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The Ins and Outs of Ring-Cleaving Dioxygenases

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ABSTRACT Ring-cleaving dioxygenases catalyze the oxygenolytic fission of catecholic compounds, a critical step in the aerobic degradation of aromatic compounds by bacteria. Two classes of these enzymes have been identified, based on the mode of ring cleavage: intradiol dioxygenases utilize non-heme Fe(III) to cleave the aromatic nucleus *ortho* to the hydroxyl substituents; and extradiol dioxygenases utilize non-heme Fe(II) or other divalent metal ions to cleave the aromatic nucleus *meta* to the hydroxyl substituents. Recent genomic, structural, spectroscopic, and kinetic studies have increased our understanding of the distribution, evolution, and mechanisms of these enzymes. Overall, extradiol dioxygenases appear to be more versatile than their intradiol counterparts. Thus, the former cleave a wider variety of substrates, have evolved on a larger number of structural scaffolds, and occur in a wider variety of pathways, including biosynthetic pathways and pathways that degrade non-aromatic compounds. The catalytic mechanisms of the two enzymes proceed *via* similar iron-alkylperoxo intermediates. The ability of extradiol enzymes to act on a variety of non-catecholic compounds is consistent with proposed differences in the breakdown of this iron-alkylperoxo intermediate in the two enzymes, involving alkenyl migration in extradiol enzymes and acyl migration in intradiol enzymes. Nevertheless, despite recent advances in our understanding of these fascinating enzymes, the major determinant of the mode of ring cleavage remains unknown.

KEYWORDS extradiol dioxygenase, intradiol dioxygenase, catechol, protocatechuate, gentisate, salicylate, hydroxyquinol, hydroquinone, aminophenol, vicinal oxygen chelate superfamily, cupin superfamily

1. INTRODUCTION

Microorganisms have an exceptional ability to utilize aromatic compounds as their sole source of energy and carbon. This capability is critical to maintaining the global carbon cycle. Aromatic compounds are planar, fully conjugated, ring-shaped molecules possessing $(4n+2)$ π electrons, where n is a non-negative integer (Hückel's rule; McMurry, 2004). Formed by a variety of biogeochemical

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processes, these compounds are widely distributed in nature and range in size from low-molecular-mass compounds, such as phenols, to biopolymers, such as lignin. Indeed, lignin is the second most abundant polymer in nature, after cellulose (Alder, 1977). Aromatic compounds are exceptionally stable due to the delocalization of their π orbitals (resonance structure). This property has contributed to the widespread production and usage of natural and non-natural aromatic compounds for a variety of industrial applications, as well as to the distribution of stable, non-natural compounds in the environment.

Microorganisms have evolved diverse catabolic pathways to degrade aromatic compounds, including anaerobic and aerobic strategies. Regardless of the specific catabolic strategy, these pathways involve two key steps: the activation of the thermodynamically stable benzene ring, and its subsequent cleavage. In aerobic microbial degradation, oxygenases activate the benzene ring by catalyzing the incorporation of oxygen-containing substituents (Mason and Cammack, 1992). The critical step of ring fission is then catalyzed by ring-cleaving dioxygenases (Dagley, 1978; Dagley, 1986; Harayama *et al.*, 1992). The efficiency of this catabolic strategy is such that it has been adapted to degrade polyalicyclic compounds (Martin and Mohn, 2000; Horinouchi *et al.*, 2001).

This review covers metalloenzymes that are involved in the oxidative ring-cleavage of aromatic compounds. Two other classes of ring-cleaving enzymes involved in the aerobic catabolism of aromatic compounds (but not covered here) are the cofactorless dioxygenases that catalyze the 2,4-cleavage of 3-hydroxy-4-quinolones (Frerichs-Deeken *et al.*, 2004; Frerichs-Deeken and Fetzner, 2005) and hydrolytic enzymes. The former possess a serine hydrolase fold and were reviewed by Fetzner (2002). The latter were initially identified as part of the CoA-driven reductive pathways responsible for the anaerobic degradation of aromatic compounds. However, it is now clear that some of these pathways function under aerobic conditions (Mohamed *et al.*, 2001; Zaar *et al.*, 2001; Gescher *et al.*, 2002; Navarro-Llorens *et al.*, 2005; Denef *et al.*, 2006).

In addition to their fundamental significance, ring-cleaving dioxygenases are of interest due to their potential utility in the degradation of environmental pollutants such as polychlorinated biphenyls (PCBs). More specifically, many bacteria are able to cometabolize xenobiotic compounds that structurally resemble naturally occurring growth substrates. These organisms and their catabolic pathways are of interest for bioremedial applications. In many instances, the ring-cleaving dioxygenase of the relevant pathway is an important determinant of the specificity of the pathway. For example, the inactivation and/or specificity of these enzymes inhibit the simultaneous degradation of chloro- and alkylaromatics (Rojo *et al.*, 1987) as well as the degradation of certain PCB congeners (Dai *et al.*, 2002). Several strategies to improve the degradation of such recalcitrant compounds have been described (Timmis *et al.*, 1994). More recently, directed evolution has been used to alter the substrate specificity of ring-cleavage dioxygenases with a view to increasing their utility for biodegradation (Okuta *et al.*, 1998; Kikuchi *et al.*, 1999; Okuta *et al.*, 2004; Ohnishi *et al.*, 2004; Fortin *et al.*, 2005).

2. OCCURRENCE WITHIN CATABOLIC PATHWAYS

In principle, microorganisms utilize a distinct catabolic pathway to degrade each type of aromatic compound. Nevertheless, the aerobic catabolism of these compounds usually proceeds via one of four intermediates (Figure 1): catechol, protocatechuate, gentisate, or hydroquinone (benzene-1,4-diol). Related compounds, such as homoprotocatechuate, dihydroxyphenyl propionates, and homogentisate, also occur as intermediates. In addition, at least two other types of compounds have been identified as substrates for ring cleavage reactions: salicylate and 2-aminophenol. These various intermediates occur in the degradation of monocyclic compounds, as described in the subsections below. Compounds containing more than one aromatic ring are degraded *via* iterations of the strategies used to degrade monocyclic compounds (Figure 1A).

The ring cleavage of catecholic compounds is performed by enzymes from one of two distinct classes: intradiol and extradiol dioxygenases, respectively (Figure 2) (Harayama and Rekik, 1989). Intradiol dioxygenases utilize non-heme Fe(III) to cleave the aromatic nucleus *ortho* to (between) the hydroxyl substituents. In contrast, extradiol dioxygenases utilize non-heme Fe(II) to cleave the aromatic nucleus *meta* (adjacent) to the hydroxyl substituents. Interestingly, a few Mn(II)-dependent extradiol dioxygenases with strong sequence similarity to Fe(II) counterparts have also been reported (Que and Reynolds, 2000; Hatta

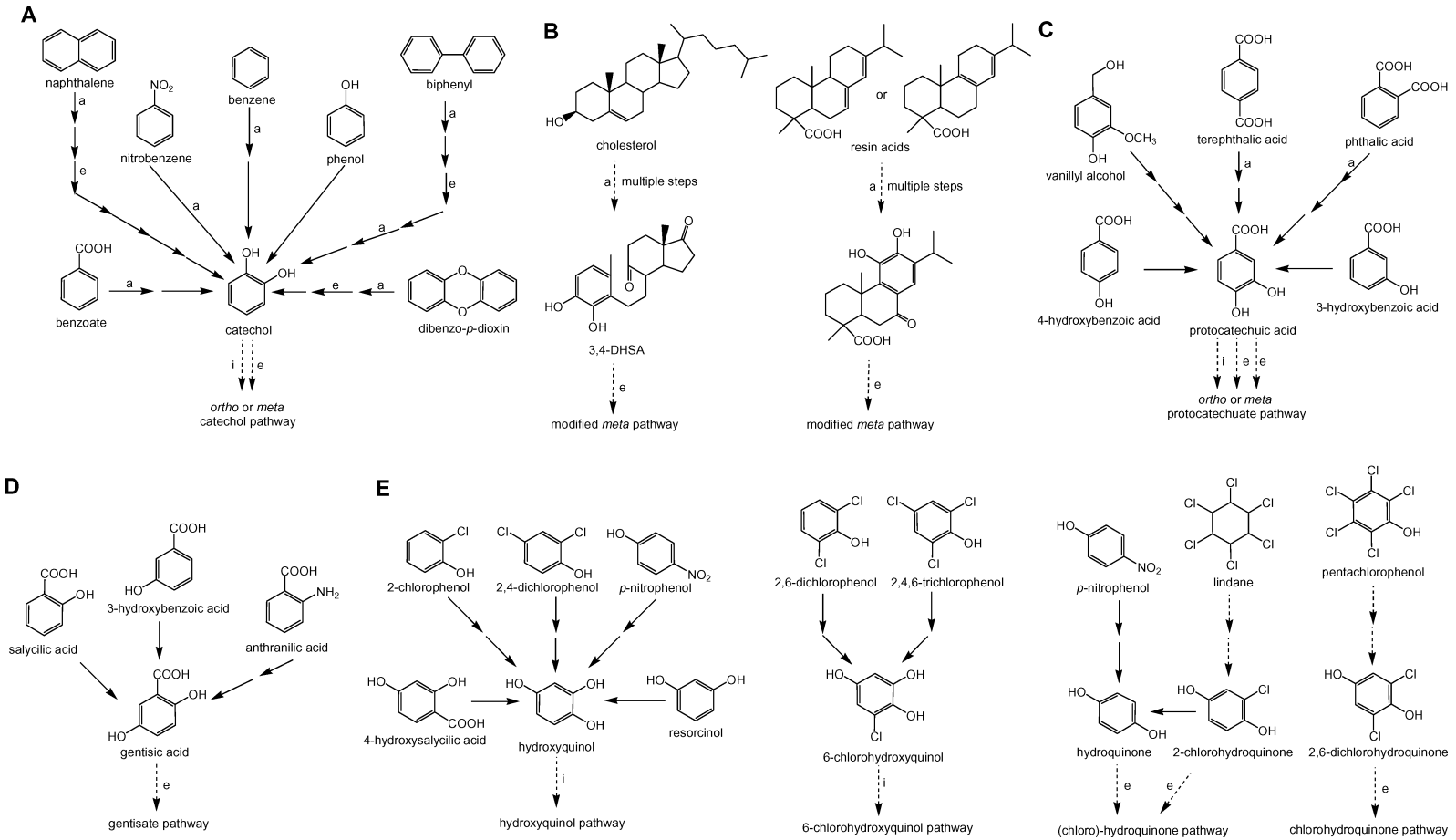

FIGURE 1 **Pathways for the catabolism of aromatic compounds leading to one of four intermediates: (A) catechol; (B) cryptic aromatic catabolism via a substituted catechol; (C) protocatechuic acid; (D) gentisic acid; and (E) (chloro)hydroxyquinol and (chloro)hydroquinone. Each solid arrow indicates a single enzyme-catalyzed reaction. Dotted arrows indicate multiple enzymatic steps. Reactions designated by "a" are catalyzed by a Rieske non-heme iron oxygenase. Reactions designated by "i" are catalyzed by an intradiol dioxygenase and reactions designated by "e" are catalyzed by an extradiol or extradiol-type dioxygenase. The summarized pathways are representative of those involving one of the four ring-cleavage intermediates. Several other pathways have been reported but are not described in detail in this review.**

