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MINIREVIEW

Ring-Cleaving Dioxygenases with a Cupin Fold

Susanne Fetzner

Institute of Molecular Microbiology and Biotechnology, Westfalian Wilhelms University Münster, Münster, Germany

Ring-cleaving dioxygenases catalyze key reactions in the aerobic microbial degradation of aromatic compounds. Many pathways converge to catecholic intermediates, which are subject to *ortho* or *meta* cleavage by intradiol or extradiol dioxygenases, respectively. However, a number of degradation pathways proceed via noncatecholic hydroxy-substituted aromatic carboxylic acids like gentisate, salicylate, 1-hydroxy-2-naphthoate, or aminohydroxybenzoates. The ring-cleaving dioxygenases active toward these compounds belong to the cupin superfamily, which is characterized by a six-stranded β-barrel fold and conserved amino acid motifs that provide the 3His or 2- or 3His-1Glu ligand environment of a divalent metal ion. Most cupin-type ring cleavage dioxygenases use an Fe^{II} center for catalysis, and the proposed mechanism is very similar to that of the canonical (type I) extradiol dioxygenases. The metal ion is presumed to act as an electron conduit for single electron transfer from the metal-bound substrate anion to O_2, resulting in activation of both substrates to radical species. The family of cupin-type dioxygenases also involves quercetinase (flavonol 2,4-dioxygenase), which opens up two C-C bonds of the heterocyclic ring of quercetin, a widespread plant flavonol. Remarkably, bacterial quercetinases are capable of using different divalent metal ions for catalysis, suggesting that the redox properties of the metal are relatively unimportant for the catalytic reaction. The major role of the active-site metal ion could be to correctly position the substrate and to stabilize transition states and intermediates rather than to mediate electron transfer. The tentative hypothesis that quercetinase catalysis involves direct electron transfer from metal-bound flavonolate to O_2 is supported by model chemistry.

Microorganisms have evolved a variety of aerobic as well as anaerobic pathways to degrade aromatic and heterocyclic compounds (40, 47, 48, 54). Since aromatic compounds, due to the delocalization of their π orbitals, are very stable, the key steps for degradation involve (i) activation of the aromatic ring by introduction of substituents and (ii) dearomatization. In aerobic microorganisms, activation of an aromatic substrate usually is achieved by hydroxylation reactions, and the critical dearomatization step is performed by ring-cleaving dioxygenases. Many pathways converge to catecholic substrates, which undergo cleavage either *ortho* to (between) the two hydroxyl substituents, catalyzed by intradiol dioxygenases, or *meta* (adjacent) to the hydroxyl substituents, catalyzed by extradiol dioxygenases. Ring-cleaving dioxygenases play important roles in the degradation of aromatic compounds by soil bacteria. They even can be key determinants of the fate of certain aromatic compounds in the environment, as in several instances their properties were shown to confine the specificity of a degradation pathway. For example, the presence of chloroaromatic compounds may prevent the degradation of methylaromatics, such as xylenes or cresols, via the *meta* cleavage pathway, because most catechol 2,3-dioxygenases are inactivated by 3-halocatechols (10, 69, 81). *Meta* cleavage also is the critical step in the degradation of polychlorinated biphenyls (PCBs), because the susceptibility of the enzyme to inactivation by *ortho*-substituted PCBs interferes with the degradation of other PCB congeners (28).

Enzymes of the intradiol dioxygenase family use a mononuclear Fe^{III} center, coordinated to two tyrosine and two histidine ligands, for catalysis. Within this family, protocatechuate 3,4-dioxygenase has been studied most extensively. Crystal structures and X-ray absorption data revealed a ferric center in a trigonal bipyramidal geometry, with a hydroxide ligand completing the coordination sphere (31, 93, 94, 123). The substrate binds as a dianion, donating both its protons to the displaced hydroxide and tyrosyl ligands (36, 60, 96). Based on spectroscopic data and electronic structure calculations, it has been proposed that the iron-catecholate interaction introduces a semiquinonate radical character to the bound substrate, which reacts directly with dioxygen to form an alkylperoxo-Fe^{III} intermediate. Rearrangement and O-O bond cleavage yield a cyclic anhydride and a metal-bound oxide or hydroxide, and the latter finally hydrolyzes the anhydride to yield the reaction product (30, 99) (Fig. 1a).

The canonical (type I) extradiol enzymes belong to the vicinal oxygen chelate (VOC) superfamily, which is characterized by paired $\beta\alpha\beta\beta\beta$ modules that provide a coordination environment for divalent metal ions. The types of reactions catalyzed by members of this superfamily are quite diverse and include isomerizations, epimerizations, nucleophilic substitutions, and oxidative C-C bond cleavage (5). Extradiol dioxygenases use an active-site Fe^{II} cofactor or, rarely, Mn^{II} for catalysis. The metal ion is coordinated by one glutamate and two histidine residues that occupy one face of a (pseudo)octahedral coordination sphere. The three adjacent coordination sites on the opposite face, usually occupied by easily displaceable solvent molecules, are available for the binding of substrates. This structural motif, termed the 2His-1carboxylate facial triad, is widespread among nonheme iron oxygenases (25, 73, 118). Whereas the intradiol enzymes appear to activate the organic substrate for electrophilic attack by O_2, extradiol dioxygenases have been proposed to simultaneously activate substrate and O_2 to form two metal-bound radical species (Fig. 1b). The reaction starts with bidentate binding of the substrate as cat-

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Address correspondence to Susanne Fetzner, fetzner@uni-muenster.de.

