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Chapter 7

Intradiol Dioxygenases — The Key Enzymes in Xenobiotics Degradation

Urszula Guzik, Katarzyna Hupert-Kocurek and Danuta Wojcieszyńska

Additional information is available at the end of the chapter

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1. Introduction

Aromatic compounds are derived from both natural and anthropogenic sources. Under natural conditions, arenes are formed as a result of the pyrolysis of organic materials at high temperatures during forest, steppe and peatland fires, and during volcanic eruptions. Biogenic aromatic compounds like aromatic amino acids and lignin, the second most abundant organic compound in the environment, are universally distributed in nature. Many species of plants, especially willow (*Salix*), thyme (*Thymus vulgaris*), camomile (*Chamomilla recutita*), bean (*Phaesoli vulgaris*) or strawberry (*Fregaria ananasa*), water plants as sweet flag (*Acorus calamus*) and many species of alga are known to produce aromatic compounds as secondary metabolites [1-4]. A lot of aromatic compounds are introduced to the environment as contaminating compounds from chemical, pharmaceutical, explosive, dyes, and agrochemicals industry. Chloro-, amino- and nitroaromatic derivatives, biphenyls, polycyclic aromatic hydrocarbons accumulate in the soil and water. They are toxic to living systems including humans, animals, and plants. Moreover, most of them may bioaccumulate in the food chain and have mutagenic or carcinogenic activity [5-8].

Due to the delocalization of π orbitals aromatic compounds are very stable [3]. For many years it has been searching for microorganism able to breakdown these kind of substrates. Among the bacteria, representatives of the genus *Pseudomonas, Sagittula, Streptomyces, Agrobacterium, Acinetobacter, Arthrobacter, Burkholderia, Bacillus, Stenotrophomonas, Brevibacterium, Sphingomonas, Geobacillus, Rhodococcus, Nocardia, Corynebacterium, Alcaligenes, Gordonia*, or *Citrobacter* are active in this respect [9-22].

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The strategy for degradation of aromatic structure comprises hydroxylation and cleavage of the aromatic ring with the usage of oxygenases [8,23,24]. These reactions are initiated by electrophilic attack of molecular oxygen and they are retarded by the presence of electron-withdrawing substituents. For this reason the velocity of reaction is connected with the kind of substituents in the aromatic ring. Substituents of the first group such as: $-NH_2$, -OH, $-OCH_3$, $-CH_3$, $-C_6H_5$ activate aromatic ring for electrophilic attack. In contrary, the substituents of the second group ($-NO_2$, $-N(CH_3)_3^+$, -CN, -COOH, -COOR, $-SO_3H$, -CHO, -COR) and the third group (-F, -Cl, -Br, -I) impede the electrophilic attack by dioxygenases [3,25-28].

Hydroxylation into the dihydroxylated intermediates, the first step in the oxidative degradation of aromatic compounds, is catalyzed by monooxygenases or hydroxylating dioxygenases (Fig. 1). Aromatic monooxygenases are divided into two groups: activated- ring monooxygenases and nonactivated- ring enzymes and usually contain FAD as a prosthetic group. These enzymes posses a dinuclear iron centre with two oxo- bridged iron atoms (Fe-O-Fe) in the active site [36-39]. Aromatic monooxygenases activate molecular oxygen through the formation of a reactive flavin (hydro)peroxide which can attack the substrate [31,33-35].

Aromatic ring hydroxylating dioxygenases that belong to the Rieske non-heme iron oxygenases are multicomponent enzyme systems. The reaction catalyzed by hydroxylating dioxygenases requires two electrons from a NAD(P)H that are consecutively transferred to the terminal oxygenase component through a electron carries such as ferredoxin and/or a reductase [36].

As a result of hydroxylation key intermediates such as catechol, protocatechuic acid, hydroxyquinol, or gentisic acid are formed. These products are substrates for ring-cleaving dioxygenases [17,37-39]. The ring-cleaving dioxygenases couple O_2 bond cleavage with ring fission of hydroxylated derivatives either between the two hydroxyl group (*ortho* cleavage) or beside one of these (*meta* cleavage) [23,40].