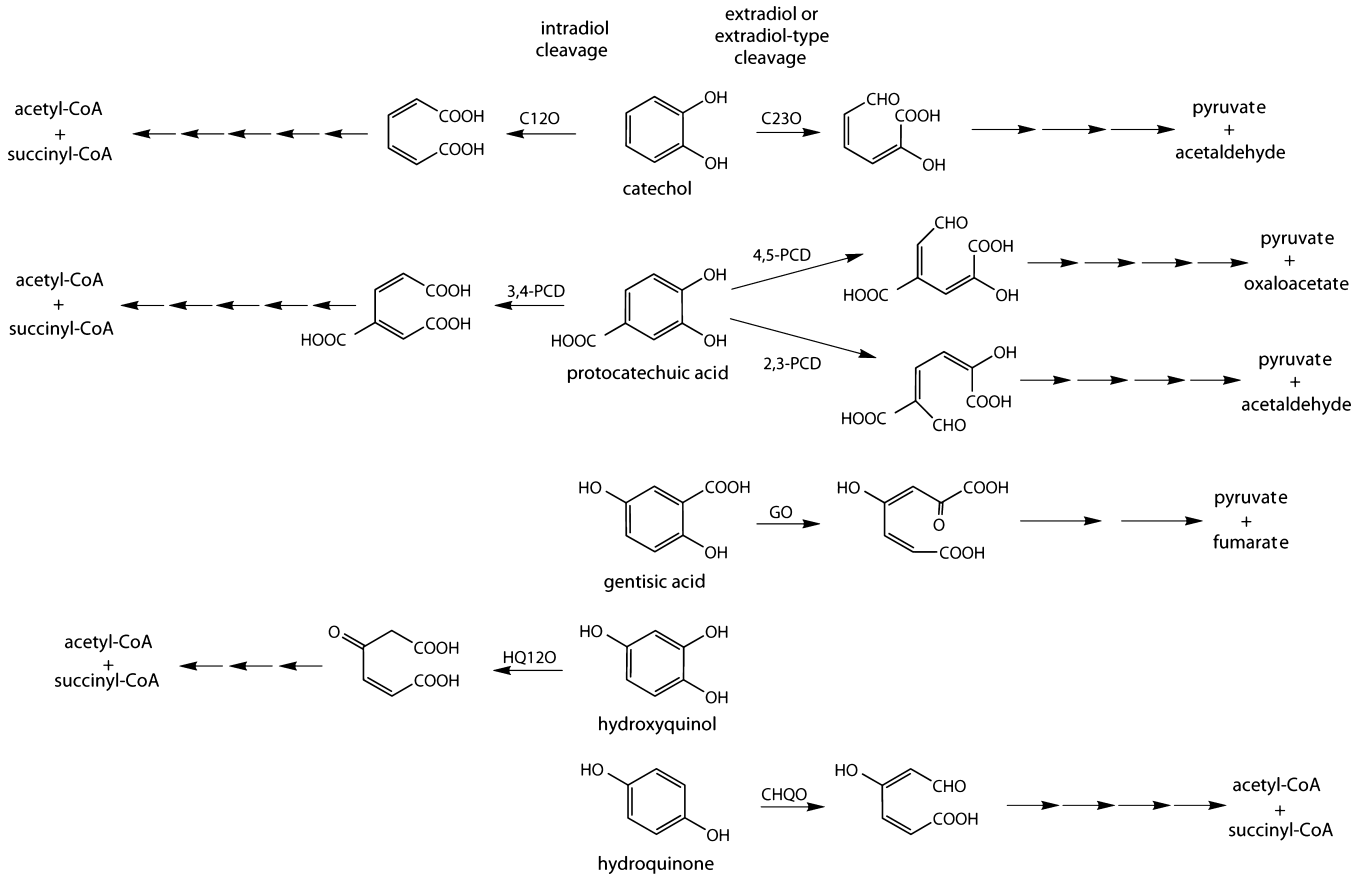

FIGURE 2 **Products of ring-cleavage dioxygenase-catalyzed reactions. Each arrow indicates a single enzyme-catalyzed reaction. Acetaldehyde enters the tricarboxylic acid cycle as acetyl-CoA. (Adapted from Dagley, 1978, 1986).**

et al., 2003; Miyazawa *et al.*, 2004), as has a Mg(II)-containing enzyme of unknown phylogeny (Gibello *et al.*, 1994). Although the distinctions between intradiol and extradiol dioxygenases may appear to be minor, they are in fact a manifestation of enzymes that have completely different structures and utilize different catalytic mechanisms (for reviews see Solomon *et al.*, 2000 and Bugg and Lin, 2001).

Overall, extradiol dioxygenases appear to be more versatile than their intradiol counterparts. Thus, the former cleave a wider variety of substrates, have evolved on a larger number of structural scaffolds, and occur in a wider variety of pathways, including some biosynthetic pathways and pathways that degrade non-aromatic compounds. This increased versatility might ultimately reflect the apparent requirement of intradiol enzymes for substrates possessing vicinal hydroxyl groups. In addition to catechol, the enzymes' only known substrates are protocatechuate and 2-hydroxyquinol (1,2,4-trihydroxybenzene), which are essentially substituted catechols. By contrast, not all aromatic compounds that are subject to extradiol-type cleavage possess vicinal hydroxyl groups. Non-catecholic compounds that are subject to extradiol-type cleavage include the other intermediates mentioned above: gentisate, hydroquinone, salicylate, and 2-aminophenol. In comparison to the substrates of typical extradiol dioxygenases, these compounds are either dihydroxylated in the *para* positions and/or possess a carboxylate or an amino group in place of the second hydroxyl group. The products of the ring cleavage of each of the four "major" intermediates listed at the outset of this section, and their further transformation to intermediates of the tricarboxylic acid cycle, are summarized in Figure 2. As discussed in section 9, the absolute requirement of intradiol dioxygenases for substrates possessing vicinal hydroxyl groups is consistent with the proposed mechanistic differences between intradiol and extradiol enzymes.

Another difference between intradiol and extradiol enzymes is that the former generally cleave catechols possessing mildly electron-withdrawing substituents *in vivo*. By contrast, extradiol enzymes cleave catechols possessing electron-donating substituents *in vivo*. There are, nevertheless, examples of extradiol enzymes that

cleave halogenated substrates. Thus, in the catabolism of chlorobenzene by *Pseudomonas putida* GJ31, a chlorocatechol 2,3-dioxgenase ($CC23O_{GJ31}$) catalyzes the cleavage of 3-chlorocatechol (Mars *et al.*, 1997). 3-Chlorocatechol may also be cleaved in a 1,6-fashion (Riegert *et al.*, 1998). However, the physiological relevance of this reaction is unclear, in part because *in vitro* studies indicate that the two classes of enzymes cleave similar ranges of substrates. Indeed, intradiol enzymes are apparently unable to transform substrates possessing strongly electron-withdrawing substituents *in vitro* (Tyson, 1975; May *et al.*, 1978), whereas extradiol enzymes can cleave compounds such as nitrocatechol at a low rate (Groce and Lipscomb, 2005).

Catechols

In many respects, catechol dioxygenases are the prototypical ring-cleavage enzymes. Thus, a metapyrocatechase, or catechol 2,3-dioxygenase (C23O) from a pseudomonad was the first identified extradiol dioxygenase (Kojima *et al.*, 1961). Studies on C23O were the first to demonstrate these dioxygenases require ferrous ion (Nozaki *et al.*, 1968) and that they utilize an ordered, ternary complex mechanism (Hori *et al.*, 1973). Similarly, one of the first identified intradiol dioxygenases was a catechol 1,2-dioxygenase involved in the catabolism of benzoates (Hayaishi and Hashimoto, 1950).

Catechol occurs in the degradation of benzene (Gibson *et al.*, 1968), benzoate (Murray *et al.*, 1972), phenol (Hughes and Bayly, 1983), and derivatives thereof. The latter include alkylated, nitrosylated, and chlorinated derivatives. Catecholic intermediates that arise in the degradation of polycyclic aromatics may be considered as substituted catechols. Accordingly, intermediates such as 1,2-dihydroxynaphthalene and 2,3-dihydroxybiphenyl, which occur in the degradation of naphthalene and biphenyl, respectively (Catelani *et al.*, 1973; Jeffrey *et al.*, 1975), are subject to extradiol cleavage.

Interestingly, catechols also occur in the degradation of diterpenoids and steroids in what may be termed "cryptic aromatic catabolism" (Figure 1B). Thus, diterpenoids and steroids are degraded via 7-oxo-11,12-dihydroxydehydroabietic acid (Martin and Mohn, 2000) and 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3,4-DHSA) (Horinouchi *et al.*, 2001), respectively. The degradation of plant-derived diterpenoids and steroids may be responsible for the multiplicity of extradiol dioxygenases in rhodococcal strains in particular. For example, *Rhodococcus* species (sp.) RHA1 appears to contain 4 steroid degradation pathways (McLeod *et al.*, 2006).

Protocatechuates

Protocatechuate occurs in the degradation of hydroxybenzoates (Crawford, 1976; Mashetty *et al.*, 1996), phthalates (Keyser *et al.*, 1976), and vanillyl alcohols (Mattevi *et al.*, 1997, Priefert *et al.*, 1997). Protocatechuate is subject to three different modes of cleavage: intradiol cleavage, catalyzed by protocatechuate 3,4-dioxygenase (3,4-PCD; Stanier and Ingraham, 1954); 2,3-extradiol cleavage, catalyzed by protocatechuate 2,3-dioxygenase (2,3-PCD; Wolgel and Lipscomb, 1990); and 4,5-extradiol cleavage, catalyzed by protocatechuate 4,5-dioxygenase (4,5-PCD; Dagley *et al.*, 1968; Ono *et al.*, 1970).

Several compounds related to protocatechuate are cleaved by extradiol dioxygenases. These include gallate (5-hydroxyprotocatechuate), produced in the degradation of syringate (Kasai *et al.*, 2005; Nogales *et al.*, 2005) and homoprotocatechuate (3,4-dihydroxyphenylacetate). The latter is cleaved by homoprotocatechuate 2,3-dioxygenase (HPCD) in the degradation of 4-chlorophenylacetate (Klages *et al.*, 1981), 3- and 4-hydroxyphenylacetate (Cooper and Skinner, 1980). Related HPCDs utilizing Fe(II) and Mn(II) have been isolated from *Brevibacterium fuscum* (Wang and Lipscomb, 1997) and *Arthrobacter globiformis* CM-2 (Whiting *et al.*, 1996), respectively. A HPCD that utilizes Mg(II) has also been purified (Gibello *et al.*, 1994). However, the phylogenetic relationship of this enzyme to other extradiol dioxygenases is not known.

Gentisates and Salicylates

The ring cleavage of gentisate is catalyzed by gentisate 1,2-dioxygenase (GO) (Crawford *et al.*, 1975; Harpel and Lipscomb, 1990), an extradiol-type dioxygenase. Gentisate has been identified as an intermediate in the catabolism of salicylate (Grund *et al.*, 1992), 3-hydroxybenzoic acid (Hopper and Taylor, 1975) and anthranilate (Cain, 1968). As noted in the section on 2-aminophenols, an important derivative of gentisate, homogentisate, occurs in the catabolism of phenylalanine and tyrosine.

A GO has been described that can also cleave salicylate (Hintner *et al.*, 2001). Although this is an interesting reaction, it is unclear whether there are any dioxygenases that preferentially utilize salicylate. Related compounds that are subject to extradiol-type cleavage include 5-aminosalicylate (Stolz *et al.*, 1992) and 1-hydroxy-2-naphthoate, which is involved in the catabolism of phenanthrene (Iwabuchi and Harayama, 1998). 1-Hydroxy-2-naphthoate dioxygenase did not detectably cleave either salicylate or gentisate.

Hydroxyquinols and Hydroquinones

Hydroxyquinols and hydroquinones are 1,4-dihydroxybenzenes (Figure 1E). 2-Hydroxyquinols and its chlorinated derivatives are cleaved by 2-hydroxyquinol 1,2-dioxygenase (HQ12O), an intradiol enzyme. This enzyme occurs in the catabolism of *p*-nitrophenol (Jain *et al.*, 1994), resorcinol (Gaal and Neujahr, 1979), 4-chlorophenol (Nordin *et al.*, 2005), and 4-hydroxysalicylic acid (Armengaud *et al.*, 1999). The related 6-chloro-HQ12O occurs in the catabolism of 2,6-dichlorophenol (Zaborina *et al.*, 1995) and 2,4,6-trichlorophenol (Zaborina *et al.*, 1995). It is unclear whether HQ12O or 6-Cl HQ12O is involved in the catabolism of 2-chlorophenol and 2,4-dichlorophenol (Zaborina *et al.*, 1995). Hydroquinones are cleaved by an extradiol-type enzyme, hydroquinone dioxygenase (HQO). This enzyme is involved in the degradation of *p*-nitrophenol (Chauhan *et al.*, 2000), pentachlorophenol (Xu *et al.*, 1999), and lindane (Miyauchi *et al.*, 1999), which is aromaticized as it is dehalogenated.

2-Aminophenols

2-Aminophenols are cleaved by Fe(II)-dependent extradiol-type dioxygenases. 2-Aminophenol 1,6-dioxygenases (APDs) are involved in the degradation of nitrobenzene (Lendenmann and Spain, 1996) and 2-aminophenol (Takenaka *et al.*, 1997). A notable derivative of 2-aminophenol is 3-hydroxyanthranilate. This compound is an intermediate in the catabolism of 2-nitrobenzoate by a pseudomonad (Muraki *et al.*, 2003) as well as in the catabolism of tryptophan.

Aromatic Amino Acids

Ring-cleavage reactions are also involved in the aerobic catabolism of aromatic amino acids. Thus, homogentisate dioxygenase (HGO) is involved in the catabolism of phenylalanine and tyrosine, and 3-hydroxyanthranilate dioxygenase (HAD) is involved in the catabolism of tryptophan in the kynurenine pathway. These dioxygenases, which are extradiol-type enzymes, occur in organisms from bacteria to man. In humans, HGO and HAD are associated with the genetic disorders alkaptonuria and Huntington's disease, respectively (La Du *et al.*, 1958; Schwarcz *et al.*, 1988).

Biosynthesis

Recent studies have revealed that ring-cleaving dioxygenases are not confined to catabolic roles: extradiol dioxygenases play a role in the biosynthesis of a variety of biologically active compounds. For example, an extradiol dioxygenase catalyzes the cleavage of L-3,4-dihydroxyphenylalanine (L-DOPA) aromatic ring during the biosynthesis of lincomycin and possibly anthramycins (Novotna *et al.*, 2004). A similar reaction occurs in the biosynthesis of the plant pigment betalaine (Christinet *et al.*, 2004).

3. CLASSIFICATION OF RING-CLEAVING DIOXYGENASES

Intradiol and extradiol enzymes share no significant sequence or structural similarities and thus belong to evolutionary distinct classes of proteins. Sequence and structural analyses further indicate that all intradiol dioxygenases characterized to date belong to a single evolutionary lineage. Thus, despite their different subunit compositions, the catalytic domains of 3,4-PCD and C12O share a common structural fold (Vetting and Ohlendorf, 2000). Moreover, these enzymes share key conserved residues with the HQ12O (hydroxyquinol 1,2-dioxygenase), including the four endogenous iron ligands (Daubaras *et al.*, 1995; see section 5).

