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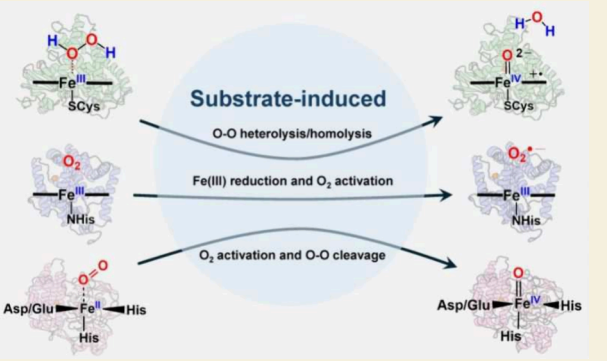
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Perspective

Substrate-Engaged Formation of Reactive Oxygen Intermediates in Metalloenzymes: Mechanisms and Implications for Biocatalysis

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ABSTRACT: Reactive oxygen intermediates in metalloenzymes are generally viewed as products of metal-centered O_2 or H_2O_2 activation that are tuned by the surrounding protein environment. Increasing mechanistic evidence, however, shows that substrates can actively participate in the generation and evolution of these oxidants. In this Perspective, we discuss a substrate-engaged framework for reactive oxygen intermediate formation in metalloenzymes. Two major modes of substrate participation are emphasized. In H_2O_2-dependent heme enzymes, substrates can functionally replace or complement distal acid−base residues by providing transferable protons, organizing hydrogen-bond networks, and/or enabling proton-coupled electron transfer, thereby promoting productive O−O bond cleavage and ferryl/Compound I formation. In O_2-activating heme and non-heme iron enzymes, substrates or substrate-derived intermediates can contribute electron density or reducing equivalents to metal−O_2 adducts, generate radical-coupled intermediates, and guide metal−oxygen species into catalytically competent geometries. These examples indicate that substrate recognition, oxygen activation, and substrate oxidation are often coupled within a single catalytic manifold. This substrate-engaged view complements the traditional metal-centered paradigm and provides mechanistic principles for discovering and designing oxidative biocatalytic reactions in which substrates help generate, control, and productively consume reactive oxygen intermediates.



KEYWORDS: *Metalloenzymes, Reactive oxygen intermediates, Substrate-engaged framework, O_2 activation, H_2O_2 activation*

1. INTRODUCTION

Metalloenzymes catalyze diverse oxidative transformations by converting O_2 or H_2O_2 into highly reactive oxygen intermediates, including metal−superoxo, metal−peroxo, metal−hydroperoxo, high-valent metal−oxo, and radical-coupled species.[1−11] These intermediates enable C−H bond activation, aromatic ring cleavage, heteroatom oxygenation, oxidative coupling, and selective bond fragmentation under mild conditions.[12−29] Traditionally, their generation has been understood mainly through a metal-centered framework: the metal ion binds and activates oxygen, while the surrounding protein matrix tunes redox potential, proton delivery, substrate access, and intermediate stability.[2−5,29−39]

Increasing mechanistic evidence, however, indicates that substrates can actively participate in O_2 or H_2O_2 activation. In many oxidative metalloenzymes, the substrate is not simply positioned near a preformed oxidant for subsequent oxidation; instead, it can directly contribute to the formation and evolution of reactive oxygen intermediates.[40−57] In H_2O_2-dependent systems, substrates bearing carboxylate,[12,40,41] phenolic,[42] hydroxyl,[43] or nucleobase[44] functionalities can organize proton-transfer networks, promote O−O bond cleavage, and functionally replace or complement canonical acid−base residues. In O_2-activating heme and non-heme systems, substrates or cosubstrates can provide reducing equivalents that drive early metal−O_2 adducts toward catalytically competent superoxo, peroxo, ferryl, or radical-coupled intermediates.[45,57−59] These observations point to a substrate-engaged mode of metalloenzyme oxidation chemistry. Here, "substrate-engaged" refers to the cases in which the substrate or a substrate-derived intermediate directly assists oxygen activation through proton transfer, electron input, geometric control, or productive oxidant coupling. In this view, the metal center remains the primary platform for oxygen binding and activation, but the substrate can serve as a proton

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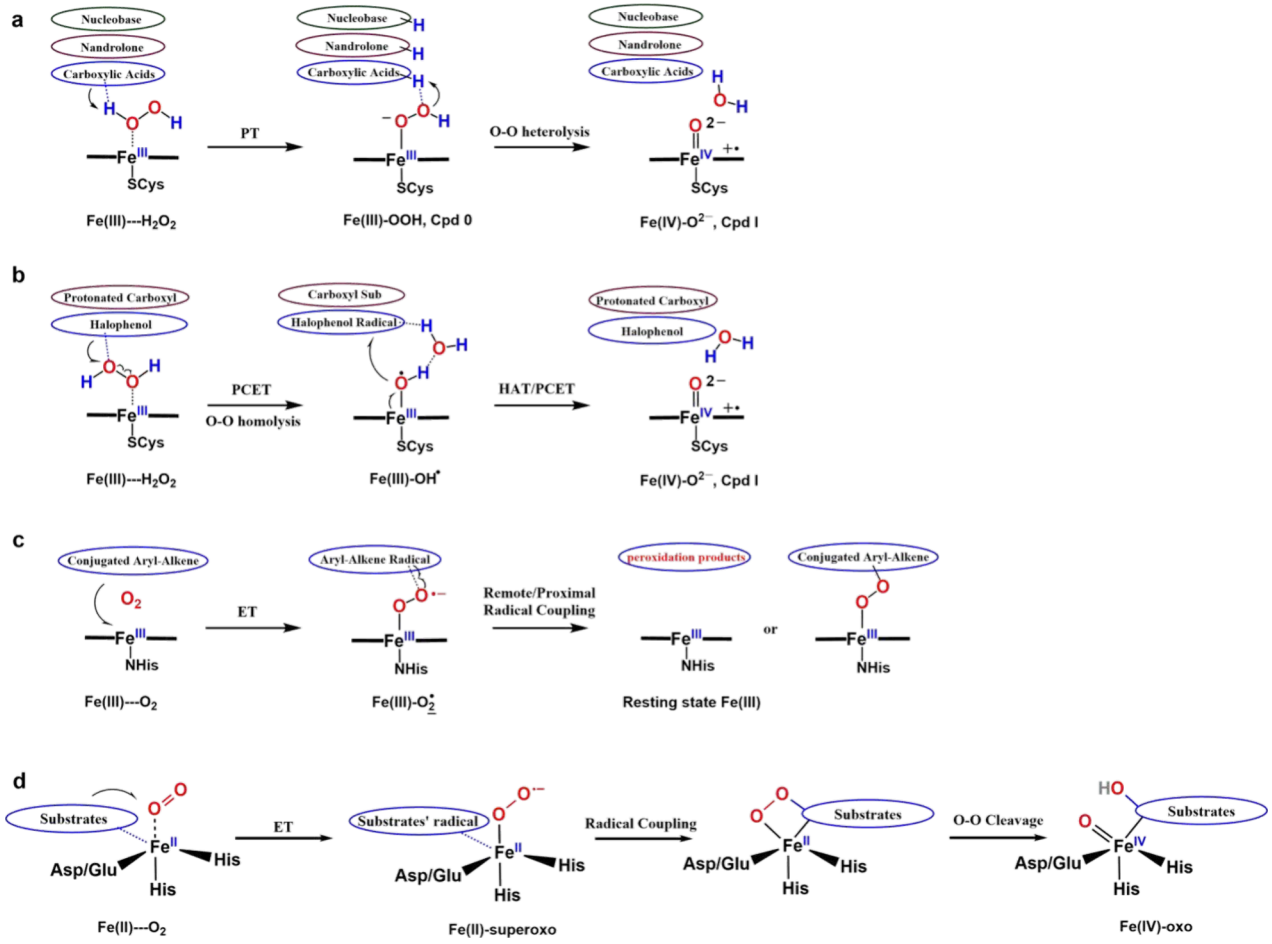


Figure 1. Substrate-engaged formation of reactive oxygen intermediates in metalloenzymes. (a) The substrate replaces catalytic residues in acid−base catalysis and promotes heterolytic H_2O_2 cleavage, leading to the formation of a Compound I-type heme Fe(IV)=O^{2-} oxidant. (b) The substrate replaces catalytic residues in acid−base catalysis and promotes homolytic H_2O_2 cleavage, leading to the formation of a ferryl heme Fe(IV)=O^{2-} oxidant together with a radical intermediate. (c) The substrate serves as an electron donor to reduce the heme Fe(III) center to Fe(II), thereby initiating O_2 activation for subsequent substrate oxidation. (d) In non-heme iron enzymes, substrate-to-O_2 electron transfer activates O_2 and concomitantly generates an activated substrate intermediate, thereby facilitating downstream oxidation and formation of the reactive Fe(IV)=O species.

relay, electron source, geometric director or radical partner. Therefore, the generation and fate of reactive oxygen intermediates are not purely mediated by the metal active center but can be dynamically coupled with the interactions of the protein environment and substrate.