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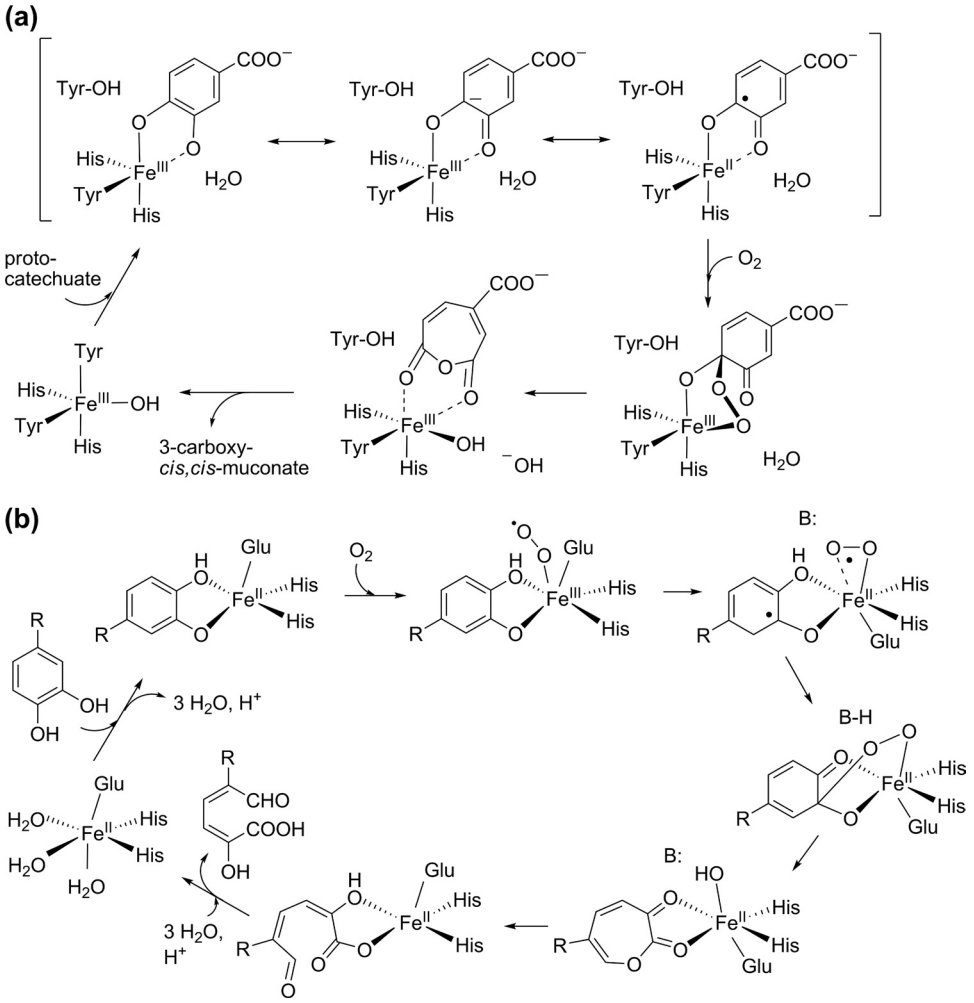

FIG 1 Proposed reaction mechanisms for intradiol and extradiol ring-cleaving dioxygenases. (a) Mechanism of protocatechuate 3,4-dioxygenase (99). (b) Mechanism of homoprotocatechuate 2,3-dioxygenase (78, 83). R, -CH_2COOH; B:, general base.

echolate monoanion to the active-site metal, displacing two or three water molecules (109, 124). Substrate binding dramatically increases the affinity of the metal center for O_2 binding (4). In the ternary complex, the two substrates are electronically connected through the metal, facilitating electron transfer from catecholate to O_2 to give a semiquinone-Fe^{II}-superoxide species. Such an activation of both substrates to radical species should allow rapid recombination to form an alkylperoxo intermediate. Subsequent rearrangement and O-O bond cleavage gives a seven-membered lactone intermediate and an Fe^{II}-bound hydroxide ion, which hydrolyzes the lactone to yield the 2-hydroxymuconate semialdehyde product (Fig. 1b) (44, 71, 72, 78, 83, 84).

A question pivotal to the understanding of the molecular mechanism of metal-dependent oxygenases refers to the role of the metal in oxygen activation. Interestingly, Fe^{II}-homoprotocatechuate 2,3-dioxygenase (Fe-HPCD) from *Brevibacterium fuscum* and the (closely related) Mn^{2+}-dependent enzyme from *Arthrobacter globiformis* (126, 128) can each be prepared with the nonphysiological metal in the active site. Since the reduction potentials of iron and manganese differ by approximately 0.7 V in the absence of redox tuning by the protein, these enzymes were used to probe the relevance of the metal oxidation state in dioxygen activation. Mn^{II}- and Fe^{II}-HPCD had superimposable structures, suggesting that the difference in redox potential of the metals should be retained in the active site (37). Because the kinetic parameters of the "physiological" and "metal-swapped" enzymes for the organic substrate and for O_2 were very similar, it has been discussed that oxygen activation and substrate oxidation steps can proceed without the requirement for an integral change in the metal redox state (37, 78). However, electron paramagnetic resonance (EPR) studies revealed the formation of a Mn^{III} radical couple in Mn-HPCD (52), and recently, an Fe^{III}-superoxo species and a [homoprotocatechuate semiquinone-Fe^{III}-(hydro)peroxo] intermediate were trapped in a protein variant of HPCD (83, 84). These findings support the hypothesis that an Fe^{III}-superoxide intermediate is formed in the catalytic cycle but has a very short lifetime. It seems that the metal center in HPCD and other extradiol dioxygenases, after having established the correct orientation of the two substrates, acts as a conduit for facile electron transfer from the catecholate to O_2. Electron transfer from the divalent metal center to O_2 is thought to elicit an immediate subsequent electron transfer from the bound substrate to the nascent trivalent metal center. The finding that Mn^{II}- and Co^{II}-substituted HPCD are fully active or even hyperactive has been rationalized by the hypothesis that even though Mn^{2+} and, especially, Co^{2+} are poorer reducing agents than Fe^{2+}, this is compensated by

