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Biophysical Investigation of Bacterial Aromatic Extradiol Dioxygenases Involved in Biodegradation Processes

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Contents

Abstract

The current knowledge of the structure—function relationships in bacterial aromatic extradiol cleavage dioxygenases involved in biodegradation processes are reviewed.

The active metal ion coordination is discussed on the basis of the biophysical studies performed to date.

Comparison between extradiol and intradiol cleaving dioxygenases allows us to discuss the differences in the active site structure and catalytic mechanism between the two classes of aromatic ring cleavage dioxygenases.

Keywords: Biodegradation; Extradiol cleaving dioxygenases; Intradiol cleaving dioxygenases

1. Introduction

Microorganisms have a key role in the breakdown and mineralization of natural and xenobiotic compounds [1,2]. Many halogenated aromatic and aliphatic sub-

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stances and several pesticides are generally very slowly degraded, but microbial communities have been observed to rapidly adapt to grow on these chemicals [3–5]. The response of such microorganisms to the presence of xenobiotics results in the selection of strains or production of mutants capable of degrading the xenobiotic substance by inducing specific enzymes [6]. In some cases it has been demonstrated that enzymes previously evolved for naturally occurring compounds can be easily recruited or rapidly adapted to metabolize xenobiotics having similar structures [7]. This rapid metabolic adaptation has been shown to be strongly aided by natural events such as gene transfer, transposition, recombination and mutation [8]. Although such biodegradative capabilities may also be present on the bacterial chromosome, more often they are located on plasmids which can be easily transmitted to similar organisms [8].

Transmissible vectors capable of extending the ability of microorganisms to utilize certain compounds as carbon sources are largely diffuse in nature [8,9]. The well-characterized TOL plasmids are self-transmissible [10]. Such catabolic plasmids, whose function was discovered in the early seventies, are required for the degradation of methylbenzenes and contain two or more operons; one operon generally codes for the upper-pathway enzymes able to transform methylbenzenes into methylbenzoates, and the other operon encodes the enzymes of the lower- or *meta*-cleavage-pathway, which convert methylbenzoates to pyruvate, acetaldehyde and acetate [10].

The aromatic degradation pathways have been studied extensively and although anaerobic (reductive) mineralization processes have important roles in the biodegradation of many pollutants [11], genetic and biochemical studies have been mainly focused on aerobic (oxidative) pathways which dominate in terms of both rate and total carbon flux [1,12–14]. A large body of literature is available on this subject which allows us to accomplish a general comparison of the major pathways for catabolism of aromatic compounds in bacteria [8,15]. As a result it has been observed that the aromatic compounds are all transformed by oxygen insertion (oxidized) into a limited number of central intermediates such as catechols, gallates, protocatechuates and gentisates (Fig. 1) [15–17].

In particular, the *ortho*-dihydroxylated derivatives are degraded following one of two possible pathways: a *meta*-cleavage (extradiol cleavage) or an *ortho*-cleavage (intradiol cleavage) pathway, depending on the position of the aromatic ring cleavage and oxygen incorporation (Fig. 2) [15–19]. Both lead to intermediates of central metabolic routes suggesting that the microorganisms can extend their substrate range by developing peripheral enzymes able to transform such compounds into one of the central intermediates.

1.1. Oxygenases

The idea that living organisms may directly incorporate into organic substrates oxygen atoms derived from atmospheric oxygen through some unknown processes of activation was suggested very early in biology [20]. Unfortunately, at that time the experimental techniques did not allow the researchers to prove unequivocally this hypothesis. Later, after the discovery that many biological oxidations occur in

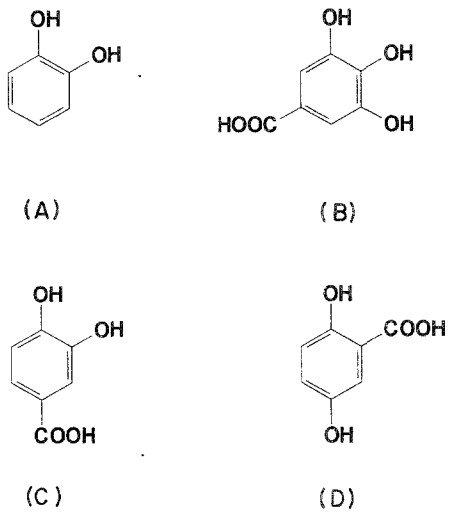

Fig. 1. Aromatic hydroxylated compounds, common products of convergent biodegradation pathways: (A) catechol; (B) pyrogallic acid; (C) protocatechuic acid; and (D) gentisic acid.

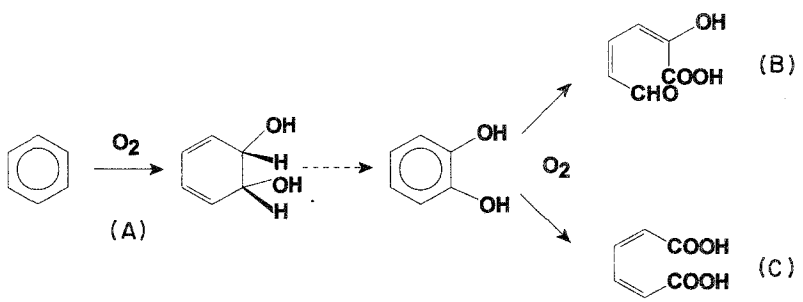

Fig. 2. Oxygenase reactions in microbial catabolic pathways for aromatic compounds. Hydroxylating dioxygenases (A); cleaving dioxygenases: extradiol (B), intradiol (C).

complete absence of oxygen, this suggestion was abandoned in favor of the idea that oxidation in biology consisted of transfer of hydrogen atoms (electrons) from a substrate to the acceptor [21]. As a consequence the oxygen atoms inserted in the organic substrate were assumed to be derived from water [22]. Only in 1955 were two independent groups able to show, by using the heavy oxygen isotope ^{18}O, that the direct insertion of molecular oxygen into substrates was a process accessible to living organisms [23,24].

The enzymes that catalyze the insertion of dioxygen into organic compounds are called oxygenases. They activate dioxygen using transition metals or reduced flavin/pteridine cofactors and are usually classified into mono- and di-oxygenases depending on whether one or both the oxygen atoms of the oxygen molecule are incorporated into the organic moiety [25,26]. In the case of mono-oxygenases the second oxygen

atom is reduced to water by a reducing agent whose presence is essential for the enzymatic reaction [27].

The role of oxygenases is extremely important, being involved in the transformation of essential metabolites such as amino acids, sugars, lipids, vitamins, hormones, neurotransmitters, etc. [25,26]. Oxygenases also play significant roles in microbial catabolic pathways, in particular in the biodegradation of aromatic compounds, initially by hydroxylating the aromatic ring and subsequently by catalyzing the ring fission reaction (Fig. 2) [28,29]. Oxygenases involved in these pathways have been extensively studied using biochemical and biophysical techniques combined with molecular genetics in order to understand their specificity, reaction mechanisms, and regulation [28,29]. The relevance of such systems in the fields of environmental and industrial biotechnology does not require further emphasis.

We want to review here the more recent studies on a particular class of oxygenases, the so-called bacterial extradiol-cleaving dioxygenases, in comparison with the best-studied intradiol enzymes.

1.2. Aromatic ring-cleaving dioxygenases

The bacterial degradation of aromatic compounds is generally performed through transformation of substrates into intermediates carrying two or more hydroxyl groups on the ring (Fig. 1) [15,29]. Such compounds are substrates for aromatic ring-cleavage dioxygenases that catalyze the ring opening, i.e. the most significant single step in the catabolism of aromatic compounds [30]. If two of the hydroxyl substituents are in the *ortho* position then the ring cleavage can occur either between the two groups (intradiol, Fig. 3) or between one hydroxyl group and an adjacent carbon atom (extradiol, Fig. 3) [31]. In the case of enzymes cleaving substituted catechols they are further subdivided into proximal and distal extradiol dioxygenases in order to distinguish the position of cleavage relative to the substituent (Fig. 3).