Intradiol dioxygenases catalyze the intradiol cleavage of the aromatic ring at 1,2-position of catechol or its derivatives (protocatechuic acid, hydroxyquinol) with incorporation of two atoms of molecular oxygen into the substrate. It leads to the production of *cis,cis*-muconic acid or its derivatives (3-carboxy-*cis,cis* muconic acid, 3-hydroxy-*cis,cis* muconic acid). The *cis,cis*-muconic acid is then subsequently transformed by a muconate cycloisomerase to muconolactone. Muconolactone isomerase shifts the double bond to form 3-oxoadipate-enol-lactone, the first common intermediate of the catechol and protocatechuate or hydroxyquinol branch (Fig. 1) [38,41,42].

Extradiol catechol and protocatechuate dioxygenases catalyze the ring fission between position C2 and C3 of catechol ring and between position C2 and C4 or C4 and C5 of protocatechuate ring respectively. Products of these reactions (2-hydroxymuconic semialdehyde or carboxy-2-hydroxymuconic semialdehyde) are transformed finally to pyruvic acid and acetaldehyde in catechol pathway and pyruvic acid and acetaldehyde or pyruvic acid and oxaloacetic acid in protocatechuate pathway (Fig. 1) [37,43].

Degradation of aromatic compounds by the *meta* pathway often leads to more toxic intermediates such as acyl chloride that is why in the degradation of some arenes the *ortho* cleavage is preferred [5,6]. Recently intradiol dioxygenases with extremely high activity were isolated.

They could be applicable not only in biodegradation but also in industry processes since products of the *ortho* cleavage are valuable intermediates in chemical synthesis [44,45].

Because some aromatics have been present in the environment for a very short time, bacteria have not evolved efficient pathways for their degradation and for that reason the construction of such pathways may be required. Therefore, a detailed understanding of the molecular bases as well as enzymes of known pathways is essential [46].

Our chapter is divided into three parts: first we classify bacterial intradiol dioxygenases, key enzymes in aromatic compounds degradation, taking under consideration phylogenetic relationships among dioxygenases from various strains and their substrate specificity. Next, we present current knowledge about molecular structure of these dioxygenases and hypothetical mechanisms for aromatic ring cleavage. And finally, we describe modifications increasing biodegradation potential of these enzymes.

2. Classification of bacterial intradiol dioxygenases

Many studies on intradiol dioxygenases concentrate on the understanding of their biochemical and structural properties. Based on the results of these studies and taking into consideration substrate specificity of the *ortho* fission dioxygenases they were divided by Vetting and Ohlendorf [47] into two families: catechol 1,2-dioxygenases and protocatechuate 3,4-dioxygenases, including hydroxyquinol 1,2-dioxygenases. In turn, Hammer et al. [12] and Contzen and Stolz [48] divided protocatechuate 3,4-dioxygenases into two types. Type I dioxygenases catalyze ring fission of protocatechuic acid and its derivatives while type II enzymes cleave *ortho* diphenols carrying a larger substituent at the 4-position of the aromatic ring [12,48].

Although Murakami et al. [14] observed partial activity of hydroxyquinol 1,2-dioxygenase towards catechol and Pandeeti and Siddavattam [49] isolated catechol 1,2-dioxygenase which was active strictly against catechol and 1,2,3-benzenotriol there is no reports about activity of catechol 1,2-dioxygenases towards hydroxyquinol [18,21,50,51]. Therefore, we propose to divide the intradiol dioxygenases into three classes: catechol 1,2-dioxygenases, protocatechuate 3,4-dioxygenases, and hydroxyquinol 1,2-dioxygenases. Representatives of these three classes are presented in Table 1. Based on the results of biochemical and genetic comparison of intradiol dioxygenases Perez-Pantoja et al. [52] distinguished hydroxyquinol 1,2-dioxygenases as a separate cluster that may support our suggestions.