TABLE 1 Biochemically or structurally characterized microbial ring cleavage dioxygenases with a cupin fold

Dioxygenase	Source	Domain structure, quaternary structure	Metal dependence	Ligand(s) to the metal ion[a]	Reference(s)
Gentisate 1,2-dioxygenase	*Pseudomonas acidovorans* ATCC 17438, *P. testosteroni* ATCC 49249	Bicupin, homotetramer	Fe^{2+}	NS	55, 56
	Escherichia coli O157:H7	Bicupin, homotetramer	Fe^{2+}	3His, $3H_2O$	1
	Silicibacter pomeroyi DSS-3	Bicupin, homotetramer	Fe^{2+}	3His	20
Salicylate 1,2-dioxygenase	*Pseudaminobacter salicylatoxidans* BN12	Bicupin, homotetramer	Fe^{2+}	3His	39, 59, 82
1-Hydroxy-2-naphthoate 1,2-dioxygenase	*Nocardioides* sp. KP7	Bicupin, homohexamer	Fe^{2+}	NS	62
3-Hydroxyanthranilate 3,4-dioxygenase	*Saccharomyces cerevisiae*	Monocupin, homodimer	Fe^{2+}	2His, 1Glu (bidentate), $2H_2O$	74, 76
	Ralstonia metallidurans	Monocupin, homodimer	Fe^{2+}	2His, 1Glu (bidentate), $2H_2O$	130
4-Amino-3-hydroxybenzoate 2,3-dioxygenase	*Bordetella* sp. 10d	Monocupin, homodimer	Fe^{2+}	NS	88, 119
Flavonol 2,4-dioxygenase (quercetinase)	*Aspergillus japonicus*	Bicupin, homodimer	Cu^{2+}	3His, $1H_2O$ (major form); 3His, 1Glu, $1H_2O$; (minor form)	49, 70, 113, 114
	Bacillus subtilis	Bicupin, homodimer	$Mn^{2+} > Co^{2+} > Fe^{2+}, Ni^{2+}, Cu^{2+}$	3His, 1Glu, $1H_2O$	51, 107
	Streptomyces sp. FLA	Monocupin, homodimer	$Ni^{2+} > Co^{2+} > Mn^{2+}, Fe^{2+}$	NS	85, 86

[a] NS, not specified.

their M^{III}-superoxo intermediates acting as stronger oxidizing agents (44).

Many biochemical and structural studies on intradiol and type I extradiol dioxygenases have contributed to a rather detailed understanding of these enzymes, which have been the subject of several comprehensive reviews (14–17, 25, 73, 78, 125). Genomic and metagenomic studies provided insight into their phylogenetic distribution, diversity, and abundance in the environment (13, 120, 127). However, in a number of bacterial degradation pathways, intermediates may be converted to gentisate, i.e., a *para*-diol, rather than to a catecholic compound. Other metabolic pathways involve monohydroxylated aromatic carboxylic acids that directly undergo dioxygenolytic ring cleavage reactions. Enzymes catalyzing these reactions have been referred to as "type III extradiol dioxygenases," even though most substrates lack the diol character. Another group of particular ring-cleaving dioxygenases open up two C-C-bonds of the flavonol heterocycle, releasing the hydroxy-substituted carbon atom at the C-3 position as carbon monoxide. These ring-cleavage enzymes active toward noncatecholic substrates, which are the main focus of this review, belong to the cupin superfamily of proteins (Table 1).

THE CUPIN SUPERFAMILY

The cupin superfamily is characterized by a conserved six-stranded β-barrel fold ("cupa" is the Latin term for small barrel). Most family members either comprise a single cupin domain ("monocupins") or have a duplicated domain structure ("bicupins") (Fig. 2). Cupins are found in all kingdoms of life. The superfamily is functionally highly diverse and includes nonenzymatic proteins, such as plant seed storage proteins, transcription regulators, and stress-related proteins, and a wide variety of enzymes. Enzymatic cupins involve isomerases and epimerases, decarboxylases, and many oxygenases, with the 2-oxoglutarate-dependent dioxygenases as the largest subset (for reviews, see references 21, 33–35, and 68).

The cupin domain comprises two conserved amino acid motifs with the consensus sequences $G(X)_5HXH(X)_{3-4}E(X)_6G$ (motif 1) and $G(X)_{5-7}PXG(X)_2H(X)_3N$ (motif 2). Each motif corresponds to two β strands separated by another two strands with an intervening loop. The two histidine residues and the glutamate residue in motif 1, together with the conserved histidine residue in motif 2, can act as ligands for the binding of a divalent metal ion. This ligand environment has been observed in many cupin structures, such as germin (129), oxalate decarboxylase (2), flavonol 2,4-di-

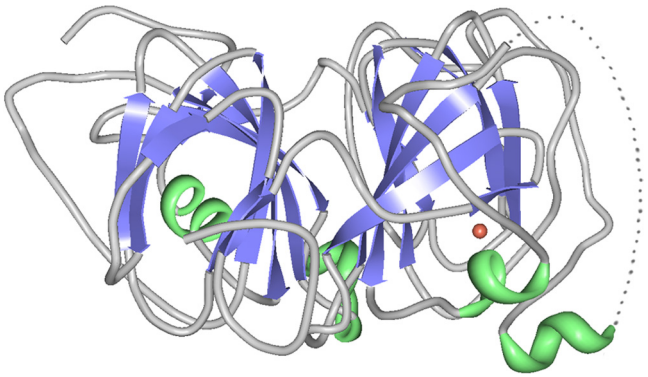

FIG 2 Ribbon diagram of the monomer of quercetinase of *A. japonicus*, illustrating the two barrels of the bicupin fold. The dotted line indicates a flexible region of the structure (residues 154 to 169) (49). The copper atom located in the N-terminal domain is shown as a red sphere. The native protein is a homodimer. The figure was generated from PDB code 1JUH using the RCSB Viewer RCSB PDB Protein Workshop 3.9 (87).