In this Perspective, we summarize recent mechanistic insights into substrate participation in the generation of reactive oxygen intermediates in metalloenzymes. We focus on two major substrate roles: replacing catalytic residues in acid−base-assisted peroxide activation (Figure 1a,b),[40−44] and replacing external reductants or acting as cosubstrate-coupled electron donors in O_2 activation and the reactive oxygen intermediate formation (Figure 1c,d).[5,45,47−53] Together, these examples support a substrate-engaged framework in which substrates govern not only whether reactive oxygen intermediates are formed, but also how O_2 or H_2O_2 activation is initiated, coupled, and directed toward productive oxidant formation.

2. SUBSTRATES REPLACE CATALYTIC RESIDUES IN ACID−BASE-ASSISTED PEROXIDE ACTIVATION

In the P450 peroxygenase mode, H_2O_2 is used directly as the sole oxygen and electron source.[60−66] This pathway bypasses the canonical O_2 activation cycle. It also avoids the requirement of external electron-transfer partners.[67,68] In a general mechanism, H_2O_2 first binds to the ferric heme center to form an Fe(III)−H_2O_2 complex. Proton transfer then generates the ferric hydroperoxo species, Fe(III)−OOH^-, known as Compound 0 (Cpd 0). Subsequent O−O bond cleavage yields Compound I (Cpd I), the high-valent Fe(IV)=O^{2-} porphyrin π-cation radical. In classical heme peroxidases, this step is controlled by distal acid−base residues.[69−72] A His/Arg pair polarizes the peroxide bond and mediates proton transfer. However, many CYP152 family members and peroxygenase-like P450s lack such a built-in distal acid−base site.[40−42,73] As a result, productive H_2O_2 activation must rely on other catalytic elements. The substrate often provides this missing functionality.

This principle is best established in CYP152 peroxygenases. $P450_{SP\alpha}$,[74] $P450_{BS\beta}$,[75] $P450_{Cla}$[76,77] and $OleT_{JE}$[78] all use fatty acid substrates. In these systems, the substrate carboxylate is

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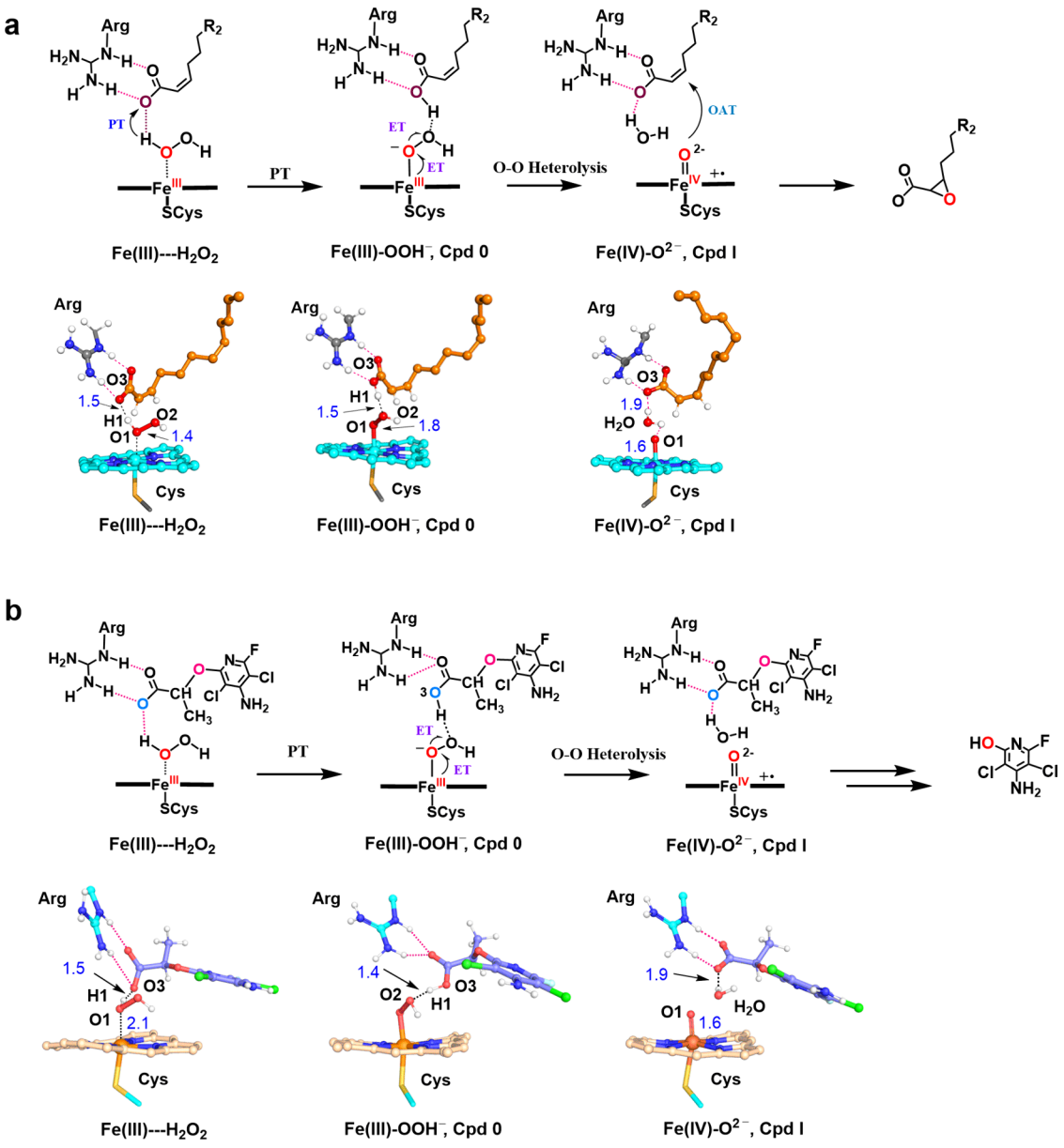


Figure 2. Carboxylate-containing substrate-mediated H_2O_2 heterolysis for Cpd I formation. (a) Unsaturated fatty acid-mediated H_2O_2 heterolysis. (b) Phenoxy carboxylic acid-mediated H_2O_2 heterolysis.

not only a binding handle, but also directly participates in peroxide activation. The carboxylate group forms a conserved interaction with an active-site Arg residue. This interaction positions H_2O_2 above the heme-iron. It also creates a substrate-derived acid−base module. Mechanistically, the deprotonated carboxylate accepts a proton from bound H_2O_2. This step promotes Cpd 0 formation (Figure 2). The protonated carboxyl group then hydrogen-bonds to Cpd 0. This interaction polarizes the O−O bond and favors heterolytic cleavage. Cpd I is then generated (Figure 2).[40,41] Thus, the native fatty acid substrate acts as a reversible proton buffer. It also serves as a proton-relay node. Productive peroxide activation is therefore achieved by the combined action of heme, H_2O_2, the conserved Arg residue and the substrate's carboxylate. This classical mechanism provides the basis for understanding non-native carboxylate-containing substrates. Phenoxy carboxylic acids are representative examples (Figure 2b).[41] These substrates are not native fatty acids. However, their carboxylate groups can still interact with the conserved Arg residue. They can also build a similar proton-transfer network for H_2O_2 activation.