In contrast to the intradiol dioxygenases, extradiol and extradiol-type dioxygenases belong to at least three evolutionarily independent families (Eltis and Bolin, 1996; Dunwell *et al.*, 2001; Table 1). Type I extradiol dioxygenases belong to the vicinal oxygen chelate superfamily (Armstrong, 2000; Gerlt and Babbitt, 2001). Type I extradiol dioxygenase includes two-domain and one-domain enzymes. The former are exemplified by 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD) from *Burkholderia xenovorans* LB400 ($DHBD_{LB400}$), $C23O_{mt2}$, $HPCD_{Bfusc,}$ and chlorohydroquinone dioxygenase (CHQO; Miyauchi *et al.*, 1999; Xu *et al.*, 1999).

TABLE 1 **Families of ring-cleavage enzymes based on structural folds**

Type	Superfamily	Prototypic members	Subunit[1]
Intradiol		3,4-PCD_{B-10}	$(\alpha\beta)_{12}$
		$C12O_{ADP1}$	α_2
		$HQ12O_{3E}$	α_2
Extradiol			
I	Vicinal oxygen chelate	$DHBD_{LB400}$	α_8
		$C23O_{mt2}$	α_4
		$HPCD_{Bfu}$	α_4
		CHQO	α_x
		$DHBD_{P6}$-III	α_2
		L-DOPA dioxygenase$_{lincolnensis}$	α_2
II	Unknown	$DHPPD_{Eco}$	α_4
		Gallate dioxygenase$_{KT2440}$	α_3
		$HPCD_{EcoC}$	α_x
		$C23O_{JMP222}$-I	α_x
		4,5-PCD_{SYK6}	$\alpha_2\beta_2$
		4,5-PCD_{T2}	$\alpha_4\beta_4$
		APD_{JS45}	$\alpha_2\beta_2$
III	Cupin	GO	α_4
		1-hydroxy-2-naphthoate dioxygenase$_{KP7}$	α_6

3,4-PCD_{B-10}, protocatechuate 3,4-dioxygenase from *P. putida* B-10 (Ohlendorf *et al.*, 1988); $C12O_{ADP1}$, catechol 1,2-dioxygenase from *Acinetobacter* sp. ADP1 (Vetting and Ohlendorf, 2000); HQ12O, hydroxyquinol 1,2-dioxygenase from *Nocardioides simplex* 3E (Ferraroni *et al.*, 2005); $DHBD_{LB400}$, 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Burkholderia* sp. LB400 (Han *et al.*, 1995); $C23O_{mt2}$, catechol 2,3-dioxygenase from *P. putida* mt-2 (Kita *et al.*, 1999); $HPCD_{Bfu}$, homoprotocatechuate 2,3-dioxygenase from *B. fuscum* (Vetting *et al.*, 2004); CHQO, chlorohydroquinone dioxygenase; $DHBD_{P6}$-III, 2,3-dihydroxybiphenyl 1,2-dioxygenase III from *R. globerulus* P6 (Asturias *et al.*, 1994); L-DOPA dioxygenase (LmbB1) from *Streptomyces lincolnensis* (Novotna *et al.*, 2004); $DHPPD_{Eco}$, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase from *E. coli* (Bugg, 1993); gallate dioxygenase from *P. putida* KT2440 (Nogales *et al.*, 2005); $HPCD_{EcoC}$, homoprotocatechuate 2,3-dioxygenase from *E. coli* C (Roper and Cooper, 1990); $C23O_{JMP222}$-I, catechol 2,3-dioxygenase I from *Alcaligenes eutrophus* JMP222 (Kabisch and Fortnagel, 1990); 4,5-PCD_{SYK6}, protocatechuate 4,5-dioxygenase from *Sphingomonas paucimobilis* SYK-6 (Sugimoto *et al.*, 1999); 4,5-PCD_{T2}, protocatechuate 4,5-dioxygenase from *Comomonas testosteroni* T-2 (Mampel *et al.*, 2005); APD_{JS45}, 2-aminophenol 1,6-dioxygenase from *P. pseudoalcaligenes* JS45 (Davis *et al.*, 1999); GO, gentisate dioxygenase from *P. testosteroni* (Harpel and Lipscomb, 1990); 1-hydroxy-2-naphthoate dioxygenase from *Nocardioides* sp. KP7 (Iwabuchi and Harayama, 1998). [1]An "x" indicates that the oligomeric state of the enzyme is not known.

One-domain enzymes are exemplified by two DHBD isozymes from *Rhodococcus globerulus* P6 ($DHBD_{P6}$-II and $DHBD_{P6}$-III) and by L-DOPA dioxygenase (LmbB1) from *Streptomyces lincolnensis*. Type II extradiol dioxygenases include enzymes consisting of one (*e.g.*, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase ($DHPPD_{Eco}$; Spence *et al.*, 1996a) or two different subunits. As discussed in Section 4, there are examples of type II enzymes in which the two different subunits are related (APD_{JS45} [Davis *et al.*, 1999]), and there are examples in which the two subunits appear to be unrelated (protocatechuate 4,5-dioxygenase (4,5-PCD), Sugimoto *et al.*, 1999). A third family of extradiol-type dioxygenases belongs to the cupin superfamily (Dunwell *et al.*, 2001). Dioxygenases belonging to this superfamily include GO, 1-hydroxy-2-naphthoate dioxygenase, HGO and HAD.

As is evident from Table 1, there is no strict correlation between substrate specificity and evolutionary origin in extradiol dioxygenases. Thus, there are examples of type I and II HPCDs, as well as type I and II C23Os. It is nonetheless noted that the substrate specificity of most ring-cleavage enzymes has not been well investigated. This shortcoming is particularly evident in cases in which multiple DHBDs have been identified in a single strain. In many instances, such enzymes were identified on the basis of plate assays performed using a single substrate at a single concentration. In the case of *Rhodococcus* sp. RHA1, genomic studies indicate that 4 of its 13 type I extradiol dioxygenases, including some annotated as BphC, are involved in steroid degradation (Gonçalves *et al.*, 2006; McLeod *et al.*, 2006).

The respective superfamilies to which type I and III extradiol dioxygenases belong are discussed below. The type II extradiol dioxygenases are not discussed in this respect, as they have not been classified in a superfamily. The structural features of each type of extradiol dioxygenase are discussed in Section 4.

Type I Extradiol Dioxygenases and the Vicinal Oxygen Chelate Superfamily

The conserved structural domain of the vicinal-oxygen-chelate superfamily (Armstrong, 2000; Gerlt and Babbitt, 2001) includes two copies of a module consisting of 4 β strands and 1 α helix that occur in the following sequence: $\beta\alpha\beta\beta\beta$. Phylogenetic analyses indicate that the type I dioxygenases share a one-domain ancestor; the evolution of type I extradiol dioxygenases therefore appears to have involved two duplication events followed by the divergence of one- and two-domain enzymes. Subsequent divergence among the two-domain dioxygenases has resulted in several

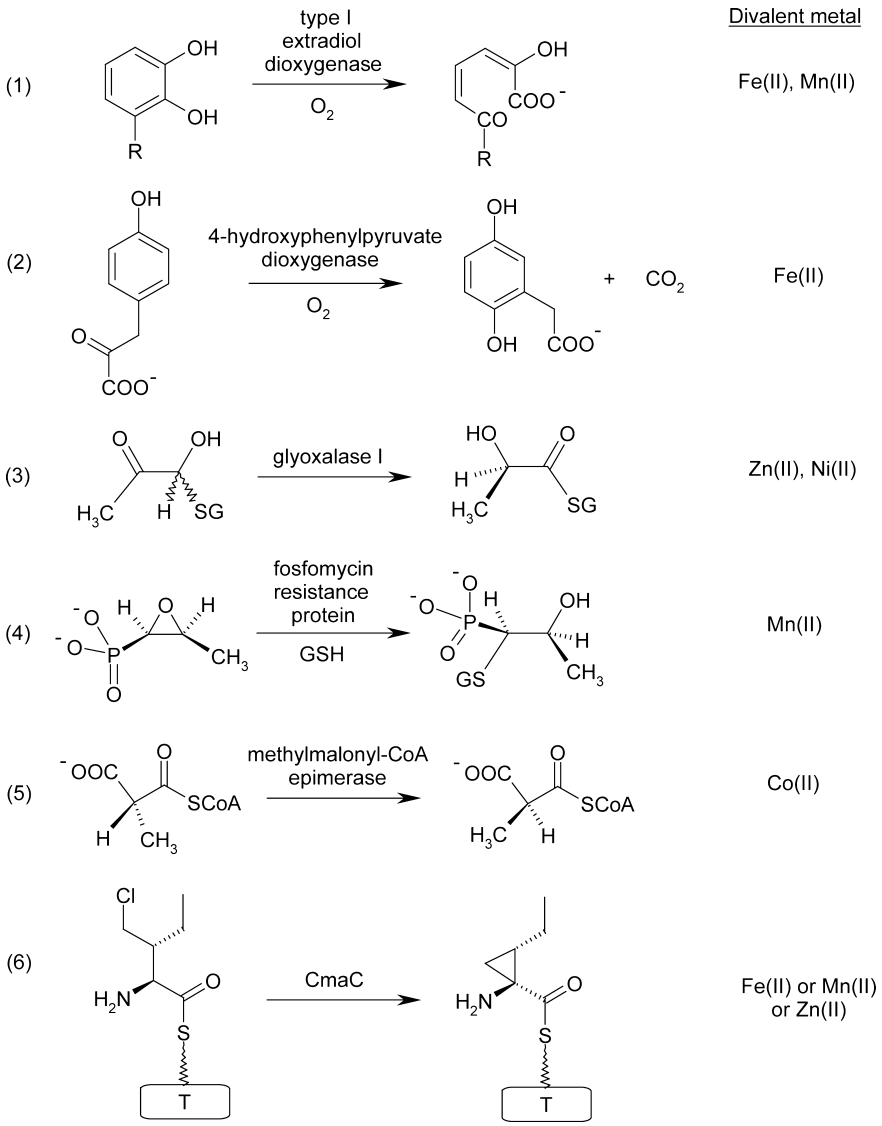

FIGURE 3 **Reactions catalyzed by members of the vicinal oxygen chelate superfamily (adapted from Gerlt and Babbitt, 2001).**

families, at least two of which are based on substrate preference (Harayama and Rekik, 1989; Eltis and Bolin, 1996). $DHBD_{LB400}$ and $DHBD_{P6}$-I belong to a family with a preference for bicyclic substrates. In contrast, $C23O_{mt2}$ belongs to a family with a preference for monocyclic substrates.

Most members of the vicinal-oxygen-chelate superfamily utilize a divalent metal ion to catalyze a reaction involving direct metal ion chelation by vicinal oxygens of the substrate or an intermediate in the reaction. The members of the superfamily identified to date are: 1. type I extradiol dioxygenases; 2. 4-hydroxyphenylpyruvate dioxygenase (Serre *et al.*, 1999, EC 1.13.11.27); 3. glyoxalases I (Cameron *et al.*, 1997, EC 4.4.1.5); 4. fosfomycin resistance proteins (Bernat *et al.*, 1997); and 5. methylmalonyl-CoA epimerases (McCarthy *et al.*, 2001, EC 5.1.99.1). The metal ion requirement of these enzymes, and the reactions they catalyze, are summarized in Figure 3. Another group of enzymes, exemplified by CmaC, are highly homologous to methylmalonyl epimerases but catalyze the transformation of chlorinated amino acids to cyclopropanes while the latter are attached to thiolation domains (Vaillancourt *et al.*, 2005b). To date, the one exception in the superfamily is the bleomycin resistance proteins (BRP; Dumas *et al.*, 1994), which form a sixth family of the vicinal-oxygen-chelate superfamily. BRPs do not bind any metal ion and appear to sequester bleomycin and related compounds without degrading or transforming them. It is thought that during the evolution of this protein, the divalent metal ion may have been shed in favor of a more hydrophobic cavity to

accommodate the antibiotic. The use of the pseudosymmetric structure provided by the pair of $\beta\alpha\beta\beta\beta$ modules offers a versatile template for metal binding.

Type III Extradiol Dioxygenases and the Cupin Superfamily

GO, 1-hydroxy-2-naphthoate dioxygenase, HGO, and HAD are part of the cupin superfamily (Dunwell *et al.*, 2001). This superfamily is composed of proteins containing at least one domain with six antiparallel β-sheets that form a β-barrel structure. Within this β-barrel are two distinct motifs. The first motif is composed of the first two β-sheets and the second, of the last two β-sheets. One of the major differences between the various classes of cupins involves variations of the two middle β-strands and the less conserved loop of variable length that separates them. Some cupins exemplified by HAD, germin (oxalate oxidase), and germin-like proteins involved in the response of plants to pathogens and stresses are composed of a single domain. Other cupins are composed of two copies of the domain that probably arose from gene duplication. The group of two-domain cupins is exemplified by GO, 1-hydroxy-2-naphthoate dioxygenase, oxalate decarboxylase, and seed storage proteins. A special class of cupins exemplified by AraC/XylS-type transcription factors and some helix-turn-helix transcription factors are composed of a cupin domain linked to a DNA binding domain. In these proteins, the cupin domain binds the effector molecule (Dunwell *et al.*, 2000, 2001).