Gentisate and homogentisate which have two *para*-hydroxyl groups are also common products of bacterial aromatic catabolism and are cleaved between the carboxyl or the acetyl group and the adjacent hydroxyl group (Fig. 4) [32].

The intradiol dioxygenases usually contain a catalytic high spin iron(III) ion in the active site. Many investigations have been performed on such systems through biochemical, spectroscopic and structural studies [33,34]. The extradiol enzymes, although more abundant than the intradiol enzymes, are less stable because of their

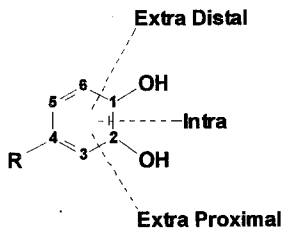

Fig. 3. Types of catalytic ring cleavage occurring for catechol (R = H) or substituted catechols.

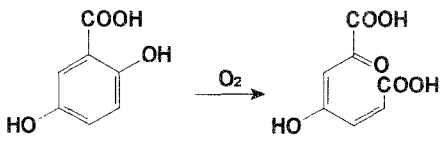

Fig. 4. Gentisate 1,2-dioxygenase catalyzed ring cleaving.

sensitivity to oxidizing compounds [35–37]. They usually contain a catalytic high spin iron(II) ion [36,38]. Little is known about the structure and the catalytic mechanism although such enzymes were first discovered in the late fifties [39].

The extradiol-cleaving dioxygenases are more abundant and assorted in their substrate specificities than the intradiol enzymes [40–42]. As reported above, they usually contain Fe(II) ions essential for catalytic activity but a few enzymes containing Mn(II) are also known [43,44].

From a genetic point of view, Fe(II) extradiol dioxygenases appear to have at least two independent origins because from a comparison of the known primary sequences it is observed that catechol 2,3-dioxygenase from *P. putida mt2*, 1,2-dihydroxynaphthalene dioxygenases, and 2,3-dihydroxybiphenyl dioxygenases belong to the same superfamily whereas the extradiol dioxygenase from *A. eutrophus* has a very different primary sequence [15,45]. In the above-mentioned superfamily a further division into monocyclic and bicyclic aromatic compounds dioxygenases is possible on the basis of the greater similarities between the amino acid sequences within each of the two subfamilies [19].

In Table 1 a summary of biochemical properties of various bacterial extradiol dioxygenases is reported.

2. The coordination chemistry of Fe(II) extradiol dioxygenases

Only a few dioxygenases containing catalytic Fe(II) ions have been spectroscopically characterized in order to clarify the coordination chemistry of the active center and its possible changes during catalysis.

Catechol 2,3-dioxygenase from *P. putida mt-2* (C2,3O), which catalyzes the conversion of catechol to α-hydroxy-muconic ε-semi-aldehyde and which contains one high spin Fe(II) ion for each of the four identical 32,000 MW subunits was the first to be isolated and crystallized [35,46].

Although this was achieved in the early sixties [35,36], the structure of the active site is still unclear. No crystallographic data are yet available and Mössbauer spectroscopy gives very limited structural information; the isomeric shift, and the large quadrupole splitting confirm the presence of high spin iron(II) ions (Table 2)[72]. The application of a magnetic field up to 75 mT as well as a change of temperature in the range 4.2–77 K did not cause significant variations in the spectra [72]. Data on the enzyme-substrate complexes were also collected and were found to be essentially identical to those for the uncomplexed enzyme, suggesting

Table 1
Some bacterial extradiol dioxygenases

Dioxygenase[a]	Organism	Subunit structure (MW)	Ref.
Catechol 2,3-D	*P. putida mt2*	$(\alpha Fe)_4$ ($\alpha = 32\,000$)	[46,47]
	Alcaligenes eutrophus JMP222	$(\alpha Fe)_n$ ($\alpha = 34\,000$)	[45]
	A. xylosoxidant KF 701	($\alpha = 39\,000$)	[48]
3-CH_3-catechol 2,3-D	*P. putida F1*	$(\alpha Fe)_n$ ($\alpha = 27\,200$–$32\,200$)	[49]
	P. putida UCC2	$(\alpha Fe)_4$ ($\alpha = 33\,500$)	[50]
3-Carboxyethylcatechol 2,3-D	*Achromabacter*		[51]
Protocatechuate 2,3-D	*B. macerans JJ1b*	$(\alpha Fe)_4$ ($\alpha = 36\,000$)	[52]
Protocatechuate 4,5-D	*P. testosteroni T2*	$(\alpha\beta)_2Fe$ ($\alpha = 17\,700$, $\beta = 33\,800$)	[53]
	P. paucimobilis SYK6	$(\alpha\beta)_nFe$ ($\alpha = 15\,000$, $\beta = 34\,000$)	[54]
3,4-di-OH-phenylacetate 2,3-D or	*B. brevis*	$(\alpha)_4Mn_2$ ($\alpha = 35\,000$)	[43]
Homoprotocatechuate 2,3-D	*B. stearothermophylus*	$(\alpha)_3X$ ($\alpha = 34\,000$)	[44]
	P. ovalis	$(\alpha Fe)_4$ ($\alpha = 35\,000$)	[55,56]
1,2-di-OH-Naphthalene D	*P. putida NCIB9816*	$(\alpha Fe)_n$ ($\alpha = 19\,000$)	[57]
	P. vesicularis BN6	$(\alpha Fe)_8$ ($\alpha = 33\,000$)	[58]
2,3-di-OH-biphenyl 1,2-D	*P. pseudoalcaligenes KF 707*	$(\alpha Fe)_8$ ($\alpha = 33\,000$)	[59]
	P. paucimobilis Q1	$(\alpha Fe)_8$ ($\alpha = 33\,000$)	[60]
	P. putida	$(\alpha\beta)_2$	[61]
	P. cruciviae S93		[62]
	Pseudomonas sp. LB400	$(\alpha Fe)_8$ ($\alpha = 32\,500$)	[63]
	Acinetobacter sp. P6	$(\alpha\beta)_2$	[64]
	Alcaligenes sp.		[65]
2,2′,3-Trihydroxybiphenyl D	*S. paucimobilis RW1*	$(\alpha Fe)_1$ ($\alpha = 32\,000$)	[66]
Steroid 4,5-D	*Nocardia restrictus*		[67]
Gentisate 1,2-D	*Moraxella osloensis*		[68]
	P. acidovorans	$(\alpha Fe)_4$ ($\alpha = 40\,000$)	[69]
	P. testosteroni	$(\alpha Fe)_4$ ($\alpha = 40\,000$)	[69]
2,5-di-OH-phenylacetate 1,2-D or homogentisate D	*P. fluorescens*		[70]
2,5-dihydroxypyridine D	*P. putida N9*	($\alpha = 39\,500$)	[71]

[a] Dioxygenase is abbreviated as D in table.