The catechol 1,2-dioxygenases family can be divided into several subclasses depending on their substrate specificity. Initially this family was divided into two subclasses: type I dioxygenases with activity against catechol and lack or weak activity against chlorocatechol and type II dioxygenases, which cleave only chlorocatechols [53]. Then, based on their amino acid sequences Liu et al. [54] divided family of catechol 1,2-dioxygenases into three groups: catechol 1,2-dioxygenases from gram-negative bacteria, chlorocatechol 1,2-dioxygenases from gram-negative bacteria, and both catechol- and chlorocatechol 1,2-dioxygenases from gram-positive bacteria. However, both of these classifications do not take into consideration substrate

specificity of these enzymes towards methylcatechols. High substrate specificity against these compounds was observed for intradiol dioxygenase isolated from genus *Rhodococcus* [55-57]. Above studies divided catechol dioxygenases into three subclasses. The first subclass comprises the enzymes with high enzymatic activity towards chlorocatechols (CCs), the second one includes enzymes with moderate activities for catechol only or catechol and 4-methylcatechol (4-MC). The third subclass consists of the enzymes with high activity for 3- and 4-methylcatechols (3-MC and 4-MC) [50,55].

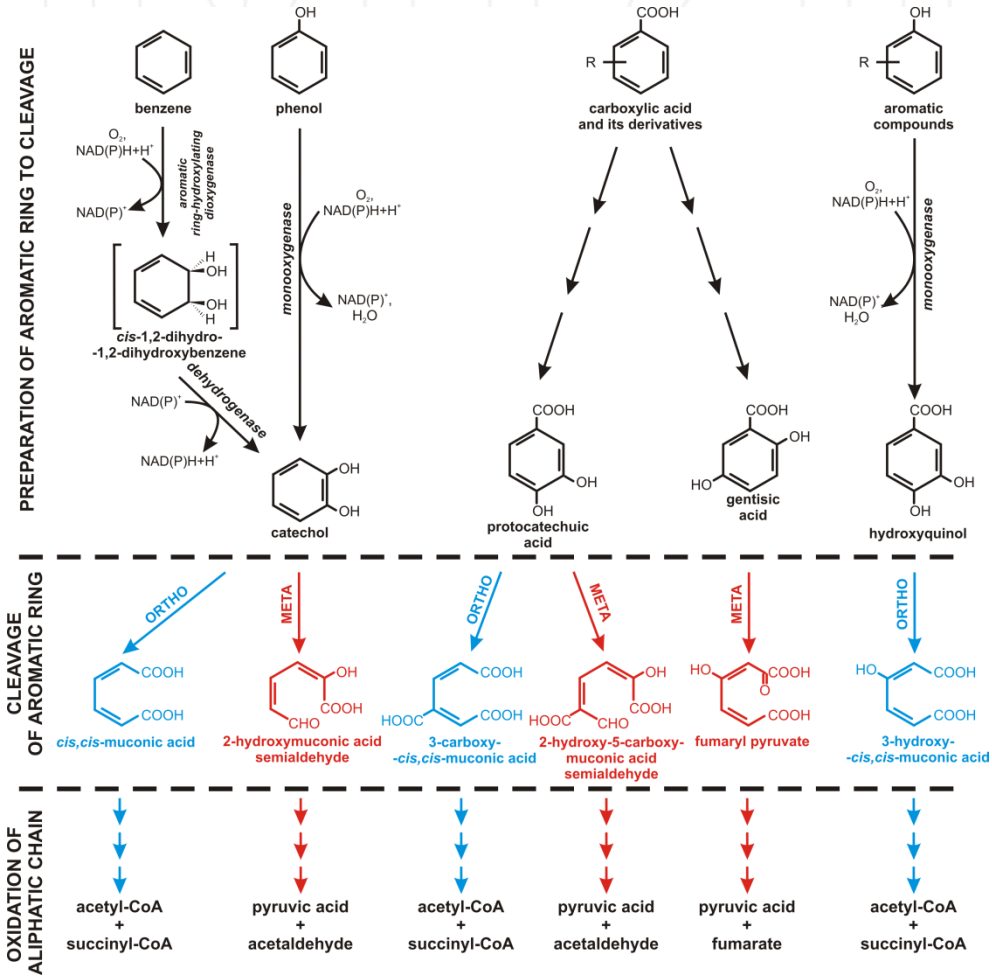

Figure 1. Pathways of aromatic compounds degradation [36,40,43].