oxygenases (49, 51), and acireductone dioxygenase (19, 27, 29, 64, 102). The majority of the enzymatic cupins use Fe^{2+} or Mn^{2+} as an active-site metal ion, but other members contain Cu^{2+}, Zn^{2+}, Co^{2+}, or Ni^{2+}. The type of metal ion and its ligand environment within the cupin fold likely are major determinants for the reaction chemistry at the active site. The metal centers usually are hexa-coordinated, adopting an octahedral (bipyramidal) or distorted octahedral geometry, with two water molecules completing the coordination sphere. Some members, however, contain a penta-coordinated metal site (32, 49, 51, 113). Interestingly, some cupin subfamilies do not contain all four of the conserved metal-binding residues. A key feature of the thiol dioxygenase family, for example, is that the glutamate residue in motif 1 is replaced by a cysteine in mammalian cysteine dioxygenases and by a glycine in bacterial thiol dioxygenases (111, 116). Other enzymes with a "reduction" of the 3His-1Glu motif are the gentisate 1,2-dioxygenases (GDOs) and 3-hydroxyanthranilate 3,4-dioxygenases (HADs) (see below). Most remarkably, the metal binding site in polyketide cyclase RemF, a monocupin protein, contains an octahedral zinc site, with four histidine residues as protein ligands to the Zn^{2+} ion (110).

Many ring cleavage dioxygenases within the cupin superfamily use a mononuclear Fe^{II} center for catalysis. Interestingly, however, flavonol-cleaving 2,4-dioxygenases from different sources exhibit different metal selectivities or even are cambialistic enzymes, capable of using several divalent metal ions as a cofactor. Such "metal promiscuity" raises the question of the role of the metal center in catalysis and the mechanistic relatedness of cupin dioxygenases and classical catechol dioxygenases.

GENTISATE 1,2-DIOXYGENASE AND RELATED ENZYMES

Gentisate (2,5-dihydroxybenzoate) is formed from 3-chloro- and 3-hydroxybenzoate or from salicylate in the bacterial degradation of many simple as well as complex aromatic compounds (for a compilation of pertinent pathways, see the University of Minnesota Biocatalysis/Biodegradation [UM-BB] Database, http://umbbd.msi.umn.edu/ [50]). Gentisate 1,2-dioxygenase (GDO), as well as 1-hydroxy-2-naphthoate and salicylate 1,2-dioxygenases (HNDO and SDO, respectively), cleave their substrates between the adjacent carbon atoms carrying a carboxylate and a hydroxyl substituent (Fig. 3a to c). The GDOs isolated from two *Pseudomonas* species are both Fe^{2+}-dependent enzymes with a high specificity for gentisate, with 5-aminosalicylate as a poor alternative substrate. Salicylate is not converted but acts as a competitive inhibitor (55, 56). All GDOs characterized to date belong to the bicupin family (1, 20). Each bicupin subunit of the homotetrameric enzyme from *Escherichia coli* contains a mononuclear iron center coordinated to three His ligands, leaving three solvent-occupied sites on the iron available for interactions with substrate (1). The conserved glutamate of the cupin consensus motif 1 is replaced by alanine (or another hydrophobic or a polar residue) in virtually all confirmed and predicted GDOs. This substitution has been discussed to possibly represent an adaptation for binding of both the aromatic carboxylate and O_2 to the metal. Earlier EPR studies on GDO from *Pseudomonas testosteroni* provided evidence that O_2 and gentisate indeed are coordinated simultaneously to the active-site iron (56). Based on spectroscopic data and supported by structural data, the catalytic cycle has been proposed to start with bidentate coordination of the substrate via its hydroxyl and carboxylate groups to Fe^{II}, which activates the Fe^{II} center for subsequent binding of O_2.