More recently, this concept has been extended to substrates lacking a carboxylate group. Nucleobase-driven H_2O_2 activation by the P450 enzyme PtmB provides a representative example.[44] The substrate does not contain a carboxylate group. Nevertheless, PtmB is still able to organize a functionally equivalent proton-transfer network through nucleobase groups and bridging water molecules. A molecular-dynamics-derived binding model shows that the 6-NH_2 and N1 sites of the nucleobase form stable hydrogen bonds with Asp299 and Lys402. These interactions not only help position the substrate, but also modulate the local electronic environment. In contrast, the N9′ position directly participates in proton transfer. Through a bridging water molecule, N9′ forms a continuous proton-relay pathway with H_2O_2 (RC, Figure 3). QM/MM calculations further clarify this process. A proton is

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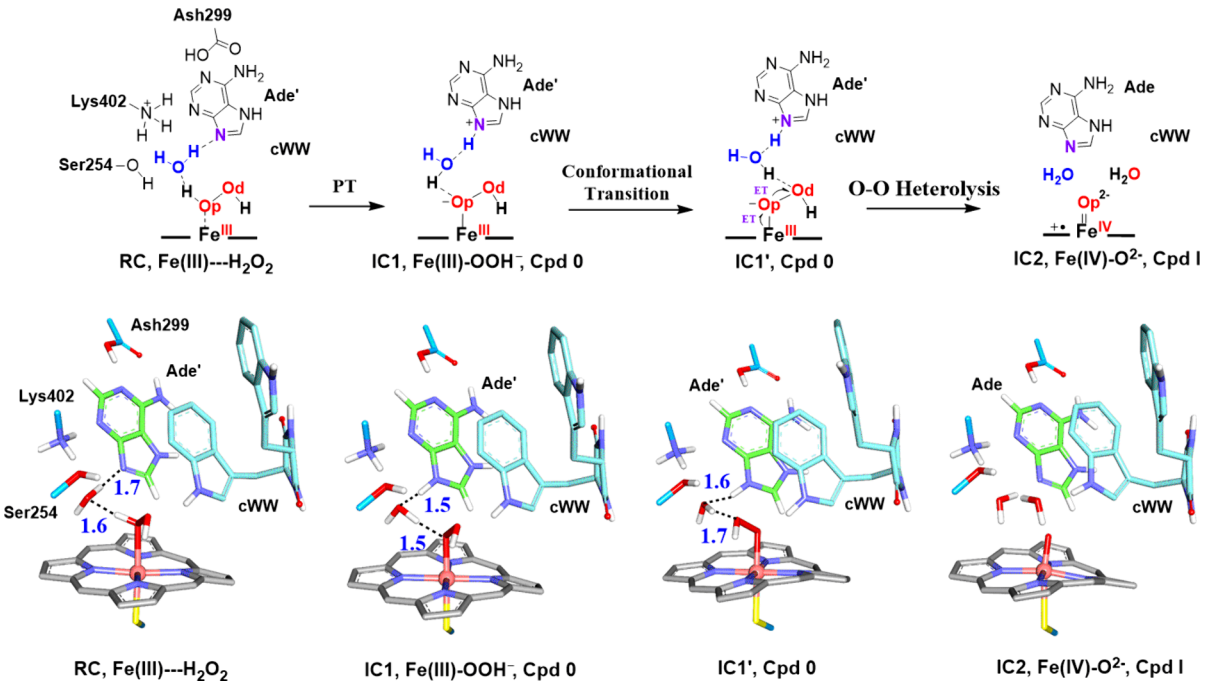


Figure 3. Nucleobase-driven H_2O_2 activation and heterolytic cleavage to generate the reactive oxygen intermediate Cpd I. Ade denotes adenine, and cWW denotes cyclo-L-Trp-L-Trp. Op denotes the oxygen atom of H_2O_2 proximal to the heme iron, whereas Od denotes the distal oxygen atom.

first transferred from H_2O_2 to the substrate nucleobase through the bridging water molecule. This step generates Cpd 0 and protonates the nucleobase site (IC1, Figure 3). The proton then migrates back within the substrate-water-peroxide network. This reverse proton migration is coupled with O−O bond polarization. As a result, heterolytic O−O cleavage occurs and Cpd I is formed (IC2, Figure 3). *Thus, even without a carboxylate, the substrate can still serve as a proton-buffering node, functionally replacing the canonical protein-based acid−base catalyst.*

The PtmB study further shows that active-site binding alone is insufficient for substrate-assisted H_2O_2 activation. Productive activation requires the bound substrate to complete a properly organized proton transfer relay. Replacement of the adenine 6-NH_2 donor by the carbonyl acceptor in guanine or hypoxanthine reverses the local hydrogen-bonding pattern, disrupts the water-mediated connection to H_2O_2, and prevents formation of a peroxide-cleavage-competent conformation. Consistently, QM/MM scans showed uphill proton transfer without a stable intermediate; purine analogues lacking the 6-NH_2 group were inactive, whereas 6-NH_2-substituted analogues restored activity.[79] Thus, substrate-assisted peroxide activation depends on the integrated match of hydrogen-bond anchoring, proton affinity, and relay geometry. When this network is absent or misaligned, the bound substrate remains catalytically inactive, and Cpd I formation is inefficient.

A related principle is observed in the CYP109B4 T245E variant (Figure 4a).[43] In this system, the hydroxyl group of the nandrolone substrate, together with a bridging water molecule, forms a hydrogen-bonded relay connecting the proximal proton of H_2O_2 to Glu245. This relay promotes proton transfer from the bound peroxide to Glu245, thereby facilitating Compound 0 (Cpd 0) formation. The resulting protonated Glu245 then hydrogen-bonds to the distal hydroperoxo group of Cpd 0, preorganizing the intermediate for productive O−O heterolysis and *in situ* generation of Cpd I and H_2O. Consistent with this mechanism, calculations show that O−O bond cleavage proceeds with a lower barrier in the T245E variant than in WT (17.5 versus 19.6 kcal mol^{-1}; Figure 4). *These results indicate that productive H_2O_2 activation can be promoted when the substrate, active-site residues, and water molecules cooperatively assemble an efficient proton-transfer network.*

Studies on halophenol hydroxylation and dehalogenation by P450 peroxygenases further expand the scope of substrate-assisted acid−base catalysis.[42] Although these substrates contain neither a carboxylate nor an obvious N−H functionality, they can still promote CYP152-mediated H_2O_2 activation. The key is that the phenolic hydroxyl group, when flanked by strong electron-withdrawing substituents such as Cl or F, exhibits a markedly lowered pK_a and thus acquires a carboxylate-like acidic character. Structural analysis combined with QM/MM calculations showed that the substrate hydroxyl group forms a stable hydrogen-bonding interaction with the conserved Arg242, thereby anchoring the substrate and positioning H_2O_2 on the distal face of the heme (RC, Figure 5). Through proton-coupled electron transfer (PCET), this organized configuration promotes homolytic O−O bond cleavage of H_2O_2, initially generating Fe(III)−$OH^{\bullet}$ and H_2O. The Fe(III)−$OH^{\bullet}$ species then undergoes spontaneous electron transfer to form Fe(IV)−OH^-. The newly formed H_2O further facilitates hydrogen atom transfer (HAT) from the Fe(IV)−OH^- moiety to the substrate radical center, converting this intermediate into the reactive oxygen intermediate Cpd I. In the later stages, bridging or solvent-derived water participates in water cleavage and hydroxylation steps, such that the newly incorporated hydroxyl oxygen ultimately originates from water rather than H_2O_2, thereby bypassing the classical oxygen rebound pathway (Figure 5). Further experimental and computational analyses showed that

