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Mechanism of extradiol aromatic ring-cleaving dioxygenases

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Abstract

The extradiol aromatic ring-cleaving dioxygenases activate molecular oxygen by binding both O₂ and the catecholic substrate to a reduced active site metal, generally Fe(II). Progress has been made in understanding the mechanism of this reaction through the combined use of kinetic, computational, biomimetic, structural, and diagnostic chemical approaches. It appears that O₂ is activated by accepting an electron transferred from the substrate through the metal, thereby simultaneously activating oxygen and substrate for reaction with each other.

Introduction

Ring-cleaving extradiol dioxygenases are found at the points of confluence of bacterial degradation pathways for both single and multi-ring aromatic compounds [1,2]. They catalyze the incorporation of both atoms of oxygen from O₂ into catechol derivatives, resulting in ring-open, muconic semialdehyde adducts that are readily degraded to TCA cycle metabolites; an example is shown in Figure 1. The extradiol dioxygenases were among the first non-heme iron-containing enzymes to be recognized, and their study has contributed to the current appreciation of the breadth and versatility of this large family [3]. While hypotheses for the metal site structure and molecular mechanism of these enzymes arose early on [4-6], only in the last few years have definitive tests for these ideas been reported. These advances have come on a broad front, encompassing the structural, kinetic, spectroscopic, chemical, and computational approaches that will be summarized here.

Roles of Fe(II) in catalysis

The pioneering X-ray crystal structures of extradiol dioxygenases by Senda and Mitsui [7] and Eltis and Bolin [8] revealed what has come to known as the 2-His-1-carboxylate facial triad motif for metal binding at the active site [9]. This motif is used in over 20 families of enzymes and exhibits remarkable catalytic versatility. Nevertheless, some common themes for oxygen activation obtain, and they were first recognized in the extradiol dioxygenase family. The advantage conveyed by the facial triad binding motif stems from the fact that the 2 His and 1 Asp or Glu residues that bind the metal occupy only one face of the coordination sphere, as illustrated in Figure 2a. Displaceable solvents occupy the opposite face, allowing several adjacent coordination sites to be used directly in the catalytic process. In the case of the extradiol dioxygenases, it is believed that the hydroxyl oxygens of the catecholic substrate occupy two

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of these positions while O₂ occupies the third (Figure 2b,c) [4]. This would serve two pivotal mechanistic purposes. First, the O₂ and substrate would be juxtaposed, presumably oriented optimally for a specific reaction. Second, these two substrates would be electronically connected through the metal, facilitating electron transfer from the catechol to the O₂. This would give both reactants radical character and allow rapid recombination to form the alkylperoxo intermediate (Figure 2c, d) in a spin-allowed reaction [4]. Once the key alkylperoxo intermediate forms, O-O bond fission and C-C bond cleavage in the ring might occur by concerted Criegee chemistry to form a 7-membered lactone. Hydrolysis of the lactone by the second oxygen from O₂, now present as a metal-bound solvent, would form the opening product ready for release from the enzyme (Figure 2e).

Intermediates in the substrate binding and O₂ activation processes

The X-ray crystal structure of 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC)-substrate complex with the O₂ surrogate NO also bound to the iron showed that the basic assumption of coincident and adjacent catecholic substrate and O₂ binding to the iron is likely to be correct [10•]. This structure and those of other extradiol dioxygenases, including homoprotocatechuate 2,3-dioxygenase (HPCD) [11], showed that the catecholic substrate binds asymmetrically to the iron, suggesting that only one of the two OH groups is deprotonated upon binding. This was definitively demonstrated through visible and UV-resonance Raman studies [12•]. The kinetics of the substrate binding process were investigated in HPCD using the slow alternative substrate 4-nitrocatechol (4NC, R = NO₂ in Figure 2) as a probe [13]. This molecule exhibits a unique chromophore in each possible ionization state, and the chromophores are sensitive to the polarity of their environment, allowing the detection of even subtly different intermediates. It was found that monoanionic 4NC binds in at least 3 steps while losing one proton to form a dianionic complex with the active site iron. Based on the spectroscopic changes, the 3 steps were hypothesized to be: binding in the hydrophobic active site, binding to the iron with ionization and solvent displacement, and a rate-limiting reorganization. This last step may be loss of solvent from the eventual site for O₂ binding because the structure of the anaerobic HPCD-substrate complex is 5-coordinate (Figure 2b) [11]. Similar substrate binding kinetics have also been noted using the alternative substrate 3-formylcatechol (3FC) with BphC [14].