In those cupins that possess a catalytic function, a metal ion is often present at the active site. When present, the metal ion ligands are found in each of the two β-sheet motifs. Cupins utilise as diverse a range of metal ions as the vicinal oxygen chelate superfamily: iron in GO (Harpel and Lipscomb, 1990), 1-hydroxy-2-naphthoate dioxygenase (Iwabuchi and Harayama, 1998), HGO (Titus *et al.*, 2000), HAD (Vescia and Di Prisco, 1962), and cysteine dioxygenase (Yamaguchi *et al.*, 1978); manganese in oxalate decarboxylase, germin (oxalate oxidase) and germin-like proteins (Woo *et al.*, 2000; Anand *et al.*, 2002); zinc in phosphomannose isomerase (Cleasby *et al.*, 1996); copper in quercetin 2,3-dioxygenase (Fusetti *et al.*, 2002); nickel in acireductone dioxygenase (Pochapsky *et al.*, 2002) and nickel or cobalt in enolase-type enzymes (Dai *et al.*, 1999).

4. STRUCTURAL ASPECTS OF EXTRADIOL DIOXYGENASES

Crystal structures of Fe(II)-dependent extradiol and extradiol-type dioxygenases are now available in the ferrous (active) form for each of $DHBD_{LB400}$ (Figure 4A, Han *et al.*, 1995), DHBD from *Peudomonas* species

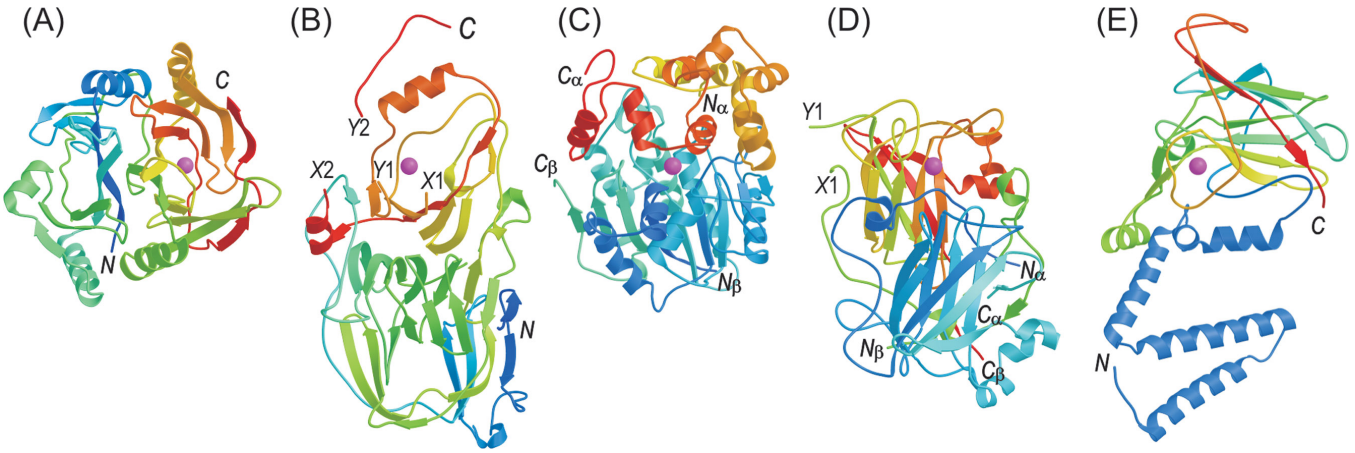

FIGURE 4 Ribbon drawings of the monomers of (*A*) $DHBD_{LB400}$ and (*B*) HGO, and the protomers of (*C*) 4,5-PCD_{SYK-6}, (*D*) 3,4-PCD_{B-10} and (*E*) $HQ12O_{3E}$. Each drawing is on the same scale and the Fe atoms, which are drawn as magenta spheres, have been aligned at the same vertical position. For $DHBD_{LB400}$ and HGO the course of the polypeptide backbone is colour-ramped from blue (N-terminus) to red (C-terminus). For 4,5-PCD_{SYK-6}, the larger β subunit is colored from blue (N-terminus) to blue-green (C-terminus), and the α chain is colored from yellow (N-terminus) to red (C-terminus). For 3,4-PCD_{B-10}, the larger α chain is colored from blue (N-terminus) to blue-green (C-terminus), and the β chain is colored from green (N-terminus) to red (C-terminus). For $HQ12O_{3E}$, residues 2-100 (dimerization domain) are colored blue, and the remainder of the residues (catalytic domain) are color-ramped from blue to red (C-terminus). The N- and C-terminal residues of each chain are labeled with *N* and *C*. In the drawings of HGO and 3,4-PCD_{B-10}, residues not resolved in the crystal structures are represented by breaks in the ribbons; symbols (*X*1, *Y*1) and (*X*2, *Y*2) mark the beginning (*X*) and end (*Y*) of breaks. Programs MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merritt and Bacon, 1997) were used to prepare the drawings.

KKS102 ($DHBD_{KKS102}$; Uragami *et al.*, 2001), $C23O_{mt2}$ (Kita *et al.*, 1999), DoxG (Neau, 2004), and $HPCD_{Bfu}$ (Vetting *et al.*, 2004), as well as in the ferric (inactive) form for $DHBD_{KKS102}$ (Senda *et al.*, 1996), 4,5-PCD_{SYK-6} (Figure 4C, Sugimoto *et al.*, 1999) and human HGO (Figure 4B, Titus *et al.*, 2000). The structure of $HPCD_{Aglob}$, an Mn(II)-dependent enzyme, is also available (Vetting *et al.*, 2004). Even though these enzymes represent each of the three families of extradiol and extradiol-type dioxygenases and possess the overall structural folds discussed in section III, they all share similar active sites and all have the same iron ligands: the two histidines and one glutamate that constitute the 2-His 1-carboxylate structural motif. Several other conserved residues identified through sequence alignments of each type of dioxygenases were observed at their respective active sites (Han *et al.*, 1995; Sugimoto *et al.*, 1999; Titus *et al.*, 2000). Further investigation is required to probe the respective roles of these residues in the catalytic mechanism. This section focuses on those structural aspects of extradiol dioxygenases that are not discussed within the context of the classification (Section 3) or catalytic mechanism (Section 6) of these enzymes.

Type I Extradiol Dioxygenases

All Type I extradiol dioxygenases identified to date consist of a single type of subunit. The size of this subunit is typically 21 and 32.5 kDa for one- and two-domain enzymes, respectively. The vast majority of type I enzymes identified to date are two-domain, suggesting that the catalytically inactive N-domain confers some sort of advantage to the host. Steady-state kinetic characterization of one- and two-domain DHBD's in *R. globerulus* P6 suggests that the latter are significantly more catalytically efficient (Vaillancourt *et al.*, 2003). It is thus tempting to speculate that the N-domain enables the C-domain to explore a greater range of sequence space, perhaps by conferring additional stability, thereby permitting the evolution of a more efficient active site. However, characterization of additional enzymes is necessary, particularly as the preferred substates of the one-domain enzymes may not yet have been identified.

The one-domain enzymes are typically dimeric (Table 1). In contrast, the two-domain enzymes exist in a range of oligomeric states. For example, 2,2′,3-trihydroxybiphenyl 1,2-dioxygenase from *Sphingomonas* species RW1 is monomeric (Happe *et al.*, 1993), $C23O_{mt2}$ is tetrameric (Kita *et al.*, 1999), and $DHBD_{LB400}$ is octameric (Han *et al.*, 1995). The physiological significance of these different oligomeric states is not clear.

Sequence alignments (Eltis and Bolin, 1996) and the structures of three different enzymes (Han *et al.*, 1995; Senda *et al.*, 1996; Kita *et al.*, 1999) indicate that the tertiary structures of two-domain type I extradiol dioxygenases are very similar, even though the sequence identities of these enzymes can be less than 15%. The crystal structure of substrate-free $DHBD_{LB400}$, determined at a resolution of 1.9 Å (Han *et al.*, 1995), revealed that each monomer comprises one chain of 297 residues, the N-terminal methionine being excised. Each monomer possesses two domains of very similar structure (Figure 4A), the ferrous ion located in the C-terminal domain (C-domain). Each domain is made-up of two $\beta\alpha\beta\beta\beta$ modules as described in Section 3: modules 1 and 2 comprise the N-domain, modules 3 and 4, the C-domain. A large, funnel-shaped space lies entirely within the domain where the active site ferrous ion is ligated deep within this space in the C-domain. The C-domain possesses two additional β-strands after the common core structure and the central funnel is slightly larger than that of the N-domain. Therefore, evolutionary adaptation of the two-domain enzymes seems to have resulted in the loss of a second active site within the N-domain.

The ferrous ion active site is located midway in the 20 Å long funnel of the C-terminal domain. This funnel is opened at both ends, the large opening is 10 Å wide and the smaller opening is 6 Å wide. Thus the iron is probably only accessible to catecholic substrates from the wide opening, but water or O_2 can access the iron through either end. The coordination geometry of the iron is that of a well-defined square pyramid, with His146 as the axial ligand, and His210, Glu260, and two waters as equatorial ligands in the basal plane (Figure 5A, Han *et al.*, 1995). Spectroscopic studies of $C23O_{mt2}$ (Mabrouk *et al.*, 1991; Shu *et al.*, 1995) and $DHBD_{LB400}$ (Davis *et al.*, 2003) provide further evidence for the five-coordinate, square pyramidal geometry of the iron in the substrate-free enzyme.

The crystal structures of $DHBD_{KKS102}$ with DHB (Uragami *et al.*, 2001; Sato *et al.*, 2002) and $DHBD_{LB400}$ with various catecholic substrates (Dai *et al.*, 2002; Vaillancourt *et al.*, 1998, 2002a) show that the latter bind to the iron inside the funnel-shaped cavity. The

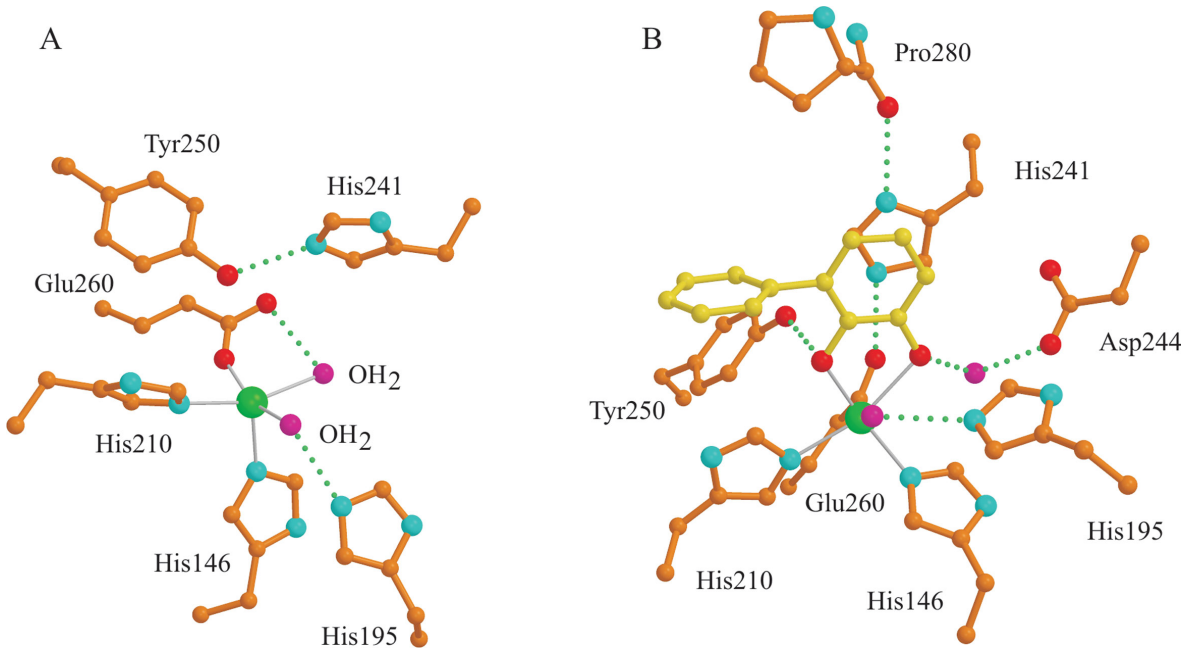

FIGURE 5 Structure of the active site of substrate free (*A*) and DHB-bound (*B*) $DHBD_{LB400}$. Carbon atoms are colored orange in protein residues and yellow in DHB. Oxygen, nitrogen, and iron atoms are colored red, cyan, and green, respectively. Fe-ligand bonds are indicated by grey sticks, and hydrogen bonds are indicated by green dotted lines. (*A*, adapted from Han *et al.*, 1995; *B*, from Vaillancourt *et al.*, 2002a).

catechol ring binds in a restricted pocket that is highly complementary in size and shape. It is generally assumed that the crystallographically observed binding mode, which is similar in all reported complexes, is the one that leads to productive catalysis. One hydroxyl group of the substrate binds in the site trans to His146, whereas the other binds trans to His210, displacing the two ordered water ligands (Figure 5B). Interestingly, a hydrogen-bonded water bridges Asp244 and the 3-hydroxyl group. In some of the ES complexes, a water is also observed between His195 and the Fe. However, the degree of occupancy of this water site seems to depend on the crystal preparation (Vaillancourt *et al.*, 2002a). Indeed, it was not occupied in a recent structure of the $DHBD_{KKS102}$:DHB complex (Sato *et al.*, 2002). Moreover, spectroscopic studies indicate that the $C23O_{mt2}$:catechol (Mabrouk *et al.*, 1991; Shu *et al.*, 1995) and $DHBD_{LB400}$:DHB (Davis *et al.*, 2003) complexes are 5-coordinate. This site is occupied by NO in the $DHBD_{KKS102}$:DHB:NO ternary complex (Sato *et al.*, 2002) and likely represents the site for O_2 binding.