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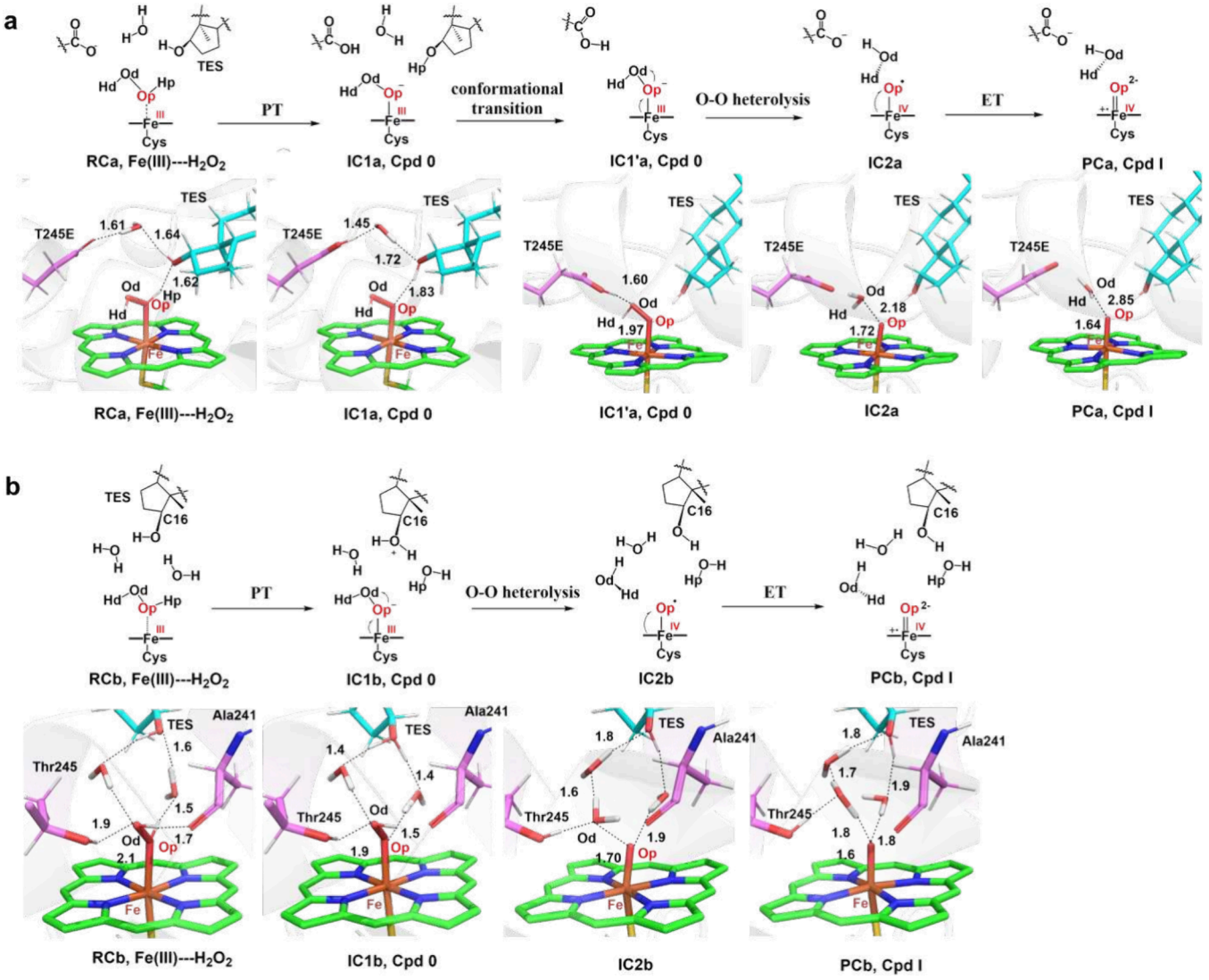


Figure 4. Comparison of substrate- and enzyme environment-driven H_2O_2 activation and heterolytic O−O cleavage for Cpd I formation in CYP109B4 T245E variant (a) and WT (b). TES denotes the testosterone substrate. Op denotes the oxygen atom of H_2O_2 proximal to the heme iron, whereas Od denotes the distal oxygen atom.

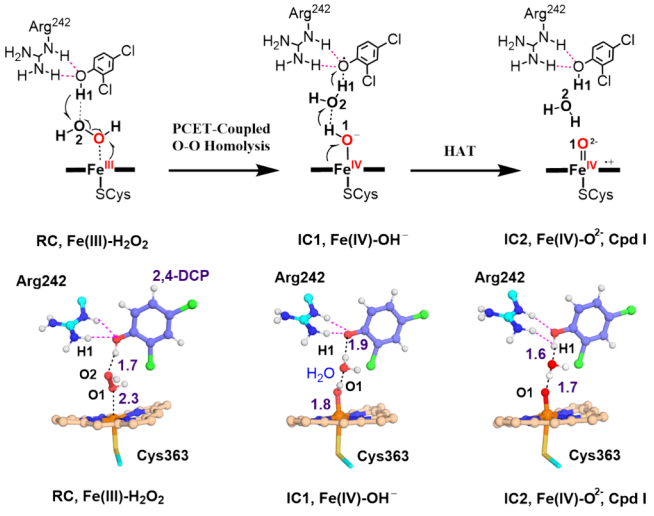


Figure 5. Halophenol-mediated homolytic cleavage of H_2O_2 to generate Cpd I in P450 peroxygenases. 2,4-DCP denotes the 2,4-dichlorophenol substrate.

less acidic substrates are markedly less effective at promoting Cpd I formation, whereas the deprotonated phenoxide form is not itself a productive reacting species. *These findings indicate that the decisive factor in H_2O_2 activation is not simply the presence of a phenolic hydroxyl group but rather the concerted match among substrate acidity, acid−base pairing with Arg242, and PCET competence.*

The distinctive feature of this mechanism is that the substrate-mimicking acid−base catalysis mediates homolytic, rather than canonical heterolytic H_2O_2 cleavage. This process is also mechanistically distinct from Fenton-type H_2O_2 activation in heme iron or copper enzymes, where electron transfer drives the generation of hydroxyl-radical-like intermediates.[57,80−82] In typical Fenton-type reactions, the substrate usually participates mainly through non-covalent positioning or subsequent radical capture, rather than directly engaging in a coupled proton−electron transfer event that controls O−O bond cleavage.[57] Thus, halophenol-dependent P450 peroxygenase catalysis represents a unique substrate-assisted PCET mode, in which substrate acidity, hydrogen-

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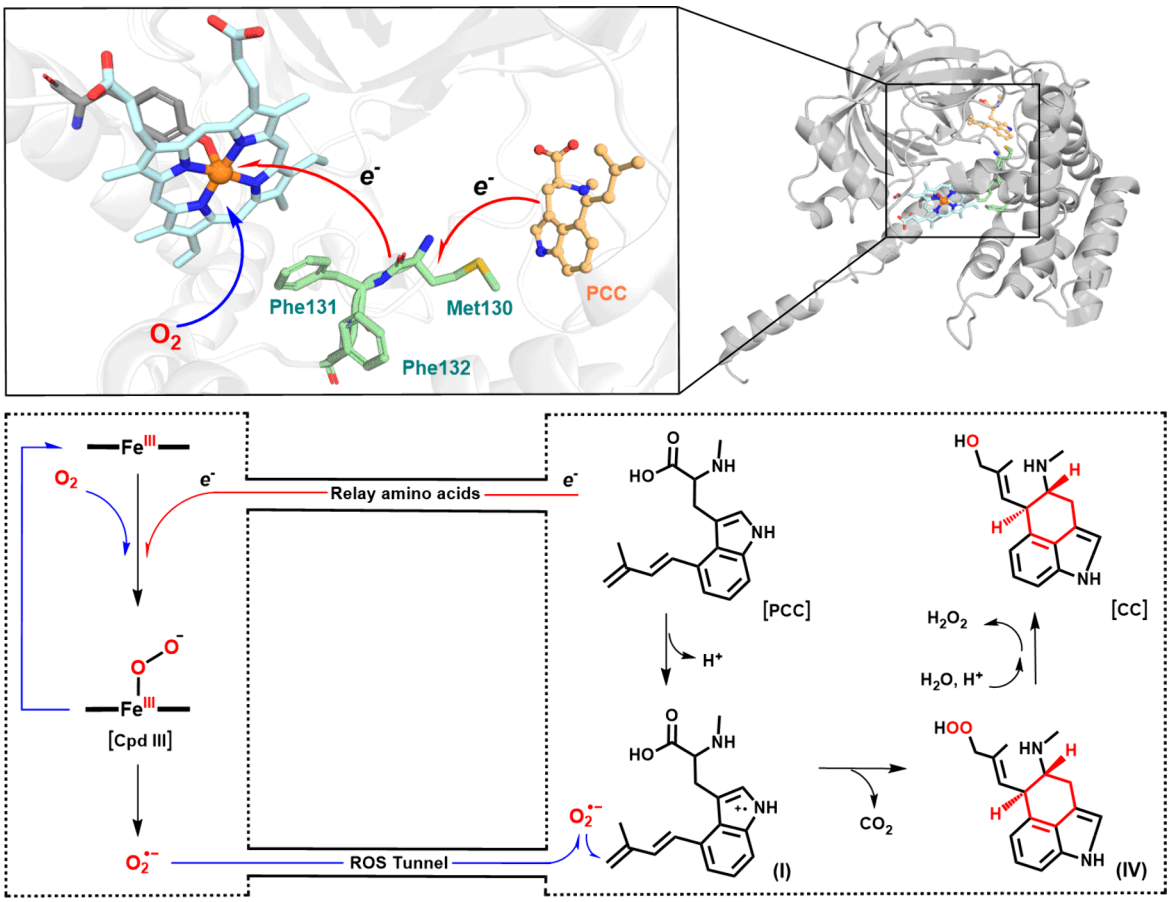


