catalytic cycle of intradiol ring-cleaving dioxygenases

displaced [84, 85]. The mechanism of the intradiol catechol dioxygenases has been proposed to be initiated via a substrate activation mechanism. The Fe(III) ion polarizes the substrate, building up the charge on one of the hydroxyl-bearing carbons. MCD and IR spectroscopies have revealed numerous charge transfer interactions between the substrate and the Fe(III) [86]. One of these interactions provides a low-energy path for electron transfer from the substrate to one of the d-orbitals of the iron, but actual electron transfer only occurs during a concerted $O_2$ binding process. Oxygen accepts one electron directly from the substrate and the second from an occupied d-orbital of the iron different from that which is accepting an electron from the substrate. As a result, two electrons of different spin are transferred to the incoming oxygen to form the alkylperoxo intermediate in one spin-allowed process [87, 88]. The collapse of the alkylperoxo intermediate and O–O bond cleavage occurs via a Criegee rearrangement to yield a cyclic anhydride intermediate and Fe(III)-bound hydroxide [89]. Then the anhydride intermediate undergoes hydrolysis by the Fe(III)–OH to produce muconic acid.

The reaction mechanisms of extradiol and intradiol catechol dioxygenases are both believed to proceed through alkylperoxo intermediates. However, the cleavage of the aromatic ring performed by these two enzymes is likely to diverge at this stage of the cycle due to the difference in the detailed structures of the intermediates. The crystal structures of both types of intermediates have been solved [64, 87]. Extradiol dioxygenases form the alkylperoxo intermediate with both of the catecholic oxygens bound to the iron. In contrast, one of the hydroxyl groups is released when the intermediate forms during the intradiol catalytic cycle. This change alters the alignment of the O–O bond of the peroxo with the bonds of the substrate ring and directs the insertions into the aligned bonds. Extradiol cleavage occurs via 1,2-alkenyl migration to give a lactone, whereas intradiol cleavage occurs via 1,2-acyl migration to give an anhydride [80]. It should be pointed out that the active site residues play critical roles to tune either the early oxygen activation steps or the subsequent O-atom transfer after formation of the common alkylperoxo intermediate. Acid–base catalysis is known to be an important part of the catalytic cycle in extradiol ring cleavage mechanism.

Intradiol oxidative cleavage activity was achieved from a directed evolution of the extradiol cleaving enzyme MhpB [90]. A single mutation of an active site histidine to phenylalanine (i.e., H200F) led to the conversion of an extradiol aromatic ring-cleaving 2,3-HPCD protein into an intradiol cleaving enzyme [91], suggesting that from chemical perspective amino acid replacements could swap these two reactions at the enzyme active site.

## Key catalytic intermediates of the dioxygenases

Catechol ring-cleaving dioxygenases are the best characterized nonheme Fe enzymes among the dioxygenases reviewed above. The common catalytic intermediate is an iron-bound alkylperoxo species (Fig. 5). Extradiol dioxygenases generate an Fe(II)-alkylperoxo intermediate (Fig. 5, **1**), while intradiol dioxygenases make an Fe(III)-alkylperoxo intermediate (Fig. 5, **2**). On the other hand, the

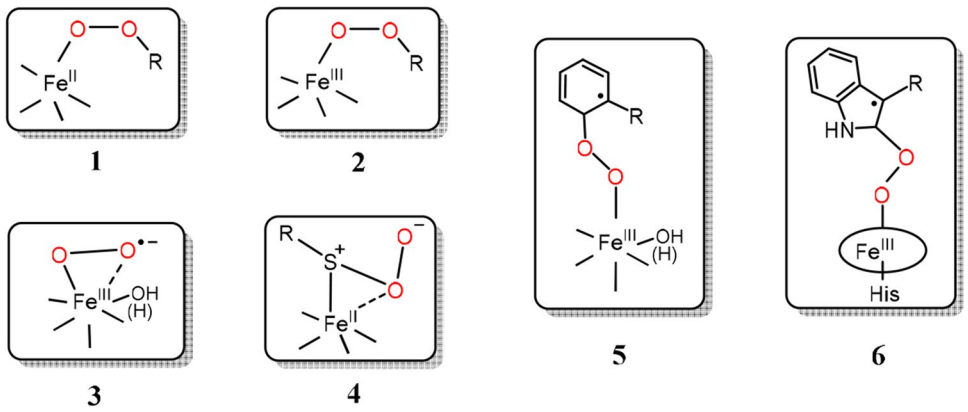



**Fig. 5** Comparison of the Fe-bound peroxo intermediates found in extradiol dioxygenases (**1**), intradiol dioxygenases (**2**), Rieske dioxygenases (**3**), and thiol dioxygenases (**4**) with proposed alkylperoxo/aryl radical intermediates for the Rieske dioxygenases (**5**) and heme-dependent tryptophan 2,3-dioxygenase (**6**)

Rieske dioxygenases produce an Fe(III)-hydroperoxo oxidant (Fig. 5, **3**). The three intermediates, although differing from each other, share similarities. The distal oxygen is the first oxygen atom to be transferred to the substrate in the subsequent steps in the catechol ring-cleaving dioxygenases (Figs. 3, 4). This is also a possible avenue for the Rieske dioxygenases through the homolytic O–O bond cleavage pathway (Fig. 1), although further research on the later reaction steps of the mechanism is needed. However, a closer look indicates that these mononuclear nonheme Fe-bound peroxo intermediates differ significantly in the oxidation state of the metal ion and the interaction between the metal and the substrates.