Table 2
Spectroscopic data on extradiol dioxygenases

Enzyme sample	Mössbauer (4.2 K)		EPR (NO complex)[a]		Ref.
	Isomer shift (mm s^{-1})	Quadrupole splitting (mm s^{-1})	g	E/D	
C2,3O	1.31	3.28	4.18/3.83/1.99	0.029	[72,73]
			4.14/3.90	0.021	
			4.09/3.97	0.10	
C2,3O + substrate	1.31	3.28	4.04/3.98/2.00	0.005	[72,73]
PC2,3O	1.29	2.57	4.11/3.95	0.014	[52]
PC2,3O + substrate	1.19	2.73	4.34/4.21/3.85	0.034	[52]
PC4,5O	1.28	2.22	4.09/3.91	0.015	[53,73]
PC4,5O + substrate	1.27/1.22	2.33/2.80	4.37/3.57	0.064	[53,73]
			4.21/3.78	0.037	
G1,2O					[69,113]
P. testosteroni			4/2.0		
P. acidovorans			4		
G1,2O + substrate					[69,113]
P. acidovorans			4.06/3.98/2.0	0.01	
P. testosteroni			4/4/2	0	
			4.19/3.85/1.99	0.032	

[a] The NO complex of native C2,3O and the substrate complexes of PC4,5O and G1,2O exhibit several $S = 3/2$ type species.

that catechol binding does not alter the coordination environment of the high spin iron(II) ion, which seems to be composed by oxygen–nitrogen donor atoms [72]. In contrast, Mössbauer data on other extradiol dioxygenases, the protocatechuate 4,5-dioxygenase from *P. testosteroni* (PC4,5O), and the protocatechuate 2,3-dioxygenase from *B. macerans* (PC2,3O) show temperature dependent quadrupole splitting resulting from thermally accessible low-lying orbital states (ΔE_Q = 2.22 mm s^{-1} at 4.2 K and 1.97 mm s^{-1} at 180 K for the PC4,5O enzyme), as in many high spin iron(II)-containing proteins [52,53]. In the presence of an external magnetic field of 6 T the spectra of PC2,3O and PC4,5O were poorly resolved but revealed magnetic patterns indicative of large positive zero-field splitting $D > 10\ cm^{-1}$. The spectra of PC4,5O and PC2,3O measured in the presence of the substrate protocatechuate reveal marked differences from the native enzymes, in contrast to C2,3O. In particular, PC2,3O shows marked changes in isomer shift and quadrupole splitting of the doublet (Table 2), whereas PC4,5O-substrate complex spectra consist of a superposition of two doublets both attributable to iron(II) species (Fig. 5). Doublet 1, accounting for 60% of the total, has parameters very similar to those of the uncomplexed enzyme; doublet 2 suggests that the substrate is bound to the iron(II) site but, surprisingly, further addition of the substrate does not increase its intensity. Therefore, the iron environments of the C2,3O, PC2,3O, and PC4,5O enzymes seem to differ significantly regardless of the similar catalyzed reactions.

The active sites of these enzymes have also been monitored through EPR on NO

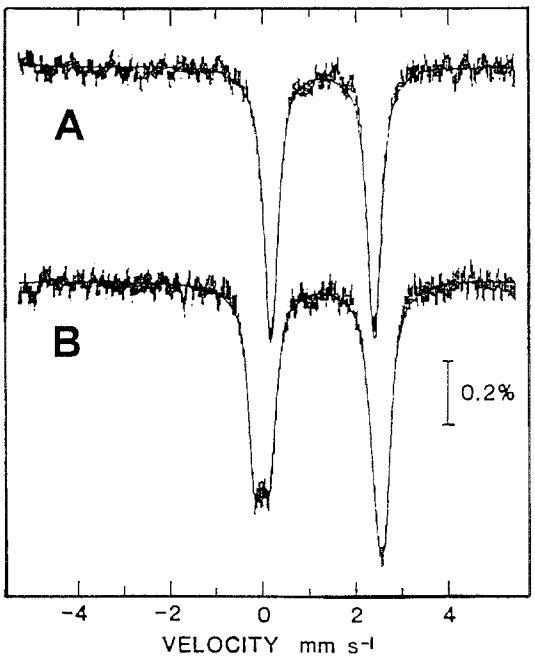

Fig. 5. Mössbauer spectra of ^{57}Fe-enriched protocatechuate 4,5-dioxygenase. Native enzyme (A) and its adduct with protocatechuate (B) (adapted from Ref. [53]).

derivatives which are easily detected by EPR [53,73,74]. The spectra are characterized by two features almost symmetrically split around $g = 4$ and one slightly below $g = 2$, indicative of an $S = 3/2$ spin state (Figs. 6(A) and 7(A); (Table 2)) [53,73,74]. Both resonance positions and line shape are altered by substrate or inhibitor binding suggesting that NO is not displaced by such compounds and that the binding of substrate to the iron(II) ion in the active site might be a prerequisite for oxygen binding to occur (Figs. 6(B) and 7(B)) [73]. In fact, the binding of the organic substrate favors the subsequent binding of NO by increasing its affinity by about two orders of magnitude [73].

Details of the iron sites of extradiol dioxygenases have also been revealed by EPR studies on the hyperfine interactions of the NO-enzyme complexes with ^{17}O labeled water, substrates and inhibitors. Hyperfine broadening is observed in the presence of ^{17}O-water, thus suggesting the presence of at least one water molecule coordinated to the metal center; such broadening is quenched by substrate binding which therefore displaces water from the active site in contrast with inhibitor-enzyme complexes where the water remains bound to the iron ion (Figs. 6 and 7) [73]. Complexes of PC4,5O with protocatechuates ^{17}O labeled either in the 3- or 4-hydroxyl group show hyperfine broadening, suggesting that both donors are simultaneously coordinated to the metal concurrently with NO (Fig. 8). Therefore three iron(II) coordination sites seem to be accessible to exogenous ligands in extradiol dioxygenases.

CD, MCD, and electronic absorption spectroscopies have also been utilized in

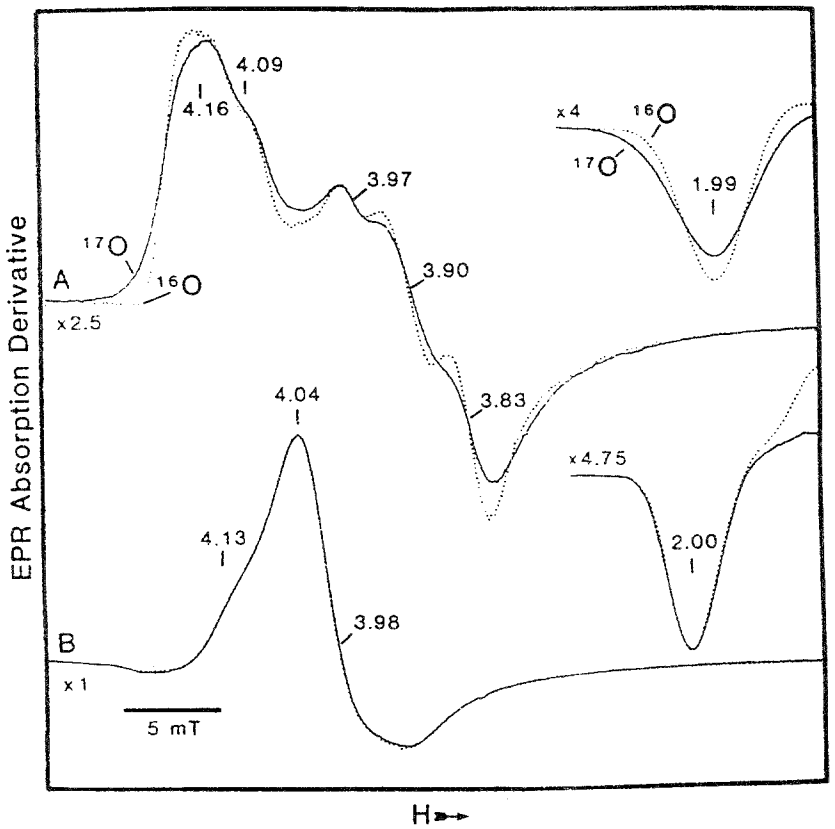

Fig. 6. EPR spectra of catechol 2,3-dioxygenase-NO complexes in the presence of ^{17}O-enriched water (—) and unenriched water (···). Native enzyme (A) and its adduct with catechol (B) (adapted from Ref. [73]).

order to characterize the coordination chemistry of the metal site of extradiol dioxygenases [75,76]. Native catechol 2,3-dioxygenase shows room temperature near-IR electronic absorption and a CD low intensity band easily assigned as an iron(II) ligand field transition (Fig. 9). The ligand field features have been analyzed resulting in a pentacoordinated square-pyramidal geometry for the iron(II) active site. The catechol substrate substantially perturbs the MCD spectra (Fig. 10) [75]. Analysis of the variations in the ligand field energies suggests a bidentate binding to the metal ion occupying the axial and one of the equatorial positions of the coordination polyhedron, displacing at least one water molecule. Azide binding was observed to occur through MCD only in the presence of substrate, thus suggesting that the oxygen molecule is directly coordinated to the metal ion only when catechol is bound [75]. Spectroscopic data for the oxidized inactive enzyme rule out the presence of cysteines and/or tyrosines because the electronic absorption spectra lack the charge transfer bands characteristic of the interaction with such groups [77,78]. Consequently, possible candidates for iron(II) coordination are histidine and carboxylate groups in aspartate and/or glutamate residues.