Figure 6. Long-range electron transfer from the substrate to heme Fe(III) in EasC activates O_2 to generate free superoxide, enabling remote substrate oxidation. PCC denotes prechanoclavine substrate, CC denotes chanoclavine, and ROS denotes reactive oxygen species.

bond organization, and radical formation are directly coupled to reactive oxygen intermediate generation.[42]

Taken together, these examples establish a stepwise expansion of substrate-assisted acid−base catalysis in P450 peroxygenases. Native CYP152 fatty acid oxidation provides a classical model. In this model, the substrate carboxylate assists H_2O_2 activation through an Arg-linked proton-relay network. Non-native carboxylate-containing substrates, such as phenoxy carboxylic acids, extend this model beyond fatty acids. These results clearly show that the fatty acid scaffold can be replaced, as long as a properly positioned acidic group is retained. More diverse non-carboxylate substrates further broaden this logic. Nucleobases, alcohol-containing substrates, and acidic halophenols can all support peroxide activation through alternative proton donors, bridging waters, or PCET-active hydroxyl groups. Therefore, the substrate-driving capacity of P450 peroxygenases is determined by whether the substrate can provide or organize transferable protons, directional hydrogen-bonding interactions, and dynamic acid−base relays. These elements channel Fe(III)−H_2O_2 toward productive Cpd 0 and Cpd I formation. In this way, substrates can functionally replace or complement missing distal catalytic residues. They can also directly participate in reactive oxygen intermediate generation.

The H_2O_2-dependent systems discussed above illustrate substrate engagement through acid−base and proton-transfer control, enabling productive O−O cleavage and Cpd I or ferryl formation. In contrast, O_2-dependent systems require stepwise reduction of early metal−O_2 adducts, allowing substrates or substrate-derived intermediates to participate through electron input, radical coupling, and coordination or geometric control. The following section therefore examines this complementary mode of substrate-coupled O_2 activation.

3. SUBSTRATES FUNCTION AS ELECTRON DONORS AND CONFORMATIONAL MODULATORS IN OXYGEN ACTIVATION

In most iron- and copper-dependent oxidative enzymes, O_2 activation is initiated by externally supplied electrons.[36,83−85] These reducing equivalents are commonly delivered by reductase partners,[30,39,86−88] such as redox proteins[36,39,89] or small-molecule electron donors,[37,83,88] thereby generating or maintaining a low-valent metal state competent for O_2 binding and further conversion into reactive metal−oxygen intermediates. In the classical view, the substrate mainly acts as the oxidation target after the reactive oxidant is generated.

However, increasing evidence indicates that substrates are not always passive recipients of oxidation. In some non-canonical metalloenzymes, the substrate or a closely coupled cosubstrate directly participates in the formation, evolution, or productive utilization of reactive oxygen intermediates.[47,53,54,59] This participation occurs through two mechanistically distinct but often interconnected modes. First, the substrate can function as an intrinsic electron donor, supplying reducing equivalents to the metal−O_2 unit and thereby promoting the formation of superoxo, peroxo, hydroperoxo, ferryl, or radical-coupled intermediates. Second, the substrate

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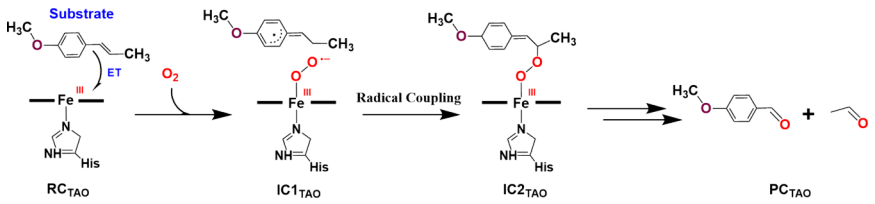


Figure 7. Proposed catalytic sequence for *trans*-anethole substrate-mediated O_2 activation in TAO.

can act as a conformational or coordination modulator, reshaping the metal coordination sphere or organizing the reactive geometry required for productive oxygen transfer or C−H activation.

Substrate-engaged electron transfer should therefore be viewed as a continuum rather than a single elementary step. At one end, redox-active substrates or substrate-derived intermediates can couple directly to the metal−O_2 unit through inner-sphere coordination, π-conjugation, or orbital overlap. At the other end, substrates may influence O_2 activation more indirectly by reorganizing active-site geometry, tuning local electrostatics, stabilizing radical character, or positioning proton/electron-transfer pathways. Key factors include substrate redox potential, metal−substrate electronic coupling, electron-transfer distance and pathway, radical stabilization, proton-coupled steps, and the lifetime of early metal−O_2 adducts. Thus, in substrate-engaged oxygen activation, the key question is not merely whether O_2 binds to the metal center, but how substrate-derived electronic input and substrate-engaged structural rearrangement are coupled with proton transfer and radical generation to yield a chemically competent oxidant.

Substrate-engaged O_2 activation does not always require direct metal coordination. Even when substrates lack obvious metal-binding determinants, their binding can reorganize the active site, exclude solvent, reposition proton/electron-transfer pathways, and reshape the local electrostatic environment around the metal−O_2 unit. These effects can tune the effective redox properties of the metal site, stabilize early dioxygen adducts, and direct them toward productive reduction rather than O_2 dissociation or off-pathway oxidant release.

Heme Systems: Substrate-Coupled Electron Input into O_2 Activation

Chanoclavine synthase (EasC) has been discussed as a possible example of this mode.[47] Initial studies showed that EasC belongs to the catalase-like heme enzyme family, which uses O_2 rather than H_2O_2 as the oxidant to catalyze the oxidative cyclization of the prechanoclavine precursor. The reaction requires NADPH as a source of the reducing equivalents. However, it does not rely on a classical P450-type external protein reductant system.[38] Subsequent work proposed a distinctive superoxide mechanism for EasC. In this model, the prechanoclavine substrate (PCC) does not bind directly above the heme. Instead, it occupies the NADPH-binding pocket and is proposed to communicate electronically with the heme pocket through key relay residues, including Met130, Phe131, and Phe132 (Figure 6). Such long-range electronic coupling could facilitate O_2 reduction at the heme iron and promote formation of a Cpd III intermediate. The resulting superoxide may be generated upon Cpd III decay. It is not confined to the heme pocket. Rather, it has been proposed to migrate through a narrow tunnel connecting the heme pocket to the substrate pocket. It eventually reaches the NADPH binding pocket, where it could contribute to substrate-centered radical oxidation and cyclization at a site remote from the metal center.