Reaction of the substrate complexes of HPCD-4NC or BphC-3FC with O₂ could also be monitored kinetically using the unique substrate chromophores. Furthermore, when the substrate ring opens during the reaction, another large chromophoric shift occurs, allowing this step to be monitored. In the case of HPCD-4NC, it was found that O₂ reacts in a series of at least 3 steps [13]. The rate constants for these steps were independent of O₂ concentration, showing that there must be at least additional one preceding step in which O₂ actually binds. Based on the optical spectra, the final two steps could be identified as ring opening and rate-limiting product release, respectively.

The roles of active site acid and base catalysts

The original mechanistic hypotheses have been supported by recent computational studies [15•-18•] based on the active site structures of BphC, HPCD, and an HPCD homolog which binds Mn(II) instead of Fe(II) in the active site (MndD) [11]. These computations suggest that following formation of the alkylperoxo intermediate, O-O bond cleavage will be facilitated by the donation of a proton to the oxygen atom bound to the Fe(II) as shown in Figure 3. The most likely acid catalyst revealed by the crystal structures is a conserved His near the O₂ binding site [8,10•,19]. This His might also serve as an active site base to deprotonate the entering substrate and/or a base to deprotonate the OH group of the substrate carbon distal to the site of ring cleavage to allow its ketonization during formation of the alkylperoxo intermediate (Figure 3) [5,19]. One or both of the latter two roles for the conserved His were supported by

mutation of a Pro residue near the relevant His (His179) in 2,3-dihydroxyphenylpropionate dioxygenase (MhpB). The mutated enzyme showed a change in the pH activity profile consistent with disruption of base catalysis. However, direct mutation of His179 led to an inactive enzyme [19]. Fortunately, this was not the case in HPCD and MndD, which retained some activity when the equivalent His200 was mutated [20,21]. This allowed the role of His200 to be investigated directly using transient kinetic techniques.

The kinetics of binding 4NC by the His200Asn, -Ala, -Glu, and -Phe mutants of HPCD were nearly unchanged relative to WT enzyme, suggesting that the role of His200 is not to deprotonate the incoming substrate [20]. In contrast, some or all of the reactions of the pre-formed HPCD-4NC complex with O₂ were slowed significantly by these mutations. Two mechanistically informative observations derived from these studies. First, a new fast step was observed that proved to be the O₂ binding reaction. The optical spectrum of an oxy intermediate of some type was observed near 610 nm during the first milliseconds of the reaction when using either 4NC or the natural substrate (HPCA). Second, HPCA and 4NC were not converted to analogous products in the reaction catalyzed by the His200Asn mutant. HPCA was converted to the normal ring-cleaved product, while a quinone with the ring intact resulted from 4NC oxidation. Thus, it appears that without His200, the Criegee rearrangement to give oxygen insertion into 4NC does not occur, supporting the role of this residue as the active site catalytic acid. The slow, but viable, ring-cleavage reaction in the case of HPCA suggests a mechanistic redundancy in which either electron donation by the HPCA side chain or proton donation by an active site acid permit the Criegee rearrangement, but the absence of both leads to substrate oxidation and release of peroxide.

Structures of intermediates

Although the mechanism shown in Figure 2 has been widely accepted, there are other possibilities including formation of a dioxetane intermediate that would guarantee dioxygenase stoichiometry, and the formation of a high-valent metal-oxo (e.g. Fe(IV)=O) species as proposed for many other members of the facial triad family [22]. An important advance in support of the mechanism described here was made possible by the fact that HPCD remains catalytically active in crystals. Moreover, when the slow substrate 4NC was used, and the crystals were exposed to a very low concentration of O₂, 3 of the 4 subunits of the tetrameric enzyme in the asymmetric unit were found to have different intermediates of the reaction cycle present in high occupancy (structures shown in Figure 2c-e) [23,24]. The first subunit contained oxygen bound side-on to the iron in the predicted ligand site next to the chelated 4NC (Figure 2c). Side-on binding was not expected, but it is the same orientation observed for the structurally characterized hydroperoxo-complex of naphthalene 1,2-dioxygenase [25]. The side-on orientation aligns the oxygen perfectly for attack at the C2 position of the substrate, ultimately providing specificity for ring opening. Importantly, the structure showed the ring of 4NC to be puckered, suggesting that it is oxidized to a semiquinone radical as the O₂ binds. Among the iron ligands available, the bound oxygen is the logical acceptor for the electron derived from the 4NC; thus, the complex is best described as the 4NC semiquinone radical-Fe(II)-superoxo species as in the postulated mechanism. The second and third subunits contained an Fe(II)-alkylperoxo species that could most easily derive from attack of the Fe-bound superoxo on the 4NC radical (Figure 2d). The intact O-O bond extending between the Fe(II) and the substrate C2 effectively rules out the mechanisms based on dioxetane and high-valent Fe-oxo formation. The final subunit contained the product complex (Figure 2e), clearly demonstrating that the complete reaction can occur in the crystal.