The crystal structure of DoxG, a 1,2-dihydroxynaphthalene dioxygenase, is available (Neau, 2004). DoxG (302 residues) and $DHBD_{LB400}$ (298 residues) share 33% sequence identity, with the identical residues distributed relatively consistently over the entire sequence. Superposition of Cα models pairs 278 residues with an rms deviation of 1.2 Å, and there are no non-terminal insertions/deletions longer than two residues. The ability of DoxG to catalyze cleavage of compounds analogous to 4-methylcatechol, such 1,2-DHN and 3,4-DHB, may be attributed to sequential variations at the surface of the extended active site. These variations affect the shape of the active site as well as the potential for adjustments to accommodate substrate binding (Neau, 2004). A key difference occurs between βQ and βR, where a single residue "deletion" in DoxG removes a proline side chain, P280 in LB400. The proline side chain is in non-bonded contact with C4 of the catechol ring in the LB400:2,3-DHB structure. Although the space occupied by P280 is partially occupied in DoxG by other side chains, crystal structures show that these side chains change position in response to the binding of 1,2-DHN and 3,4-DHB.

The structures of two 2,3-HPCDs are also available (Vetting *et al.*, 2004). Although these enzymes share 83% amino acid sequence identity, they utilize different metals: $HPCD_{Aglob}$ from *Arthrobacter globiformis* is an Mn(II)-dependent enzyme, whereas $HPCD_{Bfu}$ from *Brevibacterium fuscum* is an Fe(II)-dependent enzyme. The enzymes possess the two-domain structure typical of Type I enzymes and the active site metal is coordinated by two histidines and one glutamate. However, metals have two or three solvent ligands depending on

the crystal form of the enzymes. The structures of enzyme:homoprotocatechuate complexes reveal that the substrate binds to the metal in a bidentate fashion in both enzymes and that conserved residues interact with the acetate moiety of the substrate. One major difference with respect to other type I dioxygenases is the presence of a lid domain that closes down over the substrate as it binds. Interestingly, the structures reveal no obvious basis for metal selectivity. In particular, the first and second sphere residues are conserved in these two enzymes. Residues remote from the active site that play a role in metal selectivity have yet to be identified.

Sequence alignments reveal that the one-domain enzymes are similar in structure to the C-domain of type I extradiol dioxygenases. Some of the one-domain enzymes are approximately 65 residues larger than the typical C-domain of two-domain enzymes. The structure and function of these residues are unknown.

Sequence alignments further suggest that no type I extradiol dioxygenases possesses an active site in the N-terminal domain (Eltis and Bolin, 1996). However, an interesting module-swapping event may have occurred in the evolution of HQOs. More specifically, the first iron ligand in type I dioxygenases is a conserved histidine that is normally positioned at the beginning of the first β-strand of module 3 (His146 in $DHBD_{LB400}$). This residue is not conserved in HQOs. However, a histidine residue is conserved at a similar position at the beginning of the first β-strand of module 1. It is tempting to speculate that the two domains of the HQO subfamily of type I enzymes comprise modules 1 and 4 and modules 2 and 3, respectively, and that the former contains the active site.

Type II Extradiol Dioxygenases

As noted in Section 3, the characterized type II extradiol dioxygenases are all multimers possessing one or two different subunit types. For example, $DHPPD_{Eco}$ (Spence *et al.*, 1996a), $HPCD_{EcoC}$ (Roper and Cooper, 1990) and $C23O_{JMP222}$-I (Kabisch and Fortnagel, 1990) are homooligomers, whereas 4,5-PCD_{SYK6} (Sugimoto *et al.*, 1999) and APD (Takenaka *et al.*, 1997; Davis *et al.*, 1999) have $\alpha_2\beta_2$ composition. In the case of 4,5-PCD_{SYK6}, the subunits appear to be unrelated, and the β subunit is similar to the protomers of the homooligomeric enzymes. In contrast, the two subunits of APD share sequence similarity, but it appears that only the β subunit contains an active site.

Of the type II enzymes, the only known structures present the ferric form 4,5-PCD_{SYK6} as the free-enzyme and in a binary complex with the protocatechuate substrate (Figure 4C; Sugimoto *et al.*, 1999). The larger β subunit has 302 amino acids that form a globular α/β structure composed of 11 β stands, nine α helices and one 3_{10} helix. The 139 residues of the α subunit form 10 α-helices, which assemble into a rather non-compact plate-like structure that interacts extensively with one face of the β subunit of the same protomer and with the β subunit of the second protomer. The latter α–β contacts stabilize the α_2/β_2 dimer, which lacks α–α or β–β contacts.

The active site is located in a cleft in the β subunit on a surface that is extensively covered by the α subunit. The catalytically essential Fe is thus buried and is approximately 15 Å from the surface of the enzyme. In the substrate-free form of the enzyme, the Fe is coordinated in a distorted trigonal pyramidal geometry by His residues β12 and β61, Glu β242, and one water molecule. The protein ligands form the base of the pyramid and the Fe is displaced from the basal plane toward the water ligand. A potential weak fifth ligand, Asn β59, is located trans to the water at a distance of 2.9 Å. Although the protein ligands are identical in character to those observed for the type I enzymes, the three-dimensional arrangement of the ligands is effectively enantiomeric in that one His and the Glu ligand exchange locations relative to the positions in $DHBD_{LB400}$ and $C23O_{mt2}$. Binding of protocatechuate involves both hydroxyl groups and displaces the water ligand. The complex has a distorted trigonal bipyramidal geometry, with His β61 and the 3-hydroxyl moiety as axial ligands.

The 2-His-1-Carboxylate Metal-Binding Motif

Despite the phylogenetic diversity of extradiol-type dioxygenases, they all contain a 2-His-1-carboxylate metal-binding motif (Koehntop *et al.*, 2005). This motif is found in a wide variety of unrelated non-heme Fe(II) enzymes, including other microbial catabolic enzymes, illustrating its ability to provide a catalytic basis for diverse reactions. The active site of these

enzymes contains an Fe(II) ligated by two histidines and one carboxylate all located on one face of the Fe(II) coordination sphere. This motif therefore leaves up to three potential ligand sites for solvent species or substrate molecules on the other face of the coordination sphere. In addition to the three families of extradiol-type dioxygenases mentioned above, at least three additional families of enzymes have been identified that utilise this metal ion site: Rieske non-heme iron oxygenases (ROs), pterin-dependent hydroxylases, and α-ketoglutarate dependent enzymes. The latter include some closely related enzymes that do not utilize α-ketoglutarate as a cosubstrate. ROs include ring-hydroxylating dioxygenases, exemplified by naphthalene and biphenyl dioxygenases (Kauppi *et al.*, 1998; Imbeault *et al.*, 2000), which catalyze the NADH-mediated *cis*-dihydroxylation of an arene double bond yielding a *cis*-diol and monooxygenases such as 3-ketosteroid 9α-hydroxylase (Van der Geize *et al.*, 2002). The Fe(II) of these enzymes has two available sites as it is ligated by the two oxygens of the carboxylate group. Pterin-dependent hydroxylases, such as mammalian phenylalanine and tyrosine hydroxylases (Flatmark and Stevens, 1999), use tetrahydrobiopterin as a cofactor to hydroxylate the ring of aromatic amino acids residue in the synthesis of brain signalling molecules. A homolog in *Pseudomonas aeruginosa* hydroxylates phenylalanine to tyrosine (Zhao *et al.*, 1994). Examples of α-ketoglutarate-dependent enzymes and related enzymes (Hausinger, 2004) include enzymes involved in the synthesis of β-lactam antibiotics. Among these enzymes, isopenicillin N synthase (Roach *et al.*, 1997) requires no cofactor, whereas deacetoxycephalosporin C synthase (Valegard *et al.*, 1998) and clavaminic acid synthase (Zhang *et al.*, 2000) both require α-ketoglutarate as a cosubstrate to facilitate their respective reactions. Another member of this family that does not require α-ketoglutarate as a cosubstrate is 1-aminocyclopropane-1-carboxylic acid oxidase (Hamilton *et al.*, 1990), an enzyme involved in the formation of the plant signalling molecule ethylene. This enzyme requires ascorbate as a cofactor and CO_2 as an activator for continuous turnover. Interestingly, the enzymes in this family have been shown to be members of the cupin superfamily based on their similar distorted jelly roll β-barrel structure (Hewitson *et al.*, 2002).

The remarkable range of reactions catalyzed by this type of Fe(II) center can be explained by the mechanism of these enzymes that always involves binding of oxygen atoms to the open sites of the Fe(II) center. The 2-His-1-carboxylate motif can be seen as the counterpart of the heme cofactor where only one site is available for an endogenous ligand compared to three for this motif. The close proximity of the three open sites allows the juxtaposition of the two reactants to promote catalysis. In addition to the enzymes described here, iron superoxide dismutase (Lah *et al.*, 1995) and lipoxygenase (Minor *et al.*, 1996) also have this structural motif. However, they have an additional histidine ligand, which alters the role of the iron as it shuttles between the Fe(III) and Fe(II) oxidation states during catalysis. The contrast between these two enzymes and the Fe(II) oxygen activating enzymes shows the flexibility of the 2-His-1-carboxylate motif. The flexibility of this motif is further illustrated by the non-heme Fe(II) halogenases, which were recently shown to possess four open sites for ligation during catalysis. In these enzymes, the carboxylate ligand is replaced by a chloride ion to enable the enzymes to halogenate their substrates. A conserved alanine is present at the usual location of the Asp/Glu ligand (Blasiak *et al.*, 2006).

5. STRUCTURAL ASPECTS OF INTRADIOL DIOXYGENASES

Inspection of the crystal structures representing six distinct intradiol dioxygenases reveals two variations of a common underlying architecture. The respective protomers of 3,4-$PCD_{B\text{-}10}$ and 3,4-PCD_{ADP1} of *Acinetobacter* species strain ADP1 comprise two chains of related structure (Figure 4D), By contrast, the protomer of $C12O_{ADP1}$, $C12O_{C1}$ from *Pseudomonas arvilla* C-1, 4-chlorocatechol 1,2-dioxygenase from *Rhodococcus opacus* 1CP ($CC12O_{1CP}$) and $HQ12O_{3E}$ from *Nocardioides simplex* 3E is a single subunit (Figure 4E). Moreover, these dimeric enzymes appear to be phospholipid-binding proteins. The type member of the class, 3,4-$PCD_{B\text{-}10}$, has a large, dodecameric assembly with tetrahedral symmetry (Ohlendorf *et al.*, 1988, 1994; Ohlendorf and Vetting, 2001). The oligomer resembles a porous and hollow, truncated tetrahedron with an edge length of approximately 180 Å. Contacts between protomers are largely mediated by the β subunits, which interact extensively across tetrahedral two-fold axes to form an inner shell surrounding a central cavity of approximately 50 Å in diameter. The α subunits associate around

the three-fold axes at each apex and coincidentally lie at the corners of the opposing bases, which have a central pore outlined by β subunits. The crystal structure of 3,4-PCD_{ADP1} demonstrates the same $(\alpha\beta Fe(III))_{12}$ quaternary structure (Vetting *et al.*, 2000), whereas other 3,4-PCDs utilize the same protomer in a variety of oligomeric states (reviewed in Ohlendorf and Vetting, 2001).

The α and β subunits of 3,4-PCDs comprise approximately 200 and 230 residues, respectively, and are homologous but divergent (Ohlendorf *et al.*, 1988; Ohlendorf and Vetting, 2001). Thus, the level of identity between α and β subunits is 30% for 3,4-$PCD_{B\text{-}10}$ and 26% for 3,4-PCD_{ADP1}, whereas alignments between the two species yield 49% and 56% for the α and β subunits, respectively (Vetting *et al.*, 2000). The secondary structure of the subunits is nearly all β and is dominated by a β sandwich described as an eight-stranded sheet folded in half to form two layers. The β sandwich and two large connecting loops form a structurally conserved core such that 127 equivalent Cα atoms from the two chains of 3,4-$PCD_{B\text{-}10}$ can be superimposed with an rms deviation of 1.04 Å (Ohlendorf *et al.*, 1994). Although the β sandwich is a reasonably common structural motif, the topology of the core structure is so far unique to the intradiol dioxygenases (Ohlendorf and Vetting, 2001).