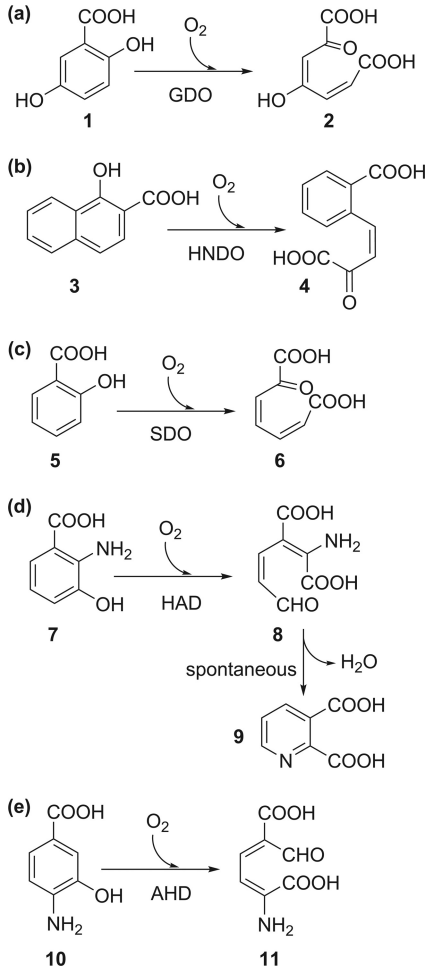

FIG 3 Reactions of cupin-type dioxygenases which catalyze the cleavage of an aromatic C-C bond. (a) Gentisate 1,2-dioxygenase (GDO); (b) 1-hydroxy-2-naphthoate 1,2-dioxygenase (HNDO), (c) salicylate 1,2-dioxygenase (SDO); (d) 3-hydroxyanthranilate 3,4-dioxygenase (HAD); (e) 4-amino-3-hydroxybenzoate 2,3-dioxygenase (AHD). 1, Gentisic acid; 2, maleylpyruvic acid; 3, 1-hydroxy-2-naphthoic acid; 4, (3Z)-4-(2-carboxyphenyl)-2-oxobut-3-enoic acid; 5, salicylic acid; 6, 2-oxohepta-3,5-dienedioic acid; 7, 3-hydroxyanthranilic acid; 8, 2-amino-3-carboxymuconic semialdehyde; 9, quinolinic acid; 10, 4-amino-3-hydroxybenzoic acid; 11, 2-amino-5-carboxymuconic semialdehyde.

Polarization of electron density from the aromatic ring toward the iron-bound O_2 in the ternary complex primes O_2 for attack at carbon 1 of the bound gentisate to form an alkylperoxo intermediate. Cleavage of the O-O bond and insertion of one oxygen atom into the ring, promoted by ketonization of the hydroxyl substituent at C-5, would generate a cyclic lactone that may be hydrolyzed by the hydroxide that after O_2 cleavage remained at the iron (1, 20, 56). The mechanism proposed for GDO is very similar to that of the type I extradiol dioxygenases (*cf.* Fig. 1b).

1-Hydroxy-2-naphthoate is an intermediate in the microbial degradation of phenanthrene and chrysene (53, 62, 90). Salicylate can be formed in the degradation of naphthalene and derivatives, polycyclic aromatic hydrocarbons, dibenzofuran, and the organophosphorous insecticide isocarbophos, as described in the UM-BB database (http://umbbd.msi.umn.edu/) (50). 1-Hydroxy-2-naphthoate 1,2-dioxygenase (HNDO) from the phenanthrene-degrading strain *Nocardioides* sp. strain KP7 and salicylate 1,2-dioxygenase (SDO) from the

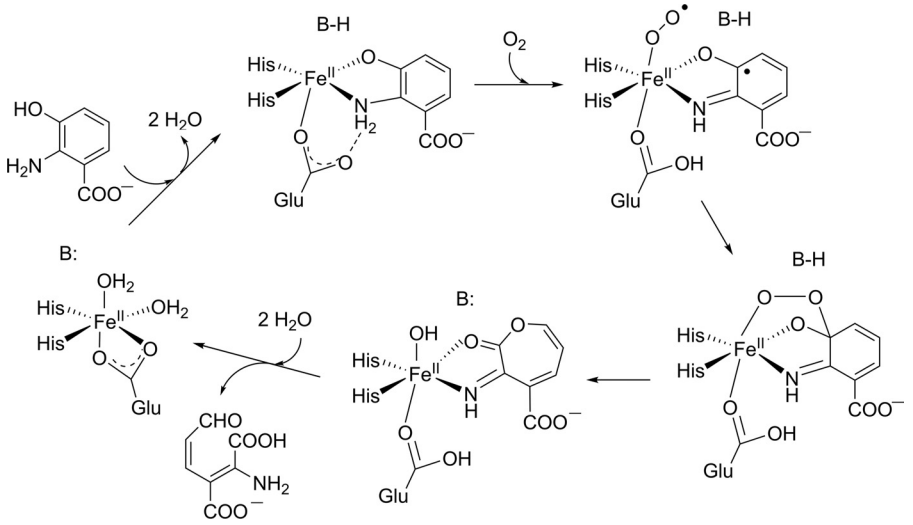

FIG 4 Proposed mechanism for the reaction catalyzed by 3-hydroxyanthranilate 3,4-dioxygenase (HAD) (76). B:, general base.

naphthalenesulfonate-degrading bacterium *Pseudoaminobacter salicylatoxidans* BN12 exhibit significant sequence similarity to GDOs (39, 59, 82). However, gentisate and salicylate were neither substrates nor competitive inhibitors of HNDO, suggesting stringent adaptation of this enzyme to its physiological substrate (62). In contrast, SDO from *P. salicylatoxidans* BN12 has a remarkably broad specificity. The highest activity was observed with gentisate, and the gentisate analogue 5-aminosalicylate, as well as 1-hydroxy-2-naphthoate, was also converted with higher activity than salicylate. SDO therefore seems to be basically a GDO that has relaxed its specificity toward the conversion of monohydroxylated substrates (39, 59, 82). Like *E. coli* GDO, SDO is a homotetramer of bicupin subunits, with one 3His-coordinated Fe^{II} site per subunit.

A 5-nitrosalicylate dioxygenase, which is distantly related to salicylate and gentisate 1,2-dioxygenases, was recently identified in the 5-nitroanthranilic acid-degrading bacterium *Bradyrhizobium* sp. strain JS329. The enzyme also catalyzes the oxidation of 5-chlorosalicylate, whereas conversion of 5-hydroxyanthranilate, 4-nitrocatechol, or 4-chlorocatechol was not observed (104, 105).