The observed intermediates and mechanistic proposal are largely in accord with structures and reactivity of biomimetic complexes. Specifically, the presence of three exchangeable and adjacent ligand sites of the metal [26], the importance of acid/base catalysis [5,27], and the

formation of the alkylperoxo intermediate in an axial orientation relative to the plane of the ring [28-30] are properties that appear to lead to extradiol ring cleavage.

Relevance of metal oxidation state

The oxidation state of the active site metal has traditionally been associated with the site of ring cleavage. All known extradiol cleaving enzyme use a reduced metal site while all intradiol cleaving dioxygenases employ Fe(III). This preference is supported by the biomimetic complexes, but the specificity for the cleavage site for these compounds is not rigorous. It has been shown recently that the absolute specificity characteristic of the extradiol dioxygenases can be compromised by mutation of residues in the second sphere of the active site [31] and beyond [32] leading to intradiol cleavage of alternative substrates. Thus, intradiol cleavage can occur in a reaction cycle beginning with iron in the ferrous state.

A question perhaps more germane to the extradiol dioxygenase mechanism is whether the oxidation state of the metal changes during the catalytic cycle. Some insight has been gained from the apparent persistence of Fe(II) in the structurally characterized oxy-complex (Figure 2c), which exhibits longer Fe-O bonds than would be expected for an Fe(III) complex. In other types of metalloenzymes, an electron from the metal is at least partially donated to the oxygen to yield a species with Fe(III) character. Extradiol dioxygenases differ from these other systems in that the substrate bound to the iron can donate the electron to stabilize the Fe-O₂ bond as part of the activation process. Accordingly, computational investigations of the mechanism predicted retention of the Fe(II) state through the formation of the activated substrate radical-Fe(II)-superoxo state [15•,18•]. However, transient formation of Fe(III) was predicted by the computations for short lived intermediates in the Criegee rearrangement reactions. In these formulations, the Criegee rearrangement does not occur as a concerted process, but rather occurs stepwise through radical gem-diol and epoxide intermediates prior to collapsing to the 7-membered lactone intermediate as shown in Figure 4.

The fixed metal oxidation state was tested by comparing the reaction kinetics and structures of HPCD and MndD and preparations of these enzymes in which the non-physiological metal had been incorporated [33•]. The structures of HPCD and Mn(II)-replaced HPCD were shown to be identical at 1.7 Å resolution, and the steady state parameters k_{cat} , $k_{\text{cat}}/K_{\text{m}}$, $k_{\text{cat}}/K_{\text{m}}^{\text{HPCD}}$ and $k_{\text{cat}}/K_{\text{m}}^{\text{O}_2}$ were all found to be the same within error for all 4 forms of the enzyme. The inherent redox potentials of Mn and Fe differ by 0.7 v, and, given the identical protein structures, this value is apparently not decreased by differential active site tuning. Thus, the most reasonable explanation for the similar kinetic parameters is a mechanism in which there is no change in redox state through the first irreversible step in the mechanism after O₂ binds, presumably formation of the Fe(II)-alkylperoxo intermediate. Transient changes in oxidation state after this step may occur as predicted by the computations, but these would not affect the steady state parameters so long they do not occur in rate limiting steps.

Roles of active site residues in catalysis

The roles of active site amino acid residues other than the conserved His near the O₂ binding site are beginning to emerge as illustrated for HPCD in Figure 5. A residue that can hydrogen bond with the substrate OH group proximal to the site of ring cleavage is always present. Examples of this are Tyr257 in HPCD and His115 in 2,3-dihydroxyphenylpropionate 1,2-dioxygenase (MphB). This residue is close to a second residue, His248 in HPCD and Asp114 in MphB, that could enhance its ability to act as a base catalyst. Consequently, in the MphB system, His115 has been proposed to deprotonate the substrate OH as part of the Criegee rearrangement and then return the proton to allow breakdown of the resulting lactone intermediate [19]. Indeed, mutagenesis of His115 gives inactive enzyme, and mutagenesis of this residue or Asp114 disrupts the ability of the enzyme to catalyze breakdown of a model