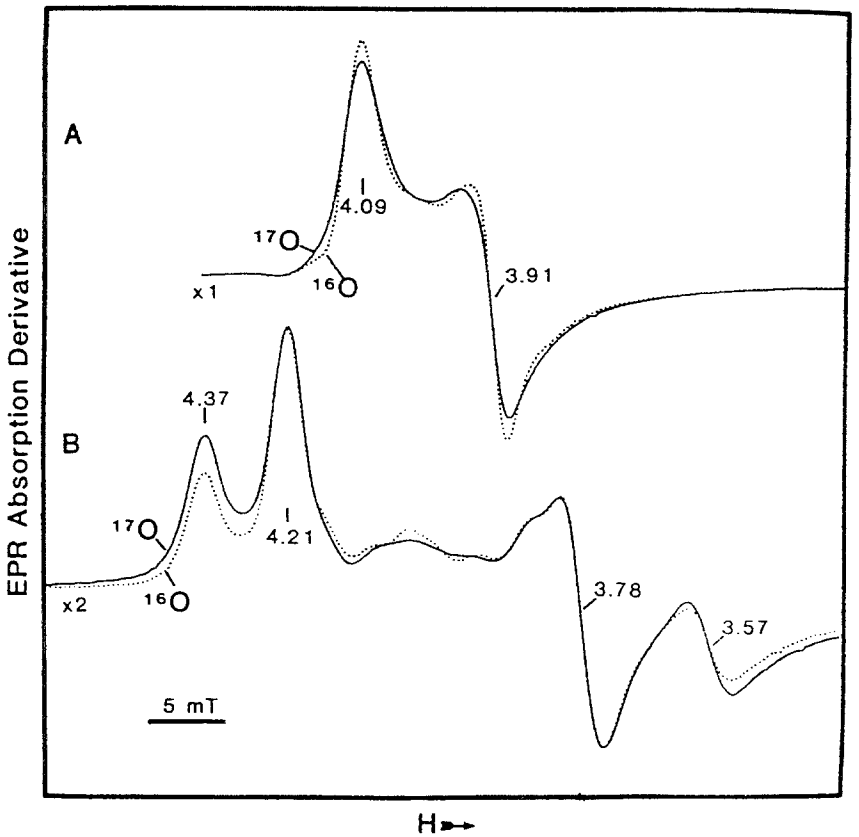

Fig. 7. EPR spectra of protocatechuate 4,5-dioxygenase-NO complexes in the presence of ^{17}O-enriched water (—) and unenriched water (···). Native enzyme (A) and its adduct with protocatechuate (b)(adapted from Ref. 73).

The active site structure of this enzyme has been recently investigated through XAS and NMR spectroscopies [79–82]. X-ray absorption spectroscopy (XAS) provides important chemical and structural information about the neighborhood of excited atoms in systems like metalloproteins. At energies just below the absorption edge, the XAS spectrum is characterized by transitions from core levels to empty bound states. This region contains information about the electronic structure of the absorbing atom and hence about the chemical environment affecting the molecular energy levels. For Fe(II) and Fe(III) ions a weak absorption can be observed at about 10 eV below the edge. This is the result of a formally forbidden transition from 1s to 3d orbitals which becomes possible because of d-p orbital mixing [83]. Studies on high-spin Fe(II) model compounds have shown that the intensity of this absorption is correlated with the deviation from centrosymmetry of the geometry of the metal center and hence can be related to the coordination number [84]. Characteristic ranges for pre-edge peak areas have been established: four coordinated

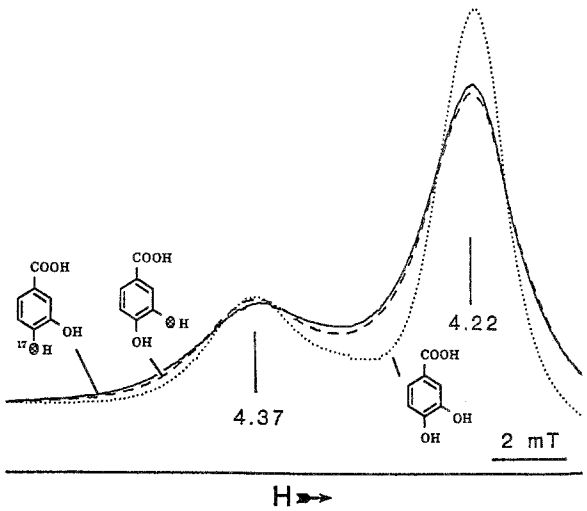

Fig. 8. Hyperfine broadening of the low field EPR resonances of the protocatechuate 4,5-dioxygenase-protocatechuic acid-NO complex resulting from the binding of ^{17}O-labeled protocatechuic acid (adapted from Ref. 74).

Fe(II) complexes have peak areas of about 0.18 eV, peak areas for five coordinated complexes range from 0.18 to 0.13 eV and for six coordinated complexes from 0.04 to 0.06 eV. The EXAFS (Extended X-ray Absorption Fine Structure) region of the spectrum refers to energies between 40 and 1000 eV above the edge. The EXAFS oscillations depend on the number, type and distances of the atoms in the near vicinity of the excited center. This part of the spectrum yield very precise structural information about the metal sites in proteins [85].

These types of XAS spectra have been recorded on the catechol dioxygenase enzyme (Figs. 11 and 12). The edge positions of the three spectra reported in Fig. 11 reflect the Fe(II) oxidation state. However, spectra (A) and (C) of Figs. 11 and 12 definitely belong to Fe(II) in different coordination environments. Spectrum (B) can be seen as a weighted average of spectra (A) and (C) in the ratio of approximately 1:1 as estimated by the pre-edge peak areas evaluation (see Fig. 11 caption).

Spectrum C has been obtained from a 1 mM protein solution in 50 mM phosphate buffer and 10% (v/v) acetone used as protectant [35]. The spectrum has been obtained twice [80,81] from independent enzyme preparations. The XAS spectra were collected on samples stored at 277 K for a few weeks. The enzymatic activity measured before data collection was found to be >300 units [46]. The pre-edge intensity has been interpreted as indicative of six-coordination being on the low end of the range for six-coordinated high spin Fe(II) as established from model compound studies [84]. The EXAFS spectrum may be equally well fitted with 5 or 6 ligands distributed over two different distances [80].