AMINOHYDROXYBENZOATE DIOXYGENASES

3-Hydroxyanthranilate 3,4-dioxygenase (HAD) is involved in tryptophan catabolism and seems to be conserved from bacteria to humans (18, 22, 32, 74, 75, 79, 89). 2-Amino-3-carboxymuconic 6-semialdehyde, the product of the HAD reaction, may either undergo further degradation via 2-aminomuconate 6-semialdehyde or spontaneously cyclize to quinolinate (23) (Fig. 3d). In the mammalian central nervous system, quinolinate binds to the *N*-methyl-D-aspartate receptor and thus has neurotoxic capacity (108, 117), but on the other hand, quinolinate is also a source for nicotinate mononucleotide, required for the biosynthesis of the NAD cofactors. HAD therefore may have a biosynthetic role in both eukaryotes and bacteria (74, 75). However, utilization of 2-nitrobenzoate by *Pseudomonas fluorescens* strain KU-7 (58, 89) and *Arthrobacter protophormiae* RKJ100 (97) proceeds via 3-hydroxyanthranilate and 2-amino-3-carboxymuconic 6-semialdehyde, with the latter subsequently undergoing decarboxylation and oxidation to 2-amino-*cis*,*cis*-muconate (89). Quite unusually, *Geobacillus thermodenitrificans* NG80-2 degrades anthranilate via 3-hydroxyanthranilate and subsequent 3,4-dioxygenolytic ring cleavage (79). In these cases, ring cleavage serves for the utilization of an aromatic compound as carbon and energy source rather than for quinolinate formation.

All HAD proteins characterized until now depend on Fe^{2+} for activity (18, 32, 89, 130). HADs from *Ralstonia metallidurans* and from yeast are homodimeric proteins, each monocupin subunit harboring a catalytic Fe^{II} site and a rubredoxin-like (FeS_4) center of unknown function located at the C-terminal periphery of the molecule (76, 130). The arrangement of the two cupin barrels in the homodimer resembles bicupin structures (130). In contrast to the bacterial HAD, the bovine and human proteins are monomeric bicupins lacking the rubredoxin-like center, with only the N-terminal cupin domain containing an active site (32). Despite these structural differences, the active-site residues considered relevant for the catalytic reaction appear to be fully conserved in the prokaryotic and eukaryotic proteins, suggesting the same catalytic mechanism. In all HAD proteins, the first conserved histidine of the consensus sequence of motif 1 is substituted, resulting in a 2His-1Glu coordination sphere. However, in contrast to the 2His-1Glu facial triad of the type I extradiol dioxygenases, the glutamate binds in a bidentate manner (130) (Fig. 4). The crystal structures of the yeast and human enzymes and of HAD from *R. metallidurans* suggest a distorted octahedral coordination geometry with Fe^{2+} bound to two His ligands, bidentate Glu, and two ligands modeled as water molecules (76, 130). 3-Hydroxyanthranilate, possibly in its phenolate form, binds as a bidentate ligand, presumably displacing one water molecule and the carbonyl oxygen of the Glu ligand. A conserved glutamate, which is not part of the first coordination sphere, was proposed to be involved in deprotonation of the substrate's hydroxy group. Structures with bound inhibitor and NO or O_2 suggest a direct interaction of the dioxygen molecule with the metal ion. The proposed reaction mechanism is analogous to that of the type I extradiol dioxygenases (24, 76, 130) (Fig. 4).

Another Fe^{2+}-containing ring-cleaving enzyme active toward a carboxy-substituted *o*-aminophenol is 4-amino-3-hydroxybenzoate 2,3-dioxygenase (AHD) from *Bordetella* sp. strain 10d

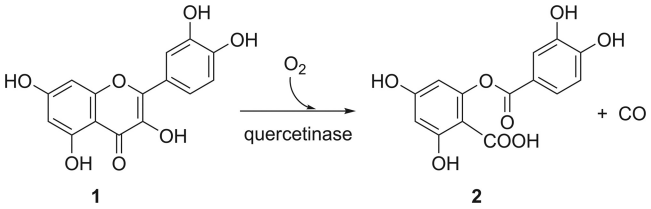

FIG 5 Reaction catalyzed by flavonol 2,4-dioxygenase (quercetinase). 1, Quercetin (3,5,7,3′,4′-pentahydroxyflavone); 2, protocatechuoylphloroglucinol carboxylic acid.

(Fig. 3e). Its amino acid sequence shows 24 to 29% identity to those of the HAD proteins; however, *Pseudomonas* HAD and *Bordetella* AHD are highly specific for their respective physiological substrates (88, 89, 119). Active-site residues which in HADs are involved in metal binding and deprotonation of the hydroxy group of 3-hydroxyanthranilate are conserved in AHD, tentatively suggesting a similar catalytic mechanism.

FLAVONOL 2,4-DIOXYGENASES (QUERCETINASES)

Flavonols are polyphenolic compounds synthesized by numerous higher plants, in most cases as *O*-glycosides (63). Major plant flavonols are quercetin (3,5,7,3′,4′-pentahydroxyflavone), kaempferol (3,5,7,4′-tetrahydroxyflavone), myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone), and isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone). Quercetin is found in considerable amounts in many vegetables and fruits, such as onions, broccoli, and apples, and thus is the major flavonol in the human diet (38, 100, 103). Quercetin exhibits antioxidative, antiinflammatory, and vasodilating effects and has been proposed to be a potential anticancer agent (38, 67, 100). It also exhibits antibacterial activity, which is at least partially due to inhibition of DNA gyrase B (26, 101). Due to release from rotting plant material and as constituents of leaf, seed, and root exudates (57, 66, 115), quercetin conjugates are widespread in soil, and various filamentous fungi and bacteria have been described to detoxify or to degrade quercetin (reviewed in reference 121). The initial step of the aerobic metabolism is a 2,4-dioxygenolytic ring cleavage to form 2-protocatechuoylphloroglucinol carboxylic acid and carbon monoxide, catalyzed by quercetinase (Fig. 5).