Recently, an Fe(II)-bound persulfenate intermediate (Fig. 5, **4**) has been identified in a crystal structure of cysteine dioxygenase (CDO), the best characterized member of the mononuclear nonheme Fe thiol dioxygenase family [92], which includes cysteamine dioxygenase (ADO) [93, 94] and 3-mercaptopropionate dioxygenase (MDO) (Table 1) [95–97]. In this intermediate, the persulfenate is bound to the iron center, forming a three-membered Fe–S–O ring, suggesting that both oxygen atoms of the dioxygen ligand may be transferred to the thiol group simultaneously, followed by an isomerization reaction to yield cysteine sulfinic acid product [92, 98]. The persulfenate intermediate is a unique peroxo intermediate structurally distinct from the carbon-based peroxo intermediates shown in Fig. 5. Although the formation of such a persulfenate species is thought to be energetically more costly [99], it was reproducibly generated in the crystalline state at different laboratories [92, 98, 100]. However, the catalytic competence of the intermediate remains controversial [100], as evidence is still lacking to prove the persulfenate to be an on-pathway intermediate of the CDO catalytic cycle. The same intermediate remains to be trapped and characterized in solution. Nonetheless, this new persulfenate intermediate, at first sight, shares similarities with the peroxo intermediates shown in Fig. 5. In the homolysis of O–O bond catalytic cycle of Rieske dioxygenase an alkylperoxo radical intermediate is proposed (Fig. 5, **5**). It should be noted that an analogous alkylperoxo radical intermediate is also proposed to be part of the catalytic cycle in heme-dependent tryptophan 2,3-dioxygenase in the form of a 2-indolenylperoxo radical intermediate, (Fig. 5, **6**) [8], analogous to that recently proposed by Rivard et al. from studies of benzoate 1,2-dioxygenase [21].

## Concluding remarks

In the past six decades, great strides have been made towards understanding the mechanisms of oxygen activation mediated by mononuclear nonheme Fe dioxygenases. Recent successful trapping and structural characterization of intermediates have helped to establish very detailed chemical understandings of each of the aromatic ring-cleaving dioxygenase enzymes described here as well as of the α-ketoglutarate-dependent dioxygenases [7]. While there are still uncertainties in the catalytic route for the Rieske dioxygenases, advances in the synthesis and characterization of iron–oxygen model compounds [101–103] have provided challenging new proposals to consider.

From the above survey of the nonheme iron enzyme dioxygenation strategies, a particular emphasis on understanding the general principles of how the iron center interacts with $O_2$ and the substrate may be reached. The active site residues, including the metal ligands and critical second sphere side chains, have been shown in numerous systems to contribute significantly to the fine-tuning of the enzyme activity and substrate specificity. A considerable amount of evidence has also shown that enzyme–substrate spectral signatures are sensitive measures of the electronic structure of the iron environment, and this data can be used to calibrate computational studies and/or validate mechanistic proposals. Several general principles have emerged from these studies: (1) the maintenance of charge at the Fe center is a dominant factor in determining how the substrate binds as well as the relative stability of intermediates;

(2) changes in the ligand set, i.e., geometry and/or coordination numbers, occur upon substrate binding to prepare the system for subsequent $O_2$ reactions; (3) the second ligand sphere is often an important factor to stabilize the oxidizing intermediates and direct the oxidizing power, and (4) homolysis or heterolysis of the O–O bond is the major divergence among oxygen transfer strategies and should be a key point of future mechanistic studies.

Looking forward, novel mononuclear nonheme Fe-dependent oxygenation reactions are likely to be discovered. Many are anticipated to share part of the oxygen activation schemes illustrated in this perspective while some catalytic steps may deviate from the common pathway to achieve novel chemical transformations due to the unique enzyme active site architecture and the chemical structure of the substrate. Time-resolved advanced spectroscopy and X-ray crystallography, site-directed alteration (i.e., substitution of a natural or unnatural amino acid) or the novel chemical mutagenesis approaches [104–106], and synthetic techniques for mechanism-based probes are expected to yield fundamental structural and chemical insights into $O_2$ activation and insertion for mechanistic enzymology of the nonheme Fe dioxygenases.

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