lactone. Unfortunately, no structural information is available for MphB, so the impact of mutagenesis on conformational integrity is impossible to interpret. In structurally characterized enzymes such as HPCD and BphC, it is known that the substrate OH group in question is deprotonated before O₂ binding, probably by departing ionized solvent ligand of the iron, so the need for another active site base to fulfill this function seems unlikely [11,12•]. In the structures of the intermediates found for the HPCD reaction carried out in a crystal, it was found that a much stronger hydrogen bond forms between Tyr 257 and the substrate C2-O⁻ substituent as this carbon becomes tetrahedral [3,23•]. Thus, hydrogen bonding from Tyr257 promotes electron transfer to O₂ and subsequent formation of the alkylperoxy intermediate, in effect, pulling the reaction through the catalytic cycle. Its purported partner residue, His248, has another potential role in stabilizing the polar side chain of the substrate. Other strong hydrogen bonding interactions are evident in the HPCD active site, notably involving Asn157 and Trp192. These are likely to play roles in stabilizing and orienting the oxy and alkylperoxy intermediates, but they have yet to be fully evaluated.

Conclusion

The versatility of the 2-His-1-carboxylate facial triad family both in terms of the types of reactions catalyzed and the mechanisms for oxygen activation is made striking clear by even a brief perusal of representative enzymes [3,34]. Indeed, mechanisms involving Fe(III)-superoxo (e.g. isopenicillin N-synthase [35-37]), Fe(III)-peroxy or the equivalent Fe(V)-oxo-hydroxo (e.g. Rieske cis-diol forming dioxygenases [29,38,39]), Fe(IV)-oxo (e.g. α -ketoglutarate-linked dioxygenases [22,40] or aromatic amino acid hydroxylases [41]), in addition to the substrate radical-Fe(II)-superoxo species of the extradiol dioxygenases described here have been proposed. Each of these enzymes catalyzes distinct chemistry, but it can be argued that they initiate catalysis in a similar manner. In each case, Fe(II) is inert to O₂ association until the organic substrate or a co-substrate binds to the enzyme, and in most cases, to the metal. This initiates solvent release, thereby opening a site for O₂ binding with consequent transfer of electron density to the oxygen. This activates oxygen for further, highly divergent chemistry. Insight into the detailed chemistry of these first few common steps is rapidly emerging from the study of the kinetics, spectroscopic properties, and structural characteristics of intermediates in the extradiol dioxygenase reaction mechanism.

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Figure 1.

Typical extradiol dioxygenase reaction. The conversion of homoprotocatechuate (HPCA) to 5-carboxymethyl-2-hydroxymuconic semialdehyde (Product) is catalyzed by homoprotocatechuate 2,3-dioxygenase. This enzyme has been isolated from different bacteria in forms that contain either active site Fe(II) (HPCD) or Mn(II) (MndD).

Figure 2.

Proposed mechanism for extradiol aromatic ring cleaving dioxygenases illustrated for HPCD. Where known, the X-ray crystal structures of the active site metal centers of intermediates are shown: (a) resting enzyme, PDB 2IG9; (b) HPCA complex, PDB 1Q0C; (c-e) the semiquinone substrate radical-Fe(II)-superoxo, Fe(II)-alkylperoxo, and product complexes found in subunits C, D, and A, respectively, of a single crystal when using 4NC as the substrate, PDB 2IGA. The chemical figures provide additional detail for proposed electron distribution or proposed steps for which intermediates have not been structurally characterized. (Color scheme: yellow, Fe(II); red, oxygen; grey, carbon; blue, nitrogen).

Figure 3.
Proposed roles of HPCD His200 as an acid/base catalyst

Figure 4.

Hypothetical steps in the Criegee rearrangement of the Fe(II)-alkylperoxo intermediate of HPCD predicted by computational studies. Some of the steps involve formal oxidation of the metal center, but none of these steps are found to be rate limiting for the Fe(II) containing enzyme [15•,16,18•].

Figure 5. Active site region of HPCD showing hydrogen bonding interactions from second sphere residues implicated in catalysis. Distances shown are in angstroms. (Color scheme: yellow, Fe (II); red, oxygen; grey, carbon; blue, nitrogen; green, catecholic substrate carbon and side chain atoms). From PDB 2IGA, subunit D.