Fungal quercetinases are extracellular glycoproteins with a bicupin fold. The reactivity of quercetinases from *Aspergillus japonicus*, other *Aspergillus* species, and *Penicillium olsonii* depends on a mononuclear Cu^{II} center (49, 61, 70, 95, 122). In the crystal structure of quercetinase of *A. japonicus*, the copper ion is mainly coordinated by three His residues and a water molecule in a distorted tetrahedral geometry; in a minor form, the metal is penta-coordinated by three His, a glutamate, and an aquo ligand in a trigonal bipyramidal geometry. The glutamate ligand has been proposed to act as a general base to generate the flavonolate anion, which binds in a monodentate fashion through its O-3 atom to the copper site (49, 70, 113, 114). A flavonoxy radical-Cu^{I} valence tautomer, arising from the flow of one electron from the flavonolate to the copper, was hypothesized to be the active species that can react with dioxygen. This proposed mechanism of substrate activation shares similarity with that of the intradiol catechol dioxygenases (113).

Whereas fungal quercetinases appear to exclusively utilize a Cu^{2+} ion for catalysis, other quercetinases are cambialistic, i.e., function with several or even a variety of metal ions. Quercetinase of *Bacillus subtilis*, at least when produced in *Escherichia coli*, is able to exchange its active-site metal ion while retaining catalytic activity. The recombinant enzyme is most active with Mn^{2+}, whereas nickel is a poor cofactor (9, 11, 107). The crystal structure of the Fe^{II} isoform shows that three His residues and one Glu residue provide the protein-based ligands for the Fe^{2+} ion (51). For the *Bacillus* quercetinase, Schaab et al. (107) suggested a mechanism similar to that of the extradiol dioxygenases, involving a [quercetin radical-M^{II}-superoxo] intermediate. However, EPR studies on the Fe form of *Bacillus* quercetinase indicated that enzyme-bound quercetin shields the Fe^{II} cofactor from interactions with the O_2 mimic nitric oxide (51), tentatively suggesting that the reaction catalyzed by *Bacillus* (Fe-)quercetinase may proceed without direct interaction of dioxygen and metal ion.

A most interesting case of a cambialistic quercetinase is the enzyme from *Streptomyces* sp. strain FLA which, in contrast to the *Bacillus* enzyme, has a monocupin fold and is most active with Ni^{2+} as a cofactor. A nickel preference is very unusual for oxygenases. Co^{2+}, Fe^{2+}, and Mn^{2+} can replace Ni^{2+} and also support catalytic activity to some extent (85, 86). Given that quercetinases are evolutionarily related and catalyze the same reaction, their variability in metal selectivity is surprising. It is also interesting to note that the metal ions that afford catalytically active enzyme have standard reduction potentials that span a range of more than 1.5 V (Table 2), suggesting that the redox properties of the bound metal are relatively unimportant for the reaction. However, a mechanism which involves initial electron transfer from the divalent metal to O_2, as proposed for the extradiol dioxygenases, requires that a M^{III}-superoxo state is thermodynamically accessible (44). On the other hand, a mechanism as discussed for copper quercetinase, with the bound substrate acting as the initial electron donor to the divalent metal center, requires accessibility of the M^{I} state. EPR spectra of the Co form of *Streptomyces* quercetinase were indicative of a high-spin Co^{II} species in a trigonal-bipyramidal or tetrahedral coordination geometry (85). Studies with model Co^{II} complexes suggested that a 3N/1O ligand environment stabilizes the Co^{II} state (106). Moreover, Ni^{II} centers in ligand environments dominated by O and N donors (in contrast to thiolate-ligated Ni^{II} sites) were proposed to be redox inert (80). Thus, for the Co^{II} and, especially, the Ni^{II} center of quercetinases, the accessibility of redox states other than M^{II} is a matter of debate. These considerations have led to the suggestion that the M^{II}-flavonolate complex is oxidized by an outer-sphere electron transfer to dioxygen, generating the superoxide anion radical along with the M^{II}-[flavonoxy radical] without valence change of the metal (Fig. 6). However, experimental evidence for intermediates of the reaction catalyzed by bacterial quercetinases is relatively scarce

TABLE 2 Standard reduction potentials of selected transition metal cations

Redox pair	eV	Reference(s)
Cu^{3+}/Cu^{2+}	2.4	12
Ni^{3+}/Ni^{2+}	2.3	12
Co^{3+}/Co^{2+}	1.92	7, 12
	1.81	3
Mn^{3+}/Mn^{2+}	1.56	12
	1.5415	77
	1.51	3
Fe^{3+}/Fe^{2+}	0.771	7, 12
Cu^{2+}/Cu^{1+}	0.153	77

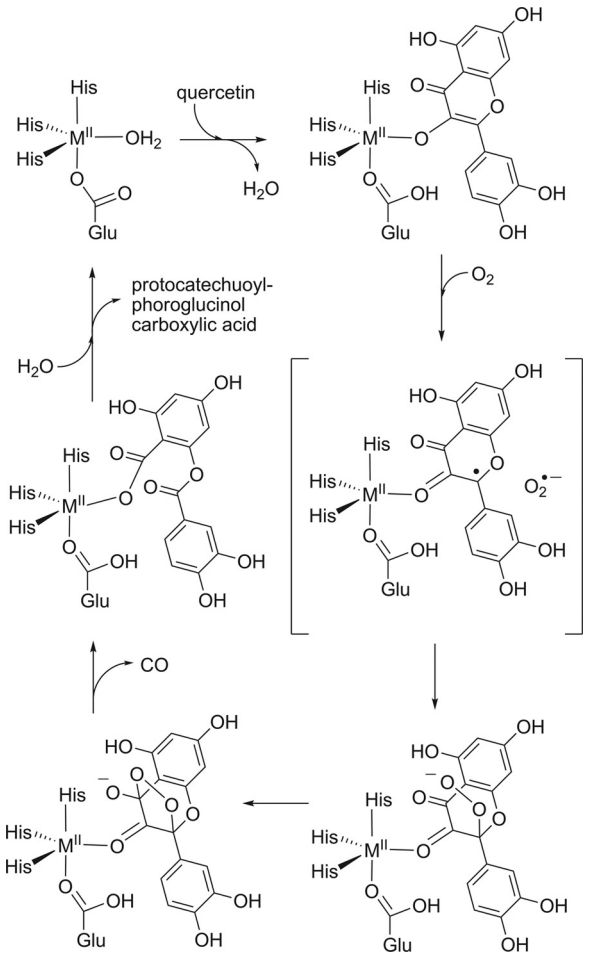

FIG 6 Hypothetical reaction mechanism of bacterial quercetinases.