The active site of each protomer is located at one end of the extensive interface between the α and β subunits near a threefold apex of the oligomer and is accessible from outside the protein. The β chain provides most of the residues in the vicinity of the Fe, although a short segment from near the N-terminus of the α chain (3-4 residues near residue 15) completes the active site. In the substrate-free enzyme, the Fe is bound by one water ligand and four protein side chain ligands supplied by the β subunit, including two tyrosines and two histidines. The ferric ion of substrate-free intradiol dioxygenase has a distorted trigonal bipyramid geometry, with a tyrosine, a histidine and a solvent species coordinated in the equatorial plane and a tyrosine and a histidine coordinated in the axial positions (Figure 6A; Ohlendorf *et al.*, 1994). The structures of a variety of binary complexes with substrates and substrate analogs indicate displacement of the axial tyrosine accompanies formation of productive complexes (Figure 6B; Orville *et al.*, 1997). The structures of a series of competitive inhibitors suggest substrate binding may involve

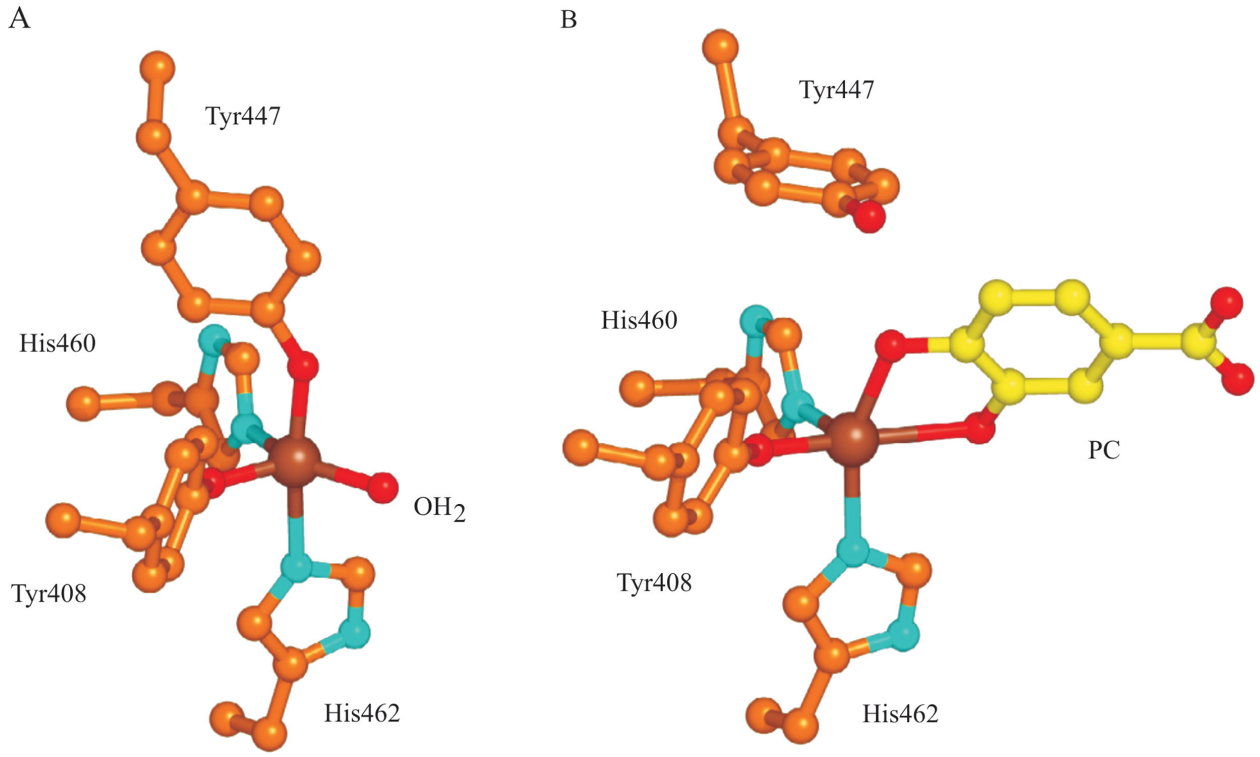

FIGURE 6 **Structure of the active site of substrate-free (*A*) and protocatechuate-bound (*B*) 3,4-$PCD_{B\text{-}10}$. Carbon atoms are colored orange in amino acids and yellow in protocatechuate. Oxygen, nitrogen, and iron atoms are colored red, cyan, and brown, respectively. (adapted from Ohlendorf *et al.*, 1994; Orville *et al.*, 1997; figure made using PyMol [DeLano, 2002]).**

several stages prior to formation of the reactive complex (Orville and Lipscomb, 1997).

$C12O_{ADP1}$ (Vetting and Ohlendorf, 2000), $C12O_{C1}$ (Earhart *et al.*, 2005), $CC12O_{1CP}$ (Ferraroni *et al.*, 2004), and $HQ12O_{3E}$ (Ferraroni *et al.*, 2005) are each homodimeric. The subunit of each protein is approximately 300 residues and is folded into two domains: a catalytic domain that replicates the basic core structure of the 3,4-PCDs and an N-terminal domain that mediates dimerization. The latter comprises approximately 100 residues that fold into five helices. The catalytic domain provides four protein ligands in locations equivalent to those provided by the β subunit in the 3,4-PCDs. An extended segment that links the dimerization domain to the catalytic domain replaces the short active site segment of the 3,4-PCD α chain. In all four structures, a phospholipid was bound at the linker domain interface. The role of the lipid remains unclear. The coordination of the Fe in the substrate-free enzyme is largely the same as in 3,4-PCD, and substrate binding displaces the axial tyrosine.

Interesting minor variations occur in the structures of each of these dimeric enzymes. Thus, $C12O_{C1}$ exists as three different isozymes: $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$. The $\beta\beta$ isozyme that was crystallized shares 53% amino acid sequence identity with $C12O_{ADP1}$. The difference between the α and β polypeptides are mostly located in part of the monomers distant from the dimer interface, consistent with the occurrence of three isozymes. The overall structure of $CC12O_{1CP}$ is similar to those of $C12O_{ADP1}$ and $C12O_{C1}$. However, the shape of the active site pocket differs and is believed to be the basis for the difference in substrate specificity. Finally, the structure of $HQ12O_{3E}$ is the first of a hydroxyquinol-cleaving intradiol dioxygenase (Figure 4E; Ferraroni *et al.*, 2005). Perhaps the most significant structural difference between this enzyme and the three others is the exposure to solvent of the upper part of the active site due to the occurrence of several openings. This, together with differences in several active residues, could explain the $HQ12O_{3E}$'s preferred binding and cleavage of hydroxyquinols *versus* catechols.

6. MECHANISM OF EXTRADIOL DIOXYGENASES

The catalytic strategy utilized by the different types of extradiol dioxygenases appears to be similar and the proposed mechanism is based on studies of members of each family (Arciero and Lipscomb, 1986; Shu *et al.*, 1995; Bugg and Lin, 2001). In the first step of this mechanism (Figure 7), the catecholic substrate binds

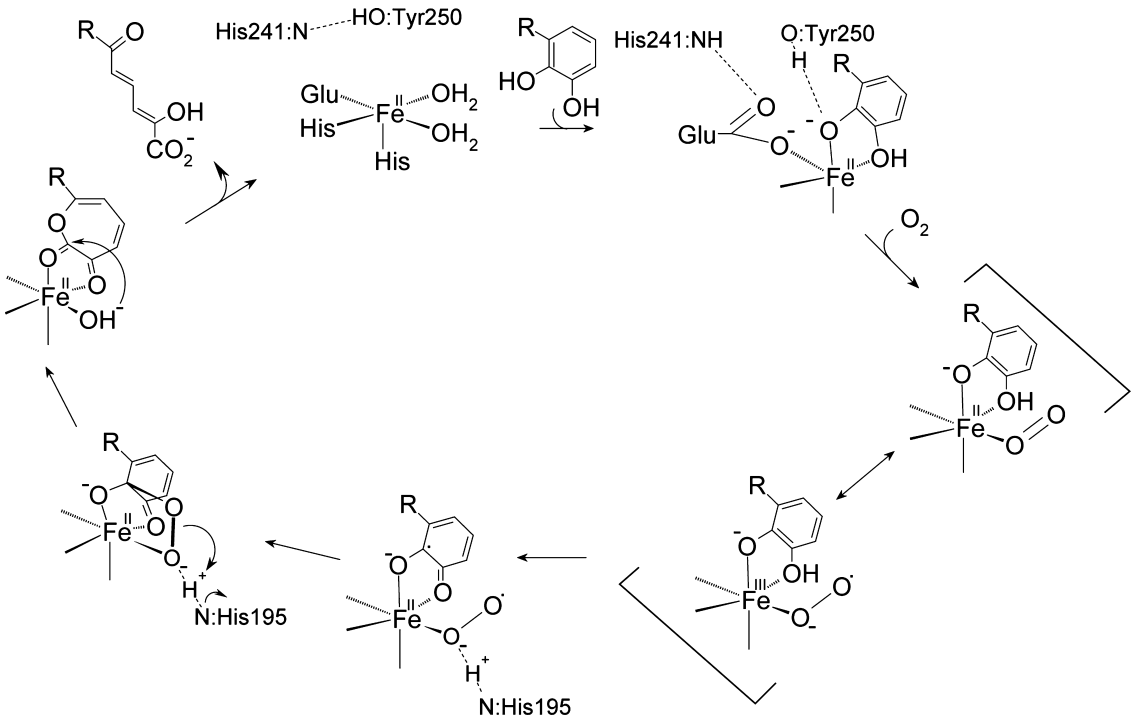

FIGURE 7 **Proposed mechanism of extradiol dioxygenases with the role of conserved active site residues (adapted from Shu *et al.*, 1995; Bugg and Lin, 2001). For clarity, the displacement of solvent species from the ferrous center is not depicted explicitly.**

to the ferrous ion in a bidentate manner, displacing the two solvent ligands (Arciero *et al.*, 1985; Arciero and Lipscomb, 1986; Mabrouk *et al.*, 1991; Uragami *et al.*, 2001; Vaillancourt *et al.*, 1998, 2002a). Spectroscopic data demonstrate that $DHBD_{LB400}$ binds its preferred substrate, DHB, as a monoanion (Vaillancourt*et al.*, 2002a) as had been inferred from XAS studies of $C23O_{mt2}$ (Shu *et al.*, 1995). The observed asymmetric binding of DHB to $DHBD_{LB400}$ ($r_{Fe-O} = 2.0$ Å and 2.4 Å; Vaillancourt*et al.*, 2002a), indicates that O-2 is deprotonated, but not O-3. The binding of the catecholic substrate to the iron activates the latter for O_2 binding (Arciero *et al.*, 1985; Mabrouk *et al.*, 1991; Shu *et al.*, 1995). Subsequent steps in the catalytic mechanism are less well substantiated. Biochemical studies provide some support for a mechanism involving iron-mediated transfer of an electron from the catechol to the O_2, yielding a semiquinone-Fe(II)-superoxide intermediate (Spence *et al.*, 1996b). This species is proposed to react to give an iron-alkylperoxo intermediate (Winfield *et al.*, 2000), which undergoes alkenyl migration, Criegee rearrangement, and O—O bond cleavage to give an unsaturated lactone intermediate and an Fe(II)-bound hydroxide ion. The latter hydrolyses the lactone to yield the reaction product (Sanvoisin *et al.*, 1995).

Catalytic roles of conserved active site residues have been proposed based on structural data, mutagenesis studies, and DFT calculations (Siegbahn and Haeffner, 2004). The substrate-induced structural changes (Figure 8, Vaillancourt *et al.*, 2002a) observed for His241 of $DHBD_{LB400}$ are consistent with its protonation, strongly suggesting that it assists in the deprotonation of the catechol in the enzyme-catalyzed reaction. Tyr250 could act as a proton shuttle between the catecholic substrate and His241 (Figure 7). Substitution of this His in $C23O_{OX1}$ with asparagine (H246N) altered the pH profile of the enzyme's k_{cat} in a manner consistent with the His playing a role in deprotonating the substrate (Viggiani *et al.*, 2004).

In the subsequent step of the proposed mechanism, O_2 binds to the ferrous ion. As shown in Figure 7, the dominant form of this species would be Fe(III)-O_2^-. Formation of Fe(III) would induce deprotonation of the 3-hydroxyl, forming the dianion chelate, as proposed in intradiol enzymes (Elgren *et al.*, 1997; Orville *et al.*, 1997; Vetting *et al.*, 2000). The proton could be picked up by the iron-bound superoxide. Interestingly, Nε2 of the conserved His195 is positioned within 3 Å of the proximal O atom in the modeled $DHBD_{LB400}$:DHB:O_2 ternary complex (Bolin and Eltis, 2001), and could thus stabilize protonation of that particular O atom of the superoxide species. The kinetic behaviour of each of four different enzymes substituted in this residue (Viggiani *et al.*, 2004; Mendel *et al.*, 2004; Groce and Lipscomb, 2005; Emerson *et al.*, 2005) is consistent with His195 having this role although this same His was also proposed to deprotonate the substrate in $DHPPD_{Eco}$, a type II dioxygenase (Mendel *et al.*, 2004). A role for this histidine in deprotonating the 3-hydroxyl group of the substrate and then stabilizing the superoxide species was suggested based on the crystal structure of a $DHBD_{KKS102}$:DHB:NO ternary complex (Sato *et al.*, 2002).