A spectrum similar to (A) has been reported recently [82] from a solution of the enzyme in 50% glycerol stored at 77 K before data collection. The sample was reported to have the same activity as that of spectrum (C). Subsequently the spectrum

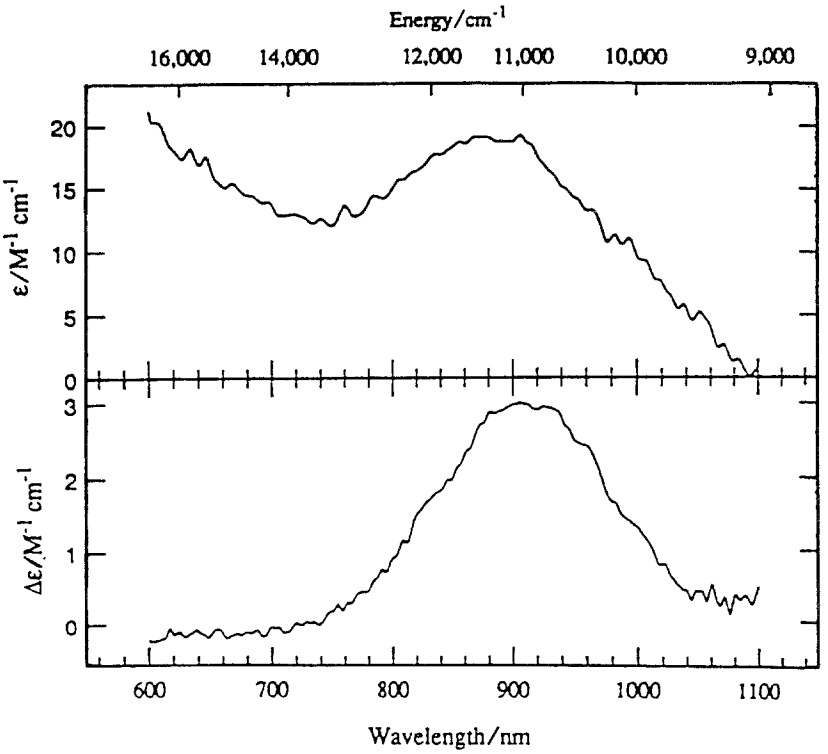

Fig. 9. Optical absorption and CD room temperature spectra of catechol 2,3-dioxygenase (adapted from Ref. 75).

(A) has been obtained also from a freshly prepared sample in 10% acetone [86]. The pre-edge peak intensity of spectrum (A) is almost twice that of spectrum (C) consistent with a 5 coordinated Fe(II) ion as previously suggested on the basis of MCD spectra [75]. Shu et al. have fitted the EXAFS spectrum of C2,30 in 50% glycerol with 5 ligands at an average distance of 2.09 Å [82]. On aging the spectrum (A) both in acetone or in glycerol slowly moves towards spectrum (C) [86]. Spectrum (B) has been obtained on a sample from the same batch of protein used for spectrum (A) in 10% acetone or 50% glycerol after 10 days of storage at 277 K. The activity of this sample was $90 \pm 5\%$ of the original activity before data collection. This 'aged' spectrum is indicative of a mixture of the species originating the (A) and (C) spectra.

The Fourier transforms of the spectra (A) and (C) show outer shell peaks at ≈ 3.0 and 4.0 Å from the iron which indicate the presence of histidine ligands. These peaks have been simulated with multiple scattering contributions from two histidines bound to the Fe(II) ion [80, 86 and refs, therein]. This has been the first determination of ligands in the extradiol dioxygenases active site as later confirmed by NMR results on a monomeric species of a related protein from a *Sphingomonas sp.* strain RW1 [83] (see later) and by the X-ray crystal structure of the 2,3 dihydroxybiphenyl 1,2-dioxygenase [87] (vide infra).

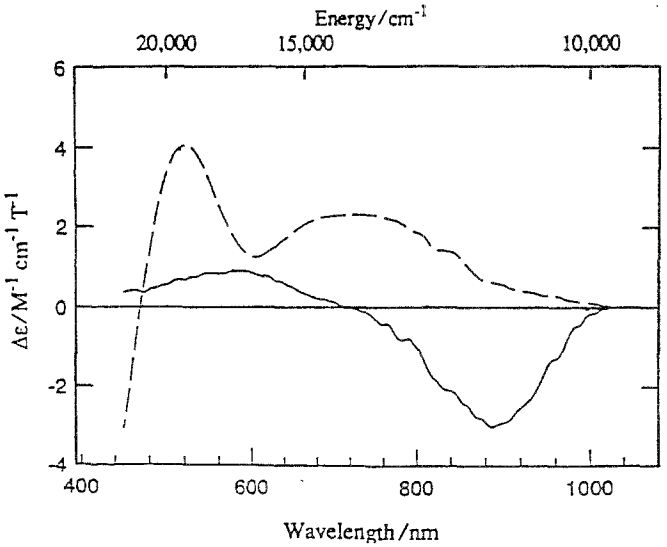

Fig. 10. MCD spectra of catechol 2,3-dioxygenase at 4.2 K and 5.9 T. Native enzyme (—) and its anaerobic adduct with catechol (– – –)(adapted from Ref. 75).

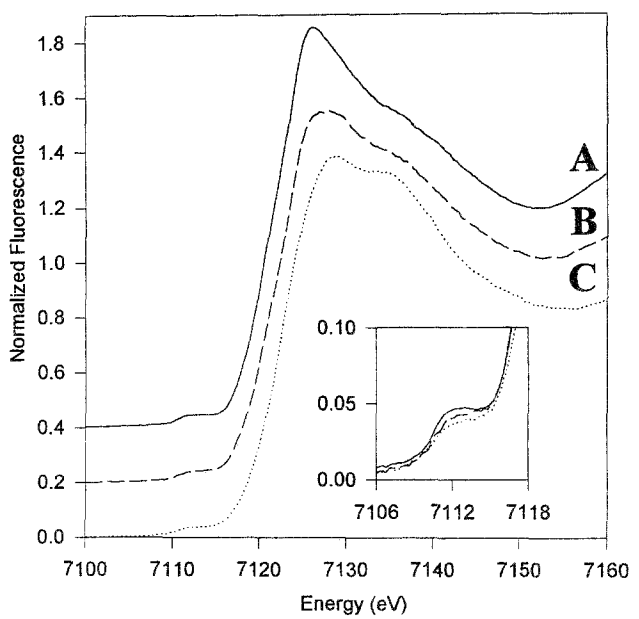

Fig. 11. X-ray absorption edge spectra for the native Fe(II)-catechol 2,3-dioxygenase: (A) freshly prepared 1 mM enzyme sample in 50 mM potassium phosphate pH 7.5 and 10% acetone (—), (B) enzyme sample after 10 days of storage at 277 K (– – –). (C) enzyme sample after few weeks of storage at 277 K (aged sample) (· · ·). In the inset the pre-edge peaks are shown (Adapted from Refs. [80,86]). The pre-edge peak areas are 0.082, 0.072, 0.049 eV for samples (A), (B) and (C) respectively.

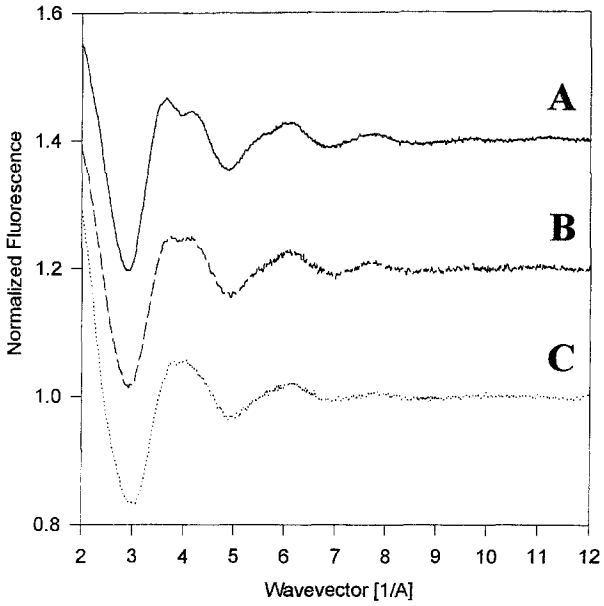

Fig. 12. EXAFS spectra for the native Fe(II)-catechol 2,3 dioxygenase samples (A), (B), and (C) as described in Fig. 11 (Adapted from Refs. [80,86]).