Thus, EasC may not simply be viewed as a conventional external electron-delivery platform. It is suggested to act as a spatiotemporal organizer that couples substrate positioning, heme-side O_2 activation, possible ROS shuttling, and the orbital reorganization required for cyclization. The conjugated π system of PCC substrate may provide an electronic framework that supports interaction with the heme/O_2 activation machinery. The resulting $O_2^{\bullet-}$ species can then become electronically coupled to the electron-rich substrate scaffold. Meanwhile, the prechanoclavine scaffold can plausibly stabilize radical character and promote the C−C/C−O bond reorganization (Figure 6). Accordingly, in EasC, substrate oxidation, superoxide formation, and cyclization should not be viewed as independent elementary processes. They form a coupled catalytic manifold jointly governed by substrate electronic structure and enzyme-imposed spatial organization.

Another relatedcomparison is provided by the heme-containing trans-anethole oxygenase from *Stenotrophomonas maltophilia* (TAO_Sm). Biochemical and structural studies have established that TAO_Sm contains a tightly bound b-type heme and catalyzes oxidative C=C bond cleavage of *trans*-anethole and related propenyl aromatics using O_2 as the only required oxidant, without external cofactor regeneration. This O_2-only reactivity is consistent with an internally redox-balanced coupling between substrate oxidation and O_2 activation, although the detailed sequence of electron transfer and oxygen−substrate coupling remains to be elucidated.[90] Within the substrate-engaged framework, one plausible working model is that the conjugated π system of the bound substrate may contribute electron density to the heme−O_2 unit, thereby promoting formation of an Fe(III)−$O_2^{\bullet-}$-like intermediate together with a substrate radical species. Our QM calculations suggest the feasibility of such a substrate-coupled pathway, in which O_2 activation, substrate radical formation, and subsequent oxygen addition and C=C bond cleavage are electronically linked (Figure 7). Nevertheless, direct substrate-to-heme−O_2 electron transfer in TAO has not yet been experimentally validated. Therefore, TAO is best regarded here as a promising but still mechanistically unresolved example of O_2-only heme-dependent oxygenation chemistry, rather than as a fully established case of direct substrate-driven heme−O_2 activation.

Non-heme Iron Enzymes: Substrate-Coupled Electron Redistribution and Coordination Control

This substrate-engaged logic is not limited to heme enzymes, but extends broadly to mononuclear non-heme Fe(II) enzymes. In these systems, O_2 binding to Fe(II) usually represents only the initial stage of oxygen activation. The subsequent evolution of the Fe−O_2 adduct depends on where the next reducing equivalent comes from and how this electron

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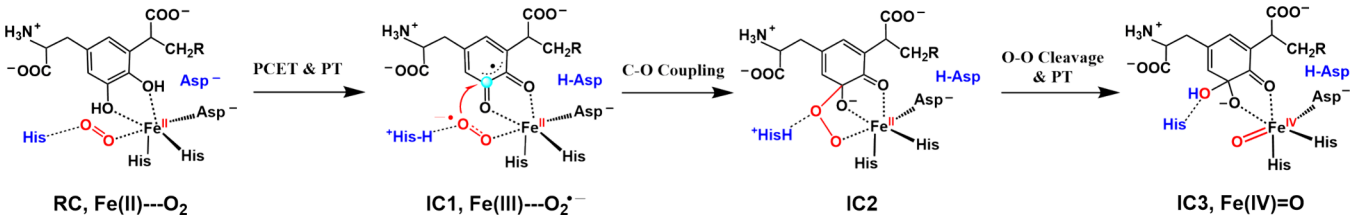


Figure 8. Substrate-radical formation coupled to O_2 reduction in PqqB. The substrate undergoes proton-coupled electron transfer to reduce O_2, generating a substrate radical that drives downstream oxidative transformation.

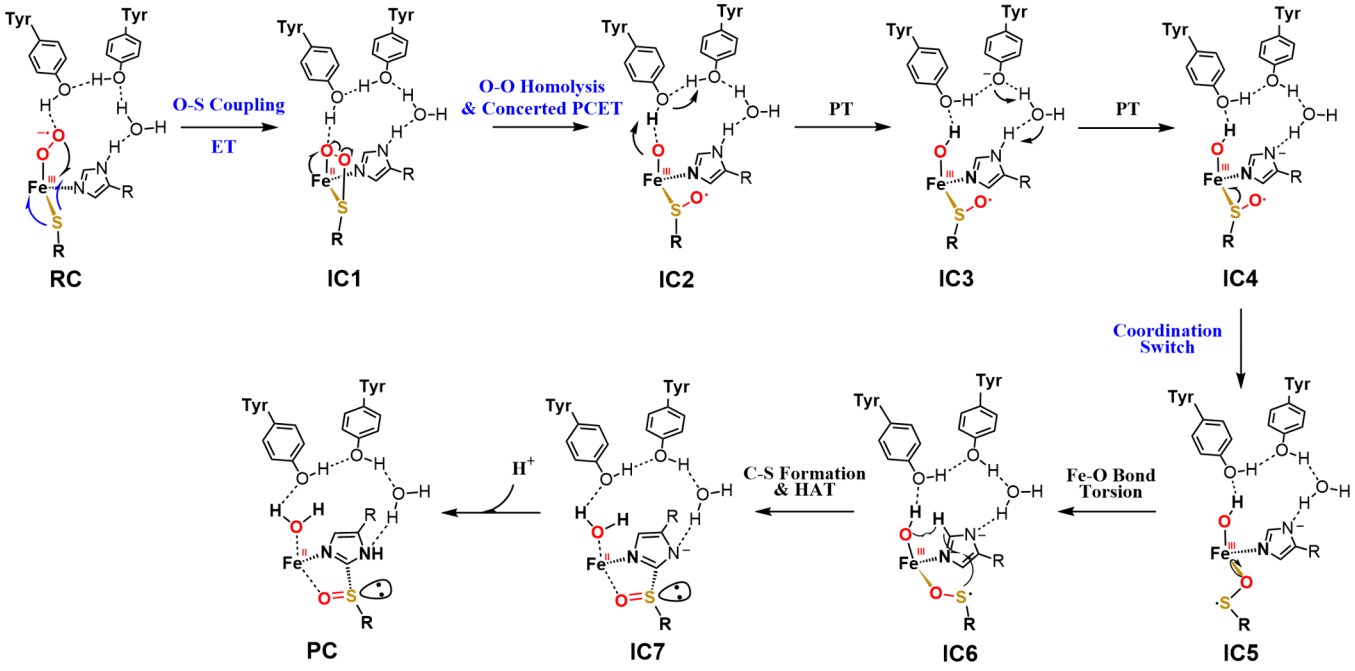


Figure 9. Proposed reaction mechanism of EgtB. The substrate undergoes oxidation while reducing Fe(III) through electron transfer, followed by C−S bond formation via intramolecular coordination replacement.

input is coupled to substrate oxidation, proton transfer, bond formation, or coordination reorganization. Compared with heme systems, non-heme iron enzymes often display more direct coupling between substrate coordination, Fe−O_2 electronic structure, and active-site geometry. As a result, the substrate can influence not only the redox level of the oxygen adduct, but also whether the resulting intermediate evolves toward oxygen insertion, C−S bond formation, oxidative cleavage, or C−H activation. This feature is evident in aromatic ring-cleaving enzymes, such as extradiol catechol dioxygenases and 3-hydroxyanthranilate-3,4-dioxygenase, as well as in sulfur-activating enzymes such as cysteine dioxygenase and EgtB. In many of these systems, the early Fe−O_2 adduct is best described as a superoxo-level intermediate. Without additional electron and/or proton coupling from the substrate or nearby catalytic groups, this species may not evolve along a productive oxidative pathway. Thus, for non-heme Fe(II) enzymes, the key mechanistic issue is not simply O_2 binding, but how the Fe−O_2 unit is further reduced, protonated, reorganized, or electronically coupled to the substrate to generate peroxo, hydroperoxo, ferryl, or radical-coupled reactive oxygen intermediates.