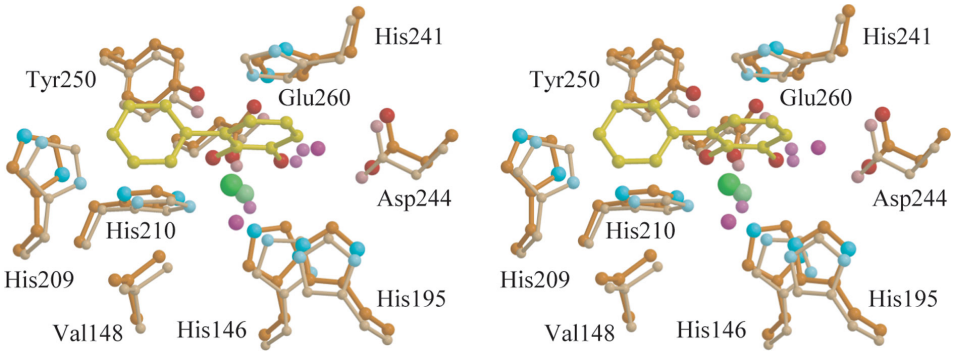

FIGURE 8 **Displacements of active site atoms/residues associated with DHB binding in $DHBD_{LB400}$. This is a (divergent) stereoscopic diagram. Atoms and bonds in the substrate-free structure are represented by smaller spheres, thinner sticks, and lighter shades. Carbon atoms are colored orange in protein residues and yellow in DHB. Nitrogen and iron atoms are cyan and green, respectively. Protein and DHB oxygen atoms are red, whereas water oxygens are magenta (from Vaillancourt *et al.*, 2002a).**

Electron transfer from the bound catechol would produce an Fe(II)-semiquinone. Attack of the activated oxygen species in a pseudo-axial position at C-2 satisfies the orbital steering requirements proposed by Bugg to be critical for extradiol cleavage (Bugg and Lin, 2001). In the subsequent step, the proton originating from the 3-hydroxyl group would assist in the heterolysis of the O–O bond in the proposed Criegee rearrangement that results in the lactone and the Fe-hydroxide. Finally, hydrolysis of the lactone by the Fe-bound hydroxide, and release of the proton on His241 complete the catalytic cycle. Although alternate roles for His195 and His241 are possible, the mechanism (Figure 7) illustrates the importance of the Fe(II)-bound monoanion in coordinating electron and proton transfer upon O_2 binding. Calculations based on density functional theory (DFT) (Siegbahn and Haeffner, 2004) are largely consistent with this mechanism. These calculations further predict that O–O bond cleavage is the rate-limiting step and that this precedes C–C bond cleavage such that an epoxide intermediate is formed. The extradiol-type cleavage of homogentisate by HGO has also been investigated using DFT calculations (Borowski *et al.*, 2005). Curiously, attack of the iron-bound superoxide was modeled at C-2 of the homogentisate and not C-1, the hydroxylated carbon equivalent to the one thought to be attacked in catechol-cleaving enzymes.

Single turnover studies promise to provide additional insights into the catalytic intermediates and roles of active site residues of extradiol dioxygenases. Two preliminary studies, one using 3-formylcatechol and $DHBD_{KKS102}$ (Ishida *et al.*, 2005), the other using 4-nitrocatechol and $HPCD_{Bfu}$ (Groce *et al.*, 2004), resolved several intermediates and indicated that ring-opening and product release are the overall rate-limiting steps, respectively. In the study using 4-nitrocatechol and $HPCD_{Bfu}$, the ring-opening step was also found to be slow relative to the other steps. Both 3-formylcatechol and 4-nitrocatechol undergo a single deprotonation upon binding to the enzyme and prior to reacting with O_2, as in the reaction with native substrates. However, in the case of 4-nitrocatechol this yields a dianion, which carries an extra charge with respect to native substrates. This may explain why the cleavage of 3-formylcatechol proceeds at a faster rate, more similar to that of the cleavage of native substrate. Overall, 3-formylcatechol appears to be a better substrate for characterizing the extradiol reaction.

7. INACTIVATION OF EXTRADIOL DIOXYGENASES

Extradiol dioxygenases are subject to two forms of substrate inhibition, reversible substrate inhibition and a mechanism-based inactivation (or suicide inhibition), as well as oxidative inactivation in the absence of substrate. As discussed below, inactivation in the absence and presence of substrate are quite similar as both involve oxidation of the active site iron.

Reversible substrate inhibition has been reported in a number of enzymes including $DHBD_{LB400}$ (Eltis *et al.*, 1993; Vaillancourt *et al.*, 1998), $DHBD_{CB15}$ (Adams *et al.*, 1992), $THBD_{RW1}$ (Happe *et al.*, 1993), $DHBD_{P6}$ (Asturias *et al.*, 1994), $DHBD_{BN6}$ (Heiss *et al.*, 1995), and $DHPPD_{Eco}$ (Spence *et al.*, 1996a). Interestingly, it has only rarely been reported for C23Os (Pascal and Huang, 1987). However, other than in $DHBD_{LB400}$, it is not clear what proportion of the decrease in the initial rate of DHB-cleavage at high concentrations of DHB is due to reversible substrate inhibition and irreversible suicide inhibition, respectively. Notably, the initial rates of cleavage of substituted catechols by $DHBD_{BN6}$ could not be fitted to a substrate inhibition equation (Heiss *et al.*, 1995). $DHBD_{LB400}$ is clearly subject to both modes of inhibition by DHB (Vaillancourt *et al.*, 1998). While the mechanism of reversible substrate inhibition is not clear, it does not appear to involve negative cooperativity between subunits as it has also been reported for a monomeric enzyme (Happe *et al.*, 1993). It is possible that DHB could occupy the auxiliary *t*-butanol binding site observed in the $DHBD_{LB400}$:DHB complex (Vaillancourt *et al.*, 1998), thereby inhibiting the cleavage reaction.

Mechanism-Based Inactivation

One physiologically significant aspect of extradiol dioxygenase function is mechanism-based inactivation. Although it was described 25 years ago in $C23O_{mt2}$ (Klecka and Gibson, 1981), the phenomenon was first recognized in mammalian HAD (Mitchell *et al.*, 1963). It has since been studied in APD_{JS45} (Davis *et al.*, 1999) and $DHBD_{LB400}$ of the *bph* pathway (Vaillancourt *et al.*, 2002b). Mechanism-based inactivation has been proposed to limit the range of toluates metabolized by the TOL pathway (Cerdan *et al.*, 1994) and the extent of transformation of PCBs by the *bph* pathway (Vaillancourt *et al.*, 2002b).

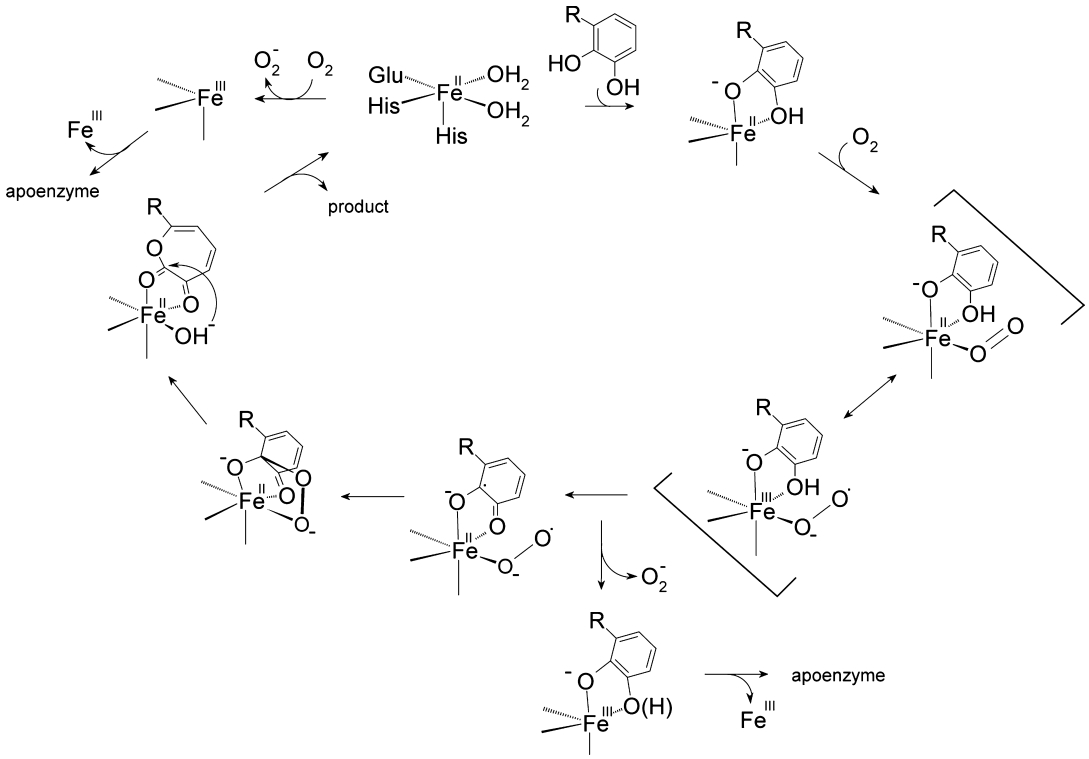

FIGURE 9 **General mechanism of inactivation of extradiol dioxygenases. The exact step at which superoxide dissociates from the ternary complex has not been determined. The ligands in the ferric form of the enzyme are unknown (adapted from. Bugg and Lin, 2001; Sato *et al.*, 2002; and Vaillancourt *et al.*, 2002b). For clarity, the displacement of solvent species from the ferrous centre is not depicted explicitly.**

The molecular basis of the mechanism-based inactivation of extradiol dioxygenases has been subject to some debate. Thus, the inactivation of $C23O_{mt2}$ by 3-chlorocatechol has been suggested to occur either through reversible chelation of the active site iron (Klecka and Gibson, 1981) or irreversible covalent modification by an acyl chloride species generated by the ring cleavage reaction (Bartels *et al.*, 1984). However, no evidence for either mechanism has been presented. In contrast, the inactivation of $C23O_{mt2}$ by alkyl catechols appears to involve the accidental oxidation of the active site Fe(II) to Fe(III) during turnover (Cerdan *et al.*, 1994). Interestingly, a halogenated substrate analog 4-chloro-3-hydroxyanthranilate, had been suggested to inhibit HAD via covalent modification by an acyl halide (Parli *et al.*, 1980), although it was subsequently shown that this analogue inhibits the enzyme reversibly *in vivo* (Walsh *et al.*, 1994).

Studies of $DHBD_{LB400}$ indicate that the mechanism-based inactivation of this enzyme in the presence of a variety of catechols, including 3-chlorocatechol and DHB, involves the dissociation of superoxide from the EAO_2 ternary complex with the concommitant oxidation of the active site Fe(II) (Figure 9; Vaillancourt *et al.*, 2002b). More particularly, *in vitro* studies demonstrated that this inactivation results in the formation of Fe(III) and was reversed by anaerobic incubation of the inactivated enzyme with Fe(II) and a reducing agent. Moreover, the mechanism-based inactivation of $DHBD_{LB400}$ does not involve covalent modification, as judged by a lack of change to the molecular mass of $DHBD_{LB400}$ inactivated by 3-chlorocatechol or other catechols. Further evidence for this conclusion comes from *in vivo* studies in which 3-chlorocatechol-inactivated $DHBD_{LB400}$ was readily reactivated in the absence of protein synthesis. The dissociation of superoxide from the EAO_2 complex prior to formation of an iron-alkylperoxo intermediate is consistent with the proposed catalytic mechanism of extradiol dioxygenases and may represent a general means by which these enzymes are inactivated during catalytic turnover. Consistent with this suggestion, catechol-inactivated APD_{JS45} can be reactivated upon incubation with Fe(II) and a reducing agent (Davis *et al.*, 1999). Moreover, one study reported that the inactivation of $C23O_{mt2}$ by 3-chlorocatechol also involves oxidation of the active site Fe(II) (Wasserfallen, 1989). While 3-chlorocatechol inactivates many catechol-cleaving

extradiol dioxygenase, $CC23O_{GJ31}$, which is related to $C23O_{mt2}$ (Mars *et al.*, 1999), catalyzes the efficient cleavage of 3-chlorocatechol (Mars *et al.*, 1997). However, how this enzyme accomplishes this remains unclear.

For both $DHBD_{LB400}$ and $C23O_{mt2}$, oxidative inactivation is more marked for poorer substrates (Cerdan *et al.*, 1995; Vaillancourt *et al.*, 2002b), suggesting that the substrate-binding pocket of these enzymes is tuned both to maximize specificity for a particular substrate and minimize inactivation during catalytic turnover. Consistent with this hypothesis, substitution of residues in the DHB-binding pocket of $DHBD_{LB400}$ altered the enzyme's specificity for catechol and the rate of oxidative inactivation during catalysis (Vaillancourt *et al.*, 2005a). Interestingly, the TOL pathway contains a 2Fe-2S ferredoxin to reactivate C23O, thereby increasing the range of substrates that the organism utilizes as sole source of carbon and energy (Polissi and Harayama, 1993). The ferredoxin is encoded by *xylT*, which is located immediately upstream of the C23O-encoding *xylE*. Homologous ferredoxins are found in a number of catabolic pathways (Hugo *et al.*, 2000; Tropel *et al.*, 2002). Interestingly, the partition ratio of C23O for catechol is 1,400,000 (Cerdan *et al.*, 1994), indicating that $DHBD_{LB400}$ is much more susceptible than is $C23O_{mt2}$ to suicide inactivation by its putative preferred substrate. Despite the higher susceptibility of $DHBD_{LB400}$ to suicide inactivation, no such ferredoxin has been associated with the *bph* pathway. However, the *in vivo* reactivation of 3-chlorocatechol-inactivated $DHBD_{LB400}$ in *B. xenovorans* LB400 and *E. coli* suggests that a non-specific electron transfer protein can play this role (Vaillancourt *et al.*, 2002b).