Shu et al. have shown that when the catechol substrate is added to the protein solution of spectrum (A), the spectrum of another five-coordinate compound is obtained [82]. The first shell data have been interpreted in terms of asymmetric bidentate binding of the substrate on the basis of the analysis of the EXAFS of a model compound. The different coordination distances used to fit the first shell EXAFS data of the C2,30-catechol complex have suggested the binding of a mono-protonated catecholate ligand in resemblance to the Fe(II)(6TLA)(DBCH)(ClO_4) model compound [82].

Addition of catechol to the solution originating spectrum (C), gives a hexacoordinated derivative as indicated by the pre-edge analysis. Analysis of the outer shells results in an orientation of the substrate aromatic ring which is compatible with bidentate binding [81]. Indeed, the orthohalogenated phenols inhibitors behave as monodentate ligands whereas the 2OH-pyridine-N-oxide substrate analogue is bidentate [80,81]. Sixfold coordination has been obtained also upon addition of catechol and nitric oxide to the spectrum (A) solution [82].

All of these studies indicate that the iron (II) is bound to the protein through two histidines and another protein ligand and that further binding positions may be occupied by the NO/O_2 or solvent molecules and by the catechol substrate.

^1H NMR as well as kinetic activity studies on the interaction of *ortho*-substituted phenols and aliphatic ketones and alcohols were also performed in order to characterize the type of interaction of such inhibitors with the active site of the enzyme [79]. T_{iM}^{-1} (i = 1, 2) paramagnetic enhancements to the relaxation rates of the protons

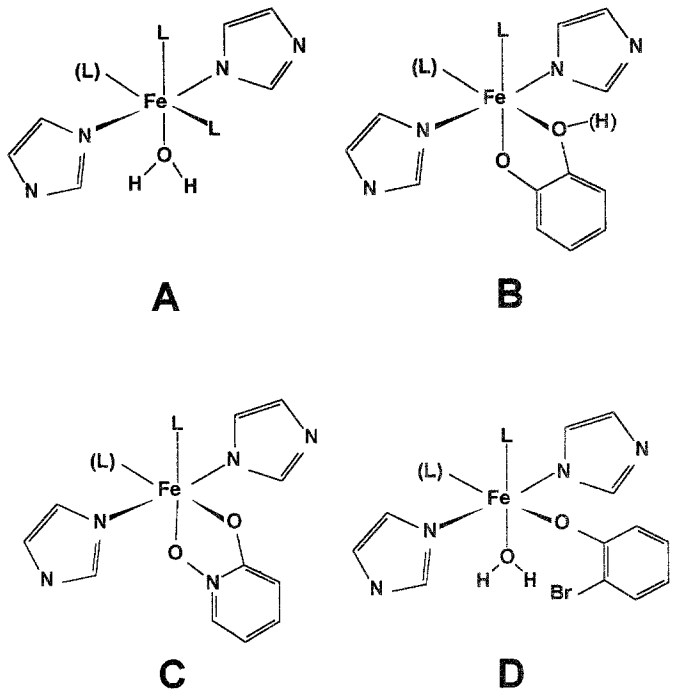

Fig. 13. Schemes of the active iron(II) coordination sites in: Native catechol 2,3-dioxygenase (C2,3O) (A), Catechol-C2,3O enzyme complex (B), 2-hydroxy-pyridine-*N*-oxide-C2,3O enzyme complex (C), and 2-bromophenol-C2,3O enzyme complex (D) as resulting from the XAS data analysis. L represents N/O donors. The trans-arrangement of the histidine ligands is just a pictorial representation (Adapted from Refs. [80,82]).

of the inhibitor were used to estimate the average distances of the inhibitors from the catalytic iron(II) ion [79]. Both phenols and ketones appear to be coordinated through their oxygen atoms to the iron(II) ion in the active site. The direct binding of 2-methoxy phenol to the iron(II) active site ion is consistent with the acidic character of the phenolate ion, but for compounds such as 2-pentanone the result is rather surprising. This suggests the presence of hydrophobic regions in the active site of the enzyme which play a significant role in binding inhibitors or substrates to the metal ion center.

Very recently the X-ray crystal structure of 2,3-dihydroxybiphenyl-1,2-dioxygenase from *Pseudomonas sp. LB400* has been announced [82]. In terms of polypeptide fold the structure shows two levels of internal repetition and topological features that appear to be different from the other known protein structures, and the active site is located within a cylindrical hydrophobic cavity different from the previously observed barrel-like structures. The Fe(II) coordination geometry appears to be square pyramidal, the metal ligands being two histidines, one glutamate, and two water molecules. The X-ray data were collected under anaerobic conditions and in the absence of substrates or inhibitors, therefore no information has been obtained

on the three sites which should be occupied by the exogenous ligands during catalysis; if the free sixth coordination position were involved in the binding of O_2 or substrates, this picture would be entirely consistent with the interpretation of the spectroscopic data previously obtained on other extradiol dioxygenases in the presence of substrates or inhibitors.

A monomeric *2,2',3-trihydroxybiphenyl-1,2-dioxygenase* has also been recently purified from *Sphingomonas sp.* strain *RW1*, a dibenzofuran- and dibenzo-*p*-dioxin-degrading bacterium [66]. The low molecular weight of this enzyme allowed high resolution NMR experiments to be performed in order to shed more light on the structure–function relationships of this class of enzymes. The resonances of last two histidines coordinated to the iron(II) active center have been observed [88].

3. Mn(II) extradiol dioxygenases

A few studies are available on extradiol dioxygenases containing Mn(II) [43,44]. 3,4-dihydroxyphenylacetate-2,3-dioxygenase from *B. brevis* is stable in the presence of H_2O_2, cyanide or azide, and is inhibited by the addition of Fe(II) ions. In fact, this enzyme has been shown to contain two Mn(II) ions per molecule. The EPR spectrum is characteristic of Mn(II) in low symmetry and it is altered upon the addition of substrate and inhibitors (Fig. 14) [43].

An enzyme exhibiting similar properties has also been isolated from the thermophilic organism *B. stearothermophilus* but no metal analysis has been performed to test whether it contains Mn [44]. This enzyme was also shown to have maximum activity at 57°C.

Mechanistic studies need to be accomplished in order to shed more light on the differences between Mn(II)- and Fe(II)-containing extradiol dioxygenases in terms of catalytic mechanism and active site structure.

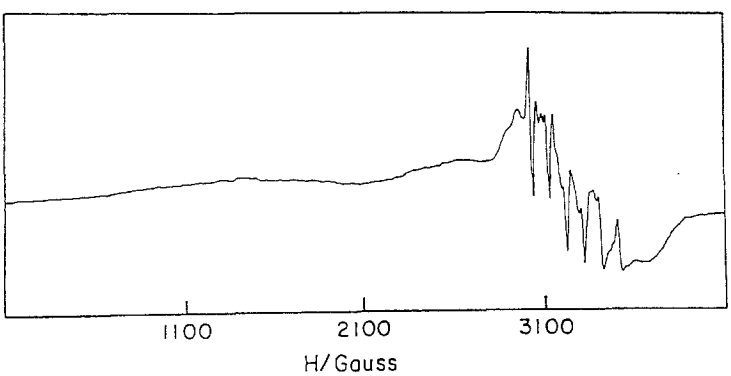

Fig. 14. EPR spectrum of the Mn(II)-containing 3,4-dihydroxyphenylacetate-2,3-dioxygenase from *Bacillus brevis* (adapted from Ref. [43]).

4. The catalytic mechanism of iron(II)-containing extradiol dioxygenases as compared to that of iron(III)-containing intradiol dioxygenases

The oxidative ring fission process catalyzed by dioxygenases must overcome large energy barriers [89,90]. In fact, the reaction between O_2 with a triplet spin ground state and organic molecules, generally with a singlet spin ground state, is spin-forbidden; further, the organic substrates are generally stable molecules so a large activation energy is required. Finally, the stoichiometry of the reaction requires that after bond cleavage the intermediates be retained for reaction completion without solvent exchange. Intra- and extra-diol dioxygenases appear to have overcome the problem of oxygen activation in different ways, although for both classes of enzymes steady state kinetic studies have revealed a similar ordered bi-uni reaction mechanism with the organic substrate binding first followed by the oxygen molecule [52,91].