The non-heme Fe-dependent hydroxylase PqqB provides a representative example of this principle.[59] PqqB catalyzes two consecutive oxygen-insertion steps during pyrroloquinoline quinone biosynthesis. After the first hydroxylation, the Glu-dihydroxyphenylalanine (Glu-DOPA) intermediate binds the Fe(II) center through a bidentate catecholate-like coordination mode. This coordination is not merely a substrate-anchoring interaction. Instead, it establishes an electronic pathway that connects the electron-rich catecholate moiety, the iron d orbitals, and the bound O_2 molecule. QM/MM calculations indicate that electron density can be transferred from the substrate intermediate to Fe-bound O_2 through the iron center, generating a substrate radical−Fe(II)−superoxo species (Figure 8). Thus, O_2 activation and substrate radical formation occur as a coupled event. PqqB therefore expands the substrate-derived electron-donation paradigm from heme-dependent oxidation to non-heme Fe-mediated aromatic hydroxylation.

Sulfur-activating non-heme Fe(II) enzymes, such as cysteine dioxygenase (CDO)[91−94] and sulfoxide synthases[95−97] follow the same substrate-coupled principle. In this class of enzymes, O_2 activation is directly coupled to the sulfur center of the bound substrate. The substrate sulfur interacts with the activated Fe−O_2 fragment and donates electron density to the metal−oxygen unit, thereby promoting the evolution of an early superoxo-level species into more reactive sulfur−oxygen intermediates.

Thus, the substrate sulfur is not merely oxidized by a preformed metal−oxygen oxidant. Instead, it participates electronically in the generation and evolution of the reactive

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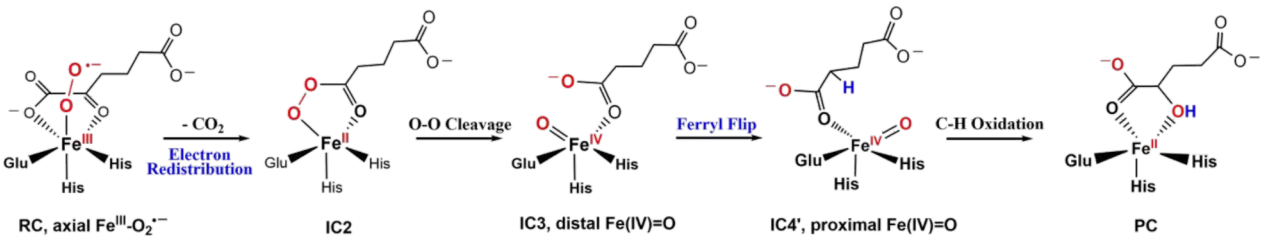


Figure 10. Proposed reaction mechanism of HglS. The substrate undergoes decarboxylation and couples with superoxide, followed by O−O bond cleavage that drives ferryl reactive oxygen intermediate formation.

oxygen intermediate. This principle is particularly evident in EgtB, where sulfur oxidation is further coupled to productive C−S bond formation rather than simple sulfur dioxygenation.[53] This distinguishes EgtB from canonical cysteine/thiol dioxygenases, in which sulfur-assisted O_2 activation typically proceeds toward direct sulfur oxygenation as the terminal outcome. In EgtB, the sulfur-containing substrate similarly participates in O_2 activation by coupling the coordinated thiolate to the Fe−O_2 unit, but the resulting sulfur-oxygenated intermediate is not simply trapped as a sulfoxide- or sulfinate-type product. Instead, O/S coordination exchange at the iron center redirects the reactive sulfur intermediate toward coupling with the imidazole-containing co-substrate, thereby enabling C−S bond formation. The surrounding active-site residues further promote this selectivity by properly positioning the two substrates, stabilizing the sulfur−oxygen intermediate, and disfavoring unproductive sulfur dioxygenation.[53]

In EgtB, the Fe(II) center binds a thiolate-containing substrate together with a histidine-derived coupling partner, assembling a substrate−Fe−O_2 ternary platform after O_2 binding. Electron density from the coordinated thiolate is coupled into the Fe−O_2 unit, allowing the early superoxo-level species to evolve toward sulfur−oxygen radical or sulfoxide-like intermediates (Figure 9).[53] Reactive oxygen intermediate generation and sulfur oxidation therefore occur as a coupled process. The resulting oxidized sulfur intermediate is then directed toward C−S bond formation with the imidazole substrate. In this sense, EgtB does not use a fully preformed metal−oxygen oxidant to oxidize a passive substrate. Instead, the coordinated sulfur substrate helps generate an Fe−O_2-derived reactive intermediate, which oxidizes the sulfur center and then channels the oxidized substrate intermediate toward productive C−S bond construction.

Hydroxyglutarate synthase (HglS) provides a complementary example in which substrate-derived electron input into O_2 activation is coupled to iron-center coordination dynamics. HglS is a mononuclear non-heme Fe(II) oxygenase that catalyzes the conversion of 2-oxoadipate and O_2 to D-2-hydroxyglutarate and CO_2. QM/MM calculations indicate that the reaction is initiated by attack of an Fe(III)−superoxo species on 2-oxoadipate, generating an Fe(II)−O_2−substrate adduct. This step reflects substrate-derived electron redistribution within the Fe−O_2−substrate adduct, rather than a simple direct substrate-to-O_2 or outer-sphere electron-transfer event. Subsequent CO_2 release further channels substrate-derived reducing equivalents into the oxygen adduct, promoting O−O bond cleavage and formation of an Fe(IV)=O species (Figure 10).[54] Thus, in HglS, the substrate does not merely undergo oxidation by a preformed ferryl oxidant; instead, its oxidative decarboxylation is directly coupled to reactive oxygen intermediate generation.

Besides, the initially formed ferryl species adopts a distal coordination mode that is not ideally positioned for C−H activation. A subsequent coordination switch converts this distal Fe(IV)=O intermediate into a proximal ferryl conformation, placing the oxo group in a productive geometry for hydrogen abstraction (Figure 10).[54] HglS therefore expands the substrate-engaged framework by linking substrate-derived electron transfer, oxidative decarboxylation, O−O bond cleavage, and coordination remodeling within a single catalytic sequence. Productive oxidant formation is not defined solely by the appearance of Fe(IV)=O, but also by whether this ferryl intermediate is reorganized into a chemically competent geometry. Thus, HglS illustrates how substrate-derived electron redistribution within the Fe−O_2−substrate adduct and dynamic remodeling of the iron coordination sphere jointly determine the formation and reactivity of a non-heme ferryl oxidant.

Taken together, these examples show that substrates can serve as intrinsic electronic contributors during O_2 activation. Rather than waiting for a preformed oxidant, the substrate can help reduce or evolve the metal−O_2 unit while simultaneously participating in its own oxidative transformation. In PqqB, a bidentate catecholate-like Glu-DOPA intermediate transfers an electron to Fe-bound O_2 through the non-heme Fe center, forming a substrate radical−Fe(II)−superoxide species.[59] In EgtB, coupling between substrate-derived sulfur and the Fe−O_2 unit controls reactive oxygen intermediate generation and directs the oxidized sulfur intermediate toward C−S bond formation.[53] In HglS, substrate-coupled oxidative decarboxylation generates the ferryl oxidant, whereas iron-center coordination dynamics determines whether this oxidant is competent for C−H activation.[54] Substrate-derived electron input can further be distinguished by redox stoichiometry. Two-electron regimes typically support partial O_2 reduction and formation of ferryl, peroxo, hydroperoxo, or radical-coupled intermediates, whereas four-electron regimes can drive deeper O_2 reduction associated with dioxygenation, oxidative cleavage, or sulfur dioxygenation. Thus, the number and timing of substrate-derived electrons help determine the fate of early metal−O_2 adducts. These systems collectively support a substrate-engaged framework in which substrates help generate, tune, and direct the reactive oxygen intermediates that define subsequent oxidative chemistry.