compared to the wealth of information on the classical intradiol and extradiol dioxygenases.

The hypothesis of direct electron transfer from the metal-bound quercetin anion to O_2 is also inspired by the intrinsic chemical reactivity of flavonolates toward dioxygen. Organic model reactions showed that basic conditions and the presence of dioxygen are sufficient for 2,4-dioxygenolytic cleavage of flavonols, demonstrating that a metal catalyst is not necessarily required for the reaction to occur. Aprotic conditions increase the rate of base-catalyzed dioxygenolysis (6, 8, 65, 91, 92). Interestingly, recent studies on model systems suggested that the presence of a carboxylate coligand on Cu^{II}-flavonolate complexes induces monodentate binding of flavonolate to the metal, resulting in high electron density on the C-2 atom of the flavonolate, which possibly facilitates the direct single electron transfer from the activated flavonolate to dioxygen (98). Thus, the major role of the divalent metal ion in the active site of quercetinases could be to correctly position the substrate and to stabilize transition states and intermediates rather than to mediate electron transfer.

In the context of a possible nonredox role of the metal centers of quercetinases, it is interesting to note that oxygenases active toward 3-hydroxy-4(1*H*)-quinolones, which, like quercetinase, catalyze a 2,4-dioxygenolytic ring cleavage reaction, neither require nor contain a metal ion for catalysis (41, 45, 46, 112). For these enzymes, which have an α/β-hydrolase fold (45, 112), and also for other cofactor-independent oxygenases from different fold families, base-catalyzed abstraction of a proton from the organic substrate seems to be the common initial step in catalysis. Subsequent catalytic steps are thought to be triggered by the intrinsic reactivity of the bound carbanion and, presumably, also by the ability of the organic anions to form resonance-stabilized radicals upon single-electron oxidation (42, 43). The biochemistry of cofactor-independent oxygenases supports the notion that the oxygenation of (carb)anionic substrates does not necessarily require a redox-active metal center.

CONCLUSIONS

Gentisate, salicylate, 1-hydroxy-2-naphthoate, 3-hydroxyanthranilate, and 4-amino-3-hydroxybenzoate occur as intermediates in the bacterial degradation of substituted benzoates, naphthalene and derivatives, and some polycyclic aromatic hydrocarbons. Ring-cleaving dioxygenases active toward these noncatecholic compounds belong to the cupin superfamily and utilize a mononuclear Fe^{II} center for catalysis. Their catalytic strategy has been proposed to involve a one-electron transfer to dioxygen, possibly via transient formation of an Fe^{III}-$O_2^{\cdot -}$ intermediate, as observed in type I extradiol dioxygenases (83, 84), which are members of the VOC superfamily (5). However, it should be emphasized that compared to the thoroughly studied extradiol dioxygenase reaction mechanism, much less experimental evidence is available on the catalytic mechanism of the cupin-type ring-cleaving dioxygenases.

Both the cupin β-barrel fold and the paired $\beta\alpha\beta\beta\beta$ modules of the VOC superfamily proteins provide a scaffold for a metal coordination environment with two or three readily accessible coordination sites, and both folds afford a remarkable breadth of catalytic diversity (5, 33–35, 68). With respect to the dioxygenases that catalyze the cleavage of aromatic C-C bonds, it seems likely that convergent evolution of the Fe^{2+}-dependent enzymes with a cupin scaffold and the extradiol dioxygenases of the VOC family has led to a similar catalytic mechanism.

The 3His- or 2- or 3His-1Glu Fe^{2+} binding motif of the cupin dioxygenases can be considered a variation of the 2His-1carboxylate facial triad motif, which is common not only in the type I extradiol dioxygenases but also in many other nonheme Fe^{II}-containing oxygenases of different fold families (25, 73, 118). However, the cupin-type motif apparently is less selective with regard to the divalent metal ion utilized than the 2His-1carboxylate center. Metal "promiscuity" in particular seems to be a distinctive feature of the few bacterial quercetinases identified until now. The enzymes from *Bacillus subtilis* and *Streptomyces* sp. can utilize several divalent metal ions for catalysis, but it is interesting to note that the *Streptomyces* enzyme is most active with Ni^{2+}, whereas the *Bacillus* enzyme prefers Mn^{2+}. In contrast to the cambialistic bacterial enzymes, fungal quercetinases seem to depend on Cu^{2+}. With respect to the biochemistry of quercetinases, a number of questions remain to be answered. The molecular basis for the observed differences in metal specificity or promiscuity and the mechanistic implications of distinct metal preferences are at present unclear. It seems that monodentate (instead of bidentate) binding of the organic substrate as a flavonolate anion is a key step in catalysis, but the mode of dioxygen binding and activation is not well understood. In particular, the question of whether or not the metal ion of prokaryotic quercetinases mediates electron

transfer is still under discussion. It is tempting to speculate that in the cambialistic enzymes, which utilize a range of divalent metal ions, the metal has a nonredox role in catalysis.

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