Extradiol dioxygenases appear to be characterized by a high specificity in ring cleavage position at variance with the intradiol enzymes which can also give rise to extradiol cleavage [92–94]. In the latter case a systematic study on 3-substituted catechols showed that steric and electronic effects play important roles in promoting such 'secondary' reactions, which seem to be favored by the presence of small electron-donating groups. As an example $3CH_3$-catechol is cleaved by catechol 1,2-dioxygenase yielding both the intra- and the extra-diol products in a 17:1 ratio [92]. Also, the cleavage of 2-aminophenol catalyzed by the same enzyme has been observed to produce picolinic acid and the α-hydroxymuconic semialdehyde in a 19:1 ratio [95]. Saeki et al. investigated the oxygenation of pyrogallol by three different dioxygenases: catechol 1,2-dioxygenase from *P. putita* (arvilla) C1, protocatechuate 3,4-dioxygenase (both intradiol enzymes) and C2,3O (extradiol enzyme) [41]. C2,3O generated uniquely the proximal extradiol cleavage product whereas the protocatechuate 3,4-dioxygenase produced the seven-membered 2-pyrone-6-carboxylic acid with the incorporation of a single oxygen atom. Catechol 1,2-dioxygenase gave instead an almost equimolar mixture of these two products. The authors proposed a mechanism including the formation of a seven-membered lactone ring intermediate as reported in Fig. 15 [41].

In the case of intradiol dioxygenases all the kinetic and spectroscopic data show that the Fe(III) does not change its oxidation state in all semistable states of the catalytic process. Further, a change in the coordination of catechol from bidentate to monodentate has been shown to occur, at least for the protocatechuate 3,4-dioxygenase, during catalysis. On these bases the following catalytic mechanism has been proposed (Fig. 16) [33,34,77,96–102]: a chelate substrate-Fe(III) complex is initially formed and one of the tyrosine ligands is probably detached, then reorientation of the substrate from bidentate to monodentate seems to occur [77,103,104]. This step appears to be essential in order to open a coordination site on the metal ion for the binding of the distal oxygen of the substrate-peroxide intermediate. Such binding should facilitate the heterolytic O–O bond cleavage by stabilizing the presumed hydroxide product. Therefore the Lewis acidity of Fe(III) seems to promote the ketonization of the substrate ring lowering the energy of the oxygen attack. The reaction is completed with the hydrolysis of the anhydride intermediate. The substrate

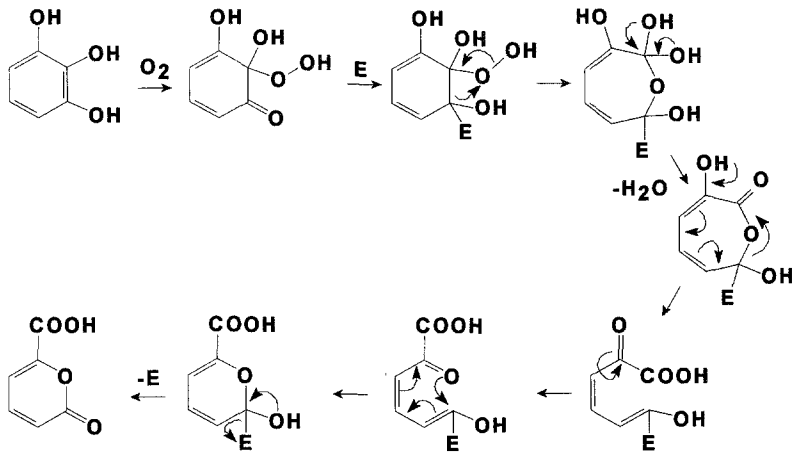

Fig. 15. Schematic representation of the reaction mechanism proposed for the pyrogallol cleavage catalyzed by protocatechuate 3,4-dioxygenase (adapted from Ref. [41]).

coordination change from bidentate to monodentate during the catalytic cycle has been proposed also in model complexes [105]

Many studies on model systems which catalyze the oxidative cleavage of catechols have also been reported and only recently an active species identical to that proposed for the intermediate of catechol oxidation has been isolated and crystallized [106]. The structure of the complex $[(triphos)Ir(OO)(DTBSQ)]^+$ is, in fact, the first example of an isolated oxygen adduct of an efficient catalyzer of catechol intradiol cleavage (Fig. 17) [106].

Only a few model systems reported to date resulted in extradiol cleaving [106–112]. In particular, studies on a $RuCl_2(Ph_3P)_3$ complex showed evidence of an α-ketolactone intermediate in the catalytic oxygenation of 3,5-di-tert-buthylcatechol resulting in both intra- and extra-diol products [109]. Selective extradiol or intradiol ring opening of 3,5-di-tert-butylcatechol has also been demonstrated to occur in mixed tetrahydrofuran–water or tetrahydrofuran–pyridine solvents in the presence of Fe^{2+} or Fe^{3+} respectively [112].

In the case of extradiol dioxygenases the spectroscopic and kinetic studies are less informative, however Lipscomb and coworkers have proposed a unifying mechanism for extradiol dioxygenases which appear to be consistent with the structural and catalytic data available to date [52,74,75,77,113,114]. As in the case of the intradiol enzymes changes to the iron(II) oxidation state have not been observed during catalysis. All such enzymes have been reported to simultaneously coordinate the organic substrate and the oxygen (or NO) molecule to the Fe(II) center in noncompeting binding sites and, in contrast to intradiol enzymes, conformational changes are not required to allow substrate chelation to the metal center. In fact, water and NO (O_2) compete for the same site in intradiol enzymes (the NO binding was studied on the reduced enzyme [104]) whereas they occupy different sites in extradiol catalysts. Fe(II) and Fe(III) in extra- and intra-diol dioxygenases, respectively, seems

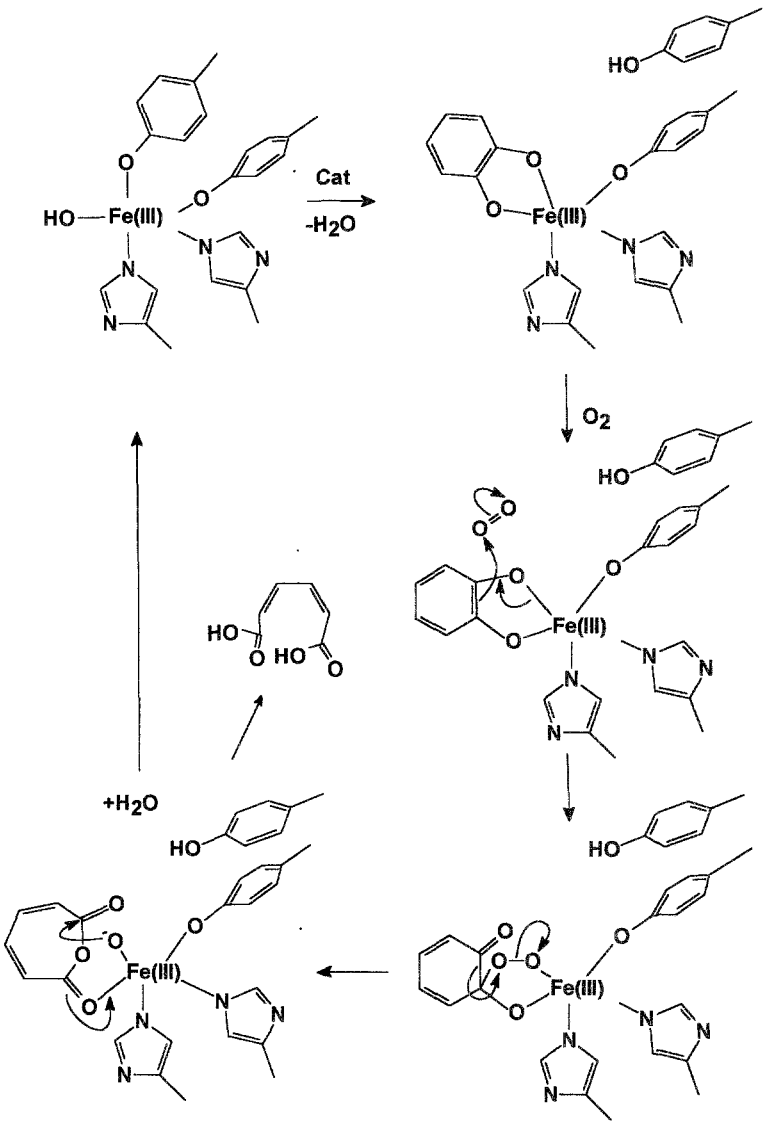

Fig. 16. Schematic representation of the reaction mechanism for intradiol cleavage catalyzed by Fe(III) dioxygenases.