Oxidative Inactivation in the Absence of Substrate

Extradiol dioxygenases are also susceptible to oxidative inactivation in the absence of substrate. This process also apparently involves binding of O_2 to the active site iron and the loss of superoxide (Vaillancourt *et al.*, 2002b). It is possible that the high K_m value of many extradiol dioxygenases for O_2 (K_{mO2}) reflects the low affinity of the free enzyme for O_2, which may have evolved as a protective adaptation against oxidative inactivation. For example, the K_{mO2} of $DHBD_{LB400}$ and 2-aminophenol dioxygenase are 1.3 mM (Vaillancourt *et al.*, 1998) and 710 μM (Lendenmann and Spain, 1996), respectively. Interestingly, $C23O_{mt2}$, which is less susceptible to O_2-dependent inactivation (Nozaki *et al.*, 1963), has a lower K_{mO2} (10 μM; Hori *et al.*, 1973; Kobayashi *et al.*, 1995). Moreover, the growth rate of *P. putida* mt-2 on benzoate at different pO_2 is limited by the K_{mO2} of C23O (Arras *et al.*, 1998). It is clear that some C23O's have evolved to function in microaerobic environments, and thus have even lower K_{mO2} (0.7 μM; Kukor and Olsen, 1996). In the latter case, there is presumably less O_2 to inactivate the C23O.

The oxidative inactivation of extradiol dioxygenases in the absence of substrate complicates their purification and characterization using aerobic buffers. This problem can be at least partially alleviated through the inclusion of organic additives such as isopropanol, *t*-butanol and acetone in solutions of the enzymes (Nozaki *et al.*, 1963; Eltis *et al.*, 1993; Kobayashi *et al.*, 1995; Vaillancourt *et al.*, 1998). These additives were also used in crystallographic studies to stabilize the enzymes. The crystallographic data from $DHBD_{LB400}$ (Han *et al.*, 1995, Vaillancourt *et al.*, 1998) and $C23O_{mt2}$ (Kita *et al.*, 1999) indicate that these additives occupy the active site, close to the catalytic iron center thereby stabilizing the active site and/or protecting the iron from direct access by oxidants or substrates. Consistent with this notion, *t*-butanol competitively inhibited $DHBD_{LB400}$ (Vaillancourt *et al.*, 1998). Moreover, *t*-butanol and isopropanol, which inhibit $DHBD_{LB400}$ more effectively than ethanol and glycerol, also stabilize the enzyme more effectively. Interestingly, acetone competitively inhibits $C23O_{mt2}$ more effectively than *t*-butanol inhibited $DHBD_{LB400}$ (Nozaki *et al.*, 1963; Kobayashi *et al.*, 1995). This suggests that $C23O_{mt2}$ has a much higher affinity for acetone than $DHBD_{LB400}$ has for *t*-butanol, and may explain why the former is so much more stable in acetone-containing buffers (Kobayashi *et al.*, 1995) than is $DHBD_{LB400}$ in *t*-butanol-containing buffers. In $C23O_{mt2}$, it was even proposed that acetone binds directly to the iron (Bertini *et al.*, 1994, Kita *et al.*, 1999). However, this direct binding remains to be clarified as the precision of the respective experiments was limited. Inspection of the *t*-butanol binding site in $DHBD_{LB400}$ and of the acetone binding site in C23O reveals that it is partly formed by non-conserved residues, suggesting that the best organic stabilizer, if any, will be isozyme-specific.

Even with the addition of organic additives, the best reported aerobic preparations of $DHBD_{LB400}$ contain at most 50% of their complement of active site Fe(II) (Eltis *et al.*, 1993). This variability in preparations of extradiol

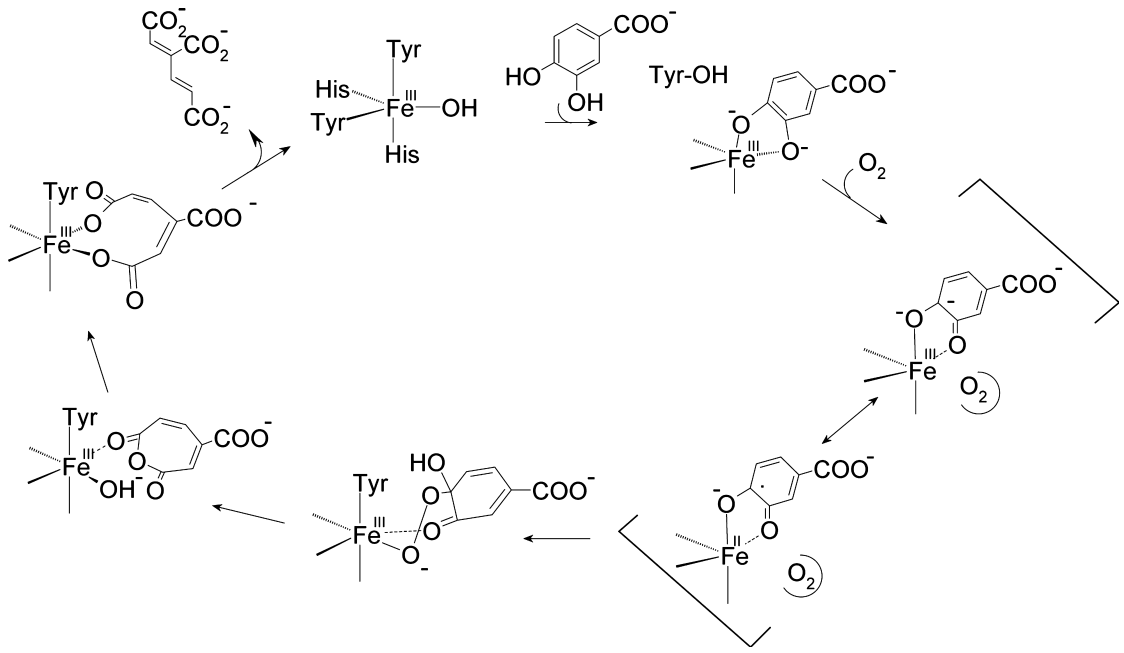

FIGURE 10 **Reaction mechanism proposed for 3,4-PCD (adapted from Orville *et al.*, 1997; and Bugg and Lin, 2001).**

dioxygenases complicates spectroscopic studies and the determination of steady-state kinetic parameters. For example, the k_{cat} of $C23O_{mt2}$ has been variously reported as 930 s^{-1} (100 mM phosphate, pH 7.5, 25°C; Cerdan *et al.*, 1995) and 278 s^{-1} (50 mM phosphate, pH 7.5, 25°C; Kobayashi *et al.*, 1995). These results demonstrate the value of anaerobic purification, and further illustrate the importance of calculating steady-state parameters as a function of the metal content of enzyme preparations (Vaillancourt *et al.*, 1998).

8. MECHANISM OF INTRADIOL DIOXYGENASES

The proposed mechanism of intradiol dioxygenases has been developed based on biochemical, spectroscopic and structural studies of 3,4-PCDs and C12Os (Figure 10; Bugg and Lin, 2001). As in extradiol dioxygenases, the intradiol enzymes utilize an ordered mechanism in which catechol binding precedes O_2 reactivity (Hori *et al.*, 1973; Bull *et al.*, 1981; Walsh *et al.*, 1983). However, whereas extradiol enzymes activate the O_2 for nucleophilic attack on the catechols, intradiol enzymes appear to activate the catechols for electrophilic attack by O_2. In intradiol dioxygenases, catechol binding is a multi-step process that ultimately results in displacement of an axial tyrosine and an equatorial hydroxide ion to yield a bidentate bound catecholate (Figure 6B; True *et al.*, 1990; Orville *et al.*, 1997; Frazee *et al.*, 1998; Vetting and Ohlendorf, 2000; Vetting *et al.*, 2000; Horseman *et al.*, 2005). The substrate binds as a dianion and the displaced tyrosyl ligand is protonated, presumably accepting a proton from the substrate (Horseman *et al.*, 2005). The other proton from the substrate is likely accepted by the displaced hydroxide ligand (Orville and Lipscomb, 1997). In the next step, O_2, thought to be sequestered in a hydrophobic pocket, attacks the bound catecholate directly, before coordinating to the iron and yielding an iron-alkylperoxo intermediate (Que *et al.*, 1977). Although recent evidence indicates that this intermediate is similar in structure to that of the extradiol reaction (Winfield *et al.*, 2000), in the case of intradiol enzymes, the Criegee rearrangement and O—O bond cleavage involve acyl migration to yield the cyclic anhydride and an iron-bound oxide or hydroxide. The latter functions as a nucleophile to hydrolyse the anhydride and yield the ring-opened product.

The protonation state of the substrate is largely based on crystallographically determined bond lengths, which indicate that the substrate is asymmetrically bound: the long Fe—O bond is *trans* to a tyrosinate ligand and the short Fe—O bond is *trans* to a neutral histidine ligand (Orville *et al.*, 1997; Vetting and Ohlendorf, 2000; Vetting *et al.*, 2000). The asymmetry is proposed to reflect ketonization of the bond *trans* to the tyrosine. A survey of the structures in the PDB database (3PCA, 1EOB, 1DLT) reveals that the Fe—O bond lengths are similar to those observed in ES complexes of extradiol

enzymes ($r_{Fe-O} = 2.0 \pm 0.1$ Å and 2.4 ± 0.2 Å). Visible resonance Raman studies using the ligand-to-metal ion charge transfer bands show that 4-nitrocatechol and 3,4-dihydroxyphenylacetate bind to 3,4-PCD as dianions (Que and Epstein, 1981; Elgren *et al.*, 1997). UV/Vis absorption spectroscopy corroborates dianionic binding of 4-nitrocatechol to 3,4-PCD and C12O (Tyson, 1975; Vetting *et al.*, 2000). 4-Nitrocatechol is an inhibitor of 3,4-PCD and 3,4-dihydroxyphenylacetate is a very poor substrate. Thus, these analog may not bind in the same manner as the preferred substrate of the enzyme, PCA. However, structural data indicate that 3,4-PCD binds 3,4-dihydroxyphenylacetate and PCA in a similar manner.

9. MECHANISTIC COMPARISON OF EXTRADIOL AND INTRADIOL DIOXYGENASES

Given the differences between extradiol and intradiol dioxygenases, it is intriguing that the respective catalytic mechanisms of these enzymes are proposed to proceed *via* similar iron-alkylperoxo intermediates. Two hypotheses have been proposed to explain why this intermediate breaks down differently in the two enzymes. According to one hypothesis, the mode of cleavage is determined by stereo-electronic factors arising from the orientation of the iron-alkylperoxo moiety relative to the organic substrate (Bugg and Lin, 2001). In particular, the extradiol dioxygenases are proposed to form a pseudo-axial iron-alkylperoxo species that would favour alkenyl migration and the intradiol dioxygenases are proposed to form a pseudo-equatorial iron-alkylperoxo species that would favour acyl migration. According to the second hypothesis, it is the degree of anionic character of the intermediate generated by O–O cleavage of the iron-alkylperoxo species that determines the mode of ring-cleavage (Siegbahn and Haeffner, 2004). Thus, a purely radical O–O cleaved intermediate was predicted to yield the intradiol cleavage product whereas an intermediate with partly anionic character was predicted to yield the extradiol cleavage product. Intriguingly, the H200F variant of $HPCD_{Bfu}$ (His195 in $DHBD_{LB400}$) catalyzed the intradiol cleavage of 2,3-dihydroxybenzoate, an alternate substrate (Groce and Lipscomb, 2003). As discussed above, this His residue is proposed to stabilize protonation of the proximal O in the iron-alkylperoxo species. It is unclear how substitution of this residue affects the orientation of the iron-alkylperoxo moiety and the character of the subsequent O–O cleaved intermediate. Nevertheless, the hypothesis that extradiol cleavage involves alkenyl migration whereas intradiol cleavage involves acyl migration is supported by the range of compounds that are known substrates for these enzymes. Thus, compounds that do not have vicinal hydroxyl groups, such as gentisate, are only known to be cleaved by extradiol-type dioxygenases. This is consistent with the breakdown of the iron alkylperoxo intermediate, which in such substrates can only proceed via alkenyl migration.

An important difference in the initial stages of the proposed extradiol and intradiol mechanisms is the protonation state of the bidentate-bound catechol in the enzyme:substrate complex (Que and Ho, 1996). Thus, in extradiol dioxygenases, an Fe(II)-bound monoanionic catecholate activates the ferrous centre for O_2-binding (Shu *et al.*, 1995; Lin *et al.*, 2001; Vaillancourt *et al.*, 2002a). By contrast, in intradiol dioxygenases, an Fe(III)-bound dianionic catecholate promotes direct electrophilic attack of the substrate by O_2, a reaction that is further favored by ketonization of the catecholate (Que *et al.*, 1977; Horsman *et al.*, 2005). Structural (Elgren *et al.*, 1997; Orville *et al.*, 1997; Vetting *et al.*, 2000; Uragami *et al.*, 2001; Vaillancourt *et al.*, 2002a) and EXAFS (Shu *et al.*, 1995; Wasinger *et al.*, 2003) data demonstrate that in both enzymes, the substrate is asymmetrically bound: one Fe–O bond is shorter than the other. In the case of intradiol dioxygenases, the *trans* tyrosinate ligand, a strong electron donor, was shown to induce the asymmetry in the substrate (Horsman *et al.*, 2005), whereas in the extradiol dioxygenases, asymmetry is induced by monoprotonation of the substrate (Vaillancourt *et al.*, 2002a).

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