Enzyme	Microorganism	Gene	AC Number	Substrates	References
Catechol 1,2-dioxygenase	*Acinetobacter calcoaceticus* ADP-96	-	-	catechol 3-isopropylcatechol 4-methylcatechol 3-methylcatechol	[58]
	Acinetobacter calcoaceticus ADP1	-	-	catechol	[47]
	Acinetobacter radioresistens	-	-	catechol 4-methylcatechol 3-methylcatechol	[59]
	Acinetobacter radioresistens S13	$catA_A$ $catA_B$	AF380158 AF182166	catechol 4-methylcatechol	[53,60]
	Acinetobacter sp. DS002	*catA*	-	catechol	[49]
	Alcaligenes eutrophus B.9	-	-	catechol 4-methylcatechol	[25]
	Alcaligenes eutrophus CH34	*catA*	NC_007973.1	catechol	[61]
	Arthrobacter BA-5-17	*catA-II*	AB109791.1	catechol 3-methylcatechol 4-methylcatechol	[62]
	Burkholderia sp. TH2	*catA1*	AB035483	catechol 3-methylcatechol 4-methylcatechol	[63]
	Burkholderia sp. TH2	*catA2*	AB035325	catechol 4-methylcatechol	[63]
	Citrobacter sp. SA01	-	-	catechol	[22]
	Gordonia polyisoprenivorans	-	-	catechol	[21]
	Pseudomonas aeruginosa TKU002	-	-	Catechol 4-methylactechol pyrogallol	[64]
	Pseudomonas putida N6	*catA*	EU000396.1	catechol	[38]
	Pseudomonas sp. B13	-	-	catechol 3-chlorocatechol 4-chlorocatechol 3-methylcatechol 4-methylcatechol	[25]
	Rhodococcus erythropolis AN-13	*catA*	D83237	catechol	[55]

Enzyme	Microorganism	Gene	AC Number	Substrates	References
	Rhodococcus opacus 1CP	*catA*	X99622.1	catechol 3-methylcatechol 4-methylcatechol	[50]
	Rhodococcus opacus 1CP	*clcA*	AF003948.1	4-chlorocatechol	[65]
	Rhodococcus opacus 1CP	*clcA2*	AJ439407.1	3-chlorocatechol	[65,66]
	Rhodococcus sp. NCIM 2891	-	-	catechol	[20]
	Sphingomonas xenophaga QYY	-	-	catechol	[67]
	Stenotrophomonas maltophilia KB2	*catA*	EU000397.1	catechol	[68]
Protocatechuate 3,4-dioxygenase	*Acinetobacter calcoaceticus*	*pcaHG*	-	protocatechuate	[10,69]
	Acinetobacter lwoffii K24	*pcaHG*	AY099487	protocatechuate	[70]
	Acinetobacter sp. ADP1	*pcaHG*	NC_005966.1	protocatechuate	[16]
	Agrobacterium radiobacter S2	*pcaH1G1* *pcaH2G2*	AF230649 AF230650	protocatechuate protocatechuate 4-sulfocatechol	[48]
	Agrobacterium radiobacter S2	-	-	protocatechuate4-sulfocatechol	[12]
	Brevibacterium fuscum	-	-	protocatechuate	[71]
	Burkholderia sp. NCIMB 10467	*pcaHG*	YP552570 YP552569	protocatechuate	[19]
	Geobacillus sp.	-	-	protocatechuate	[18]
	Hydrogenophaga palleronii S1	-	-	protocatechuate 4-sulfocatechol 3,4-dihydroxyphenyl-acetate 3,4-dihydroxy-cinnamate	[12]
	Moraxella sp.GU2	-	-	protocatechuate	[11]
	Pseudomonas cepacia	-	-	protocatechuate	[72-74]
	Pseudomonas cepacia DBO1	-	-	protocatechuate	[75]

Enzyme	Microorganism	Gene	AC Number	Substrates	References
	Pseudomonas fluorescens A.3.12	-	-	protocatechuate	[76]
	Pseudomonas marginata	*pcaHG*	U33634	protocatechuate	[77]
	Pseudomonas putida ATCC23975	*pcaHG*	L14836.1	protocatechuate	[78-80]
	Pseudomonas sp. HR199	*pcaHG*	Y18527	protocatechuate	[81]
	Rhizobium trifolii	-	