to play a different role in oxygen binding and activation; in the latter class of enzymes the Lewis acidity of Fe(III) is likely to be adequate to activate the organic substrate for the direct attack by O_2. In extradiol enzymes the Fe(III)/Fe(II) redox potential is probably reasonably high to prevent a strong binding of the O_2 molecule and substrate coordination is predicted to lower the potential resulting in stronger bonds with electrophilic ligands such as O_2 or NO. The observation that the iron(II) center

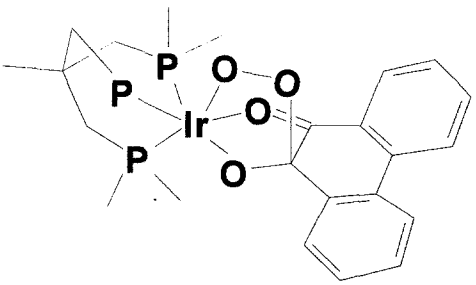

Fig. 17. Schematic drawing of the X-ray structure of the complex cation [(triphos)Ir(OO)(DTBSQ)]$^+$ (the phenyl rings of the triphos ligand are omitted for clarity) (adapted from Ref. [106]).

of protocatechuate 2,3-dioxygenase is not rapidly oxidized by exposure to high O_2 pressures seems to support this hypothesis [114]. The concurrent binding of the organic substrate and O_2 could result in electron density polarization on the distal oxygen atom, which could attack the positively charged ring coordinated to the metal ion (Fig. 18). Such an attack would result in the formation of a peroxy intermediate with sp^3 hybridization at the carbon atom. Subsequent O_2 heterolytic cleavage and insertion of the second oxygen atom into the ring would complete the catalytic process (Fig. 18).

With the exception of the cooperative binding of oxygen and organic substrates to the metal ion center there is no definite proof for the above-reported subsequent steps of the catalytic mechanism. In particular, nothing is known about the structure of the oxygen adduct, which could be different from that proposed for the extradiol enzymes, as shown in Fig. 18. In fact the molecular oxygen could initially bind to the atom C(1) of the catechol ring [112] instead of C(6). Systematic analyses of kinetic and spectroscopic data of binary and ternary adducts of such enzymes are probably needed in order to rationalize the complete catalytic process performed by extradiol dioxygenases.

5. Concluding remarks

The aromatic ring cleaving dioxygenases are metalloenzymes which generally contains iron in the active site. The two principal classes of bacterial dioxygenases differ in both the mode of ring fission and the oxidation state of the active iron ion: +2 in the case of the extradiol, +3 in the case of the intradiol enzymes.

The origin of the differences in ring opening specificity is not clear at the moment. These could arise from the chemical properties of iron(III) compared with iron(II) [112], and also from the steric control that the protein exerts on the substrate. Both of these factors are probably important in determining the site of oxygen attack and/or the stabilization of different products of ring cleavage. Interestingly, some intradiol enzymes also show a substantial formation of products deriving from an extradiol ring opening mechanism.

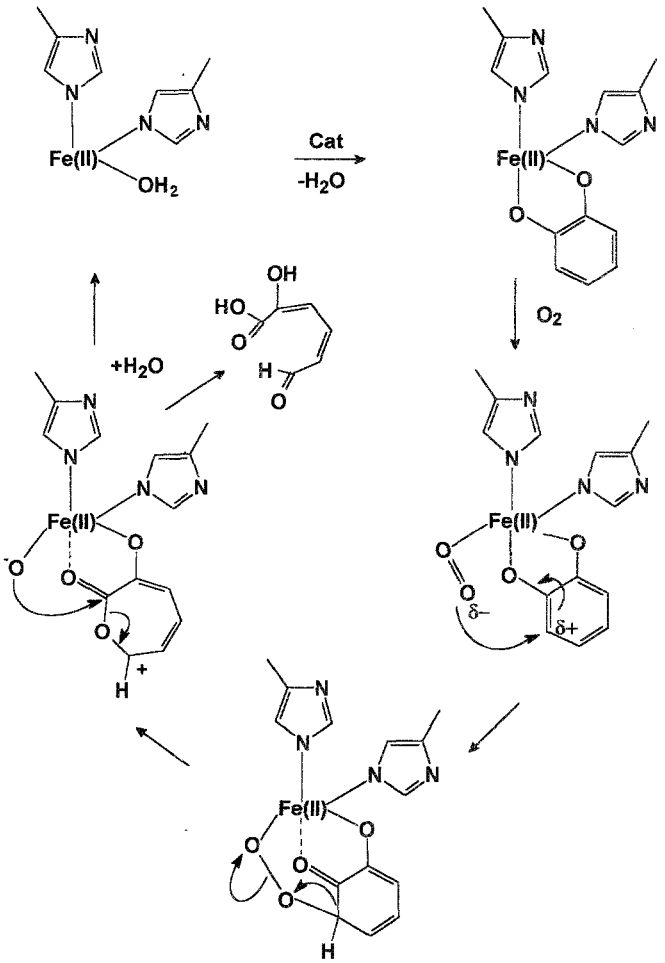

Fig. 18. Schematic representation of the reaction mechanism for extradiol cleavage catalyzed by Fe(II) dioxygenases.

It must be also remembered that the iron ions in the two classes of dioxygenases have different sets of donor ligands which stabilize the oxidation state typical of each class. In the intradiol enzymes, in addition to histidines, tyrosinate ligands, which are known to stabilize iron(III) ions, are present. Conversely, in the extradiol enzymes the iron(II) active site is coordinated to two histidine residues and one carboxylate group resulting in a loosely bound ion which completes its coordination sphere with water molecules. Therefore, it is not surprising that the two classes cannot interconvert, i.e. the reduced form of the intradiol and the oxidized form of the extradiol enzymes are both inactive.

Strict similarities between the two classes can also be found concerning the site of oxygen interaction, the differences being ascribable to the chemical properties of iron(II) compared to the iron(III) ions. So, nitric oxide, which is a competitive

inhibitor at the oxygen binding site in extradiol dioxygenases, binds directly to the metal, as easily demonstrated through ESR spectroscopy. The iron(III) in the intradiol class does not bind oxygen (nor NO) but again the reduced (inactive) form, is able to bind NO to the metal ion. Therefore, the behavior toward oxygen is determined by the electronic properties of the metal ion. In any case, the oxygen binds after the organic substrate binding, indicating for both classes a concerted mechanism of activation.

In conclusion, although dihydroxylated phenyl compounds are generally reactive molecules, the study of ring-cleaving dioxygenases is important for the understanding of the basis of oxygen activation and of the high specificity in ring fission. This would be useful in order to develop a new chemistry which leads to selective oxygen insertions making use of a more environmentally benign oxidant.

Acknowledgements

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