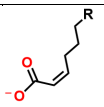
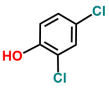
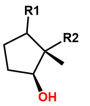
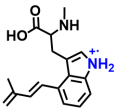
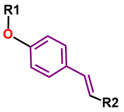
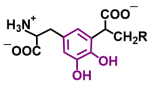
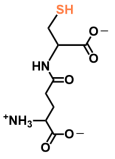
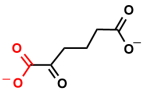
4. CONCLUSION AND OUTLOOK

Reactive oxygen intermediates in metalloenzymes have traditionally been interpreted within a metal-centered framework, in which the metal ion binds O_2 or H_2O_2 and the protein environment tunes redox potential, proton delivery, substrate

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Table 1. Substrate-Engaged Mechanisms and Design Principles for Reactive Oxygen Intermediate Generation[a]

Enzyme/ Catalytic center	Substrate 2D structure	Substrate engaged mode	Key reactive oxygen intermediate	Key descriptors	Design implications
CYP152 peroxygenases Cys-ligated heme Fe		Arg-linked proton relay; H_2O_2 polarization	Cpd 0, Cpd I	Carboxylate position; proton affinity; Arg-H_2O_2 geometry	Install acidic handles for peroxide heterolysis
CYP152 peroxygenases Cys-ligated heme Fe		PCET-coupled homolytic H_2O_2 cleavage	Fe(III)–OH•, Fe(IV)–OH⁻, Cpd I	Phenolic pK_a; EWG effect; Arg pairing; PCET ability	Use acidic phenols to enable peroxygenase reactivity
PtmB Cys-ligated heme Fe		Water-mediated proton relay	Cpd 0, Cpd I	6-NH_2 anchoring; N9′ relay; water connectivity	Screen for proton-relay topology
CYP109B4 T245E Cys-ligated heme Fe		Substrate–water–Glu245 proton-transfer network	Cpd 0, Cpd I	Substrate OH orientation; water bridge; O–O polarization	Engineer substrate–water–residue triads
EasC b-type heme Fe		Long-range ET; superoxide shuttling	Cpd III free $O_2^{\bullet-}$ ROS	π-Conjugation; ET pathway; ROS tunnel	Couple substrate electronics with spatial oxidant delivery
TAO_Sm b-type heme Fe		Putative substrate-coupled O_2 activation	Putative Fe(III)–$O_2^{\bullet-}$ substrate radical	π-Electron donation; radical stability; redox balance	Explore O_2-only substrate-coupled heme chemistry
PqqB non-heme Fe(II)		Catecholate-to-Fe–O_2 electronic coupling	Substrate radical–Fe(II)–superoxo, ferryl	Bidentate coordination; catecholate redox ability	Use redox-active metal-binding motifs
CDO / EgtB/sulfoxide synthases non-heme Fe(II)		S-to-Fe–O_2 coupling	Superoxo-level and sulfur–oxygen intermediates	Thiolate coordination; S–Fe–O_2 coupling	Redirect sulfur oxidation to bond construction
HglS non-heme Fe(II)		Decarboxylation-coupled Fe–O_2 evolution	Fe(III)–superoxo, Fe(IV)=O	Electron redistribution; ferryl orientation; coordination switch	Control ferryl formation and geometry

[a]The colored moieties in the "Substrate 2D structure" column represent the functional groups directly involved in the catalytic reaction.

access, and intermediate stability. The examples discussed in this Perspective support a complementary substrate-engaged view (Table 1). In this view, the substrate is not merely an oxidation target positioned near a preformed oxidant. Instead, it can contribute to reactive oxygen intermediate generation by donating or organizing protons, supplying electron density, shaping metal−oxygen geometry, and coupling radical or peroxide intermediates to productive chemical steps. Thus, substrate recognition, oxygen activation, and substrate oxidation should often be considered as coupled events within a single catalytic manifold.

Here, two mechanistic themes emerge. In H_2O_2-dependent heme enzymes, substrates can replace or complement distal acid−base residues by establishing proton-transfer or PCET networks that promote productive O−O bond cleavage and ferryl/Compound I formation. This function is not restricted to a single chemical motif, such as a carboxylate, but can arise from any substrate feature capable of providing appropriate acidity, hydrogen-bond directionality, and proton-transfer connectivity. In O_2-activating heme and non-heme iron enzymes, substrates or substrate-derived intermediates can provide electron density to metal−O_2 adducts, generate radical-coupled species, and reorganize metal−oxygen intermediates into catalytically competent geometries. These cases show that substrates can help determine not only which reactive oxygen intermediate is formed but also whether that intermediate is productively coupled to bond formation, bond cleavage, or C−H activation.

Looking forward, a central challenge is to define substrate-driven oxygen activation with greater mechanistic precision. Many substrate-coupled oxygen intermediates are short-lived, electronically delocalized, and involve simultaneous changes in metal oxidation state, O−O bonding, proton position, substrate radical character, and coordination geometry. Future studies should combine rapid freeze-quench spectroscopy, isotope labeling, EPR/ENDOR, Mössbauer spectroscopy, resonance Raman spectroscopy, time-resolved crystallography, and QM/MM or QM/MM molecular dynamics to distinguish whether a substrate primarily functions as a proton relay, electron donor, radical partner, coordination modulator, or a combination of these roles. Such assignments will be essential

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for moving from descriptive case studies to general mechanistic rules.

A second priority is to establish quantitative descriptors for substrate-driving capacity. For H_2O_2-dependent systems, relevant descriptors include substrate pK_a, proton affinity, hydrogen-bond-network persistence, water-relay connectivity, local electric-field projection along the O−O bond, and the population of peroxide-cleavage-competent conformations. For O_2-activating systems, key descriptors include substrate redox potential, π-electron delocalization, metal−substrate orbital overlap, substrate-to-O_2 electronic coupling, radical stabilization energy, and the ability of the substrate to promote productive metal−oxygen coordination rearrangement. Connecting these descriptors to O−O cleavage barriers, ferryl/Compound I formation, intermediate lifetime, product selectivity, and off-pathway oxidant release will allow substrate-engaged reactivity to be predicted rather than inferred retrospectively.

These descriptors should also be integrated into the substrate-screening and design workflows. From this viewpoint, future substrate screening should not only ask whether a candidate substrate binds to the enzyme or undergoes turnover but also whether it can assist oxidant generation in a reaction-compatible manner. The substrate-driving capacity should therefore be evaluated together with binding affinity, product selectivity, and coupling efficiency. Conventional substrate screening in oxidative biocatalysis often emphasizes binding affinity, turnover, and product profile. A substrate-engaged framework suggests an additional layer: candidate substrates should be evaluated to see whether they can actively assist oxidant generation. For peroxide-dependent enzymes, libraries can be prioritized by acidic or proton-relaying motifs that are geometrically compatible with distal pockets. For O_2-dependent enzymes, substrates or substrate analogues can be selected on the basis of redox accessibility, conjugation, coordination ability, or decarboxylation-driven electron release. Computational workflows combining cheminformatics, docking, molecular dynamics, local electric-field analysis, and QM/MM reaction profiling could preselect substrates that are not only binders but also potential drivers of reactive oxygen intermediate formation.

An important future direction is the codesign of substrate, reaction type, and enzyme scaffold. In this regard, the target is not simply to find a substrate that fits an enzyme pocket, but to identify substrate−enzyme pairs in which oxygen activation, reactive intermediate evolution, and product-forming chemistry are mutually compatible. This requires matching three elements: a substrate motif capable of driving or assisting oxidant generation, an active-site environment that positions this motif within an effective proton-transfer or electron-transfer network, and a reaction trajectory that converts the resulting intermediate into a high-value product. Such a framework could accelerate the discovery of oxidative transformations that are difficult to achieve by conventional substrate scope expansion, including selective C−H oxygenation, oxidative coupling, heteroatom oxidation, dehalogenation, ring cleavage, and late-stage functionalization.

Finally, substrate-engaged mechanisms provide opportunities for product-oriented biocatalyst development. Many high-value oxygenated molecules require the selective activation of strong or electronically biased bonds under mild conditions. Designing substrates or substrate analogues that participate in oxidant formation may help to lower the dependence on external reductants, improve coupling efficiency, reduce nonproductive oxidant decay, and expand the accessible chemical space of metalloenzyme catalysis. In this context, future platforms should combine mechanism-guided substrate libraries, enzyme variant panels, high-throughput product detection, and active learning models that iteratively optimize substrate-driving capacity, reaction compatibility, and product value. Such an approach would transform substrate screening from an empirical search for accepted substrates to a rational strategy for discovering reaction-engaged substrates that generate, control, and consume reactive oxygen intermediates in a productive manner.

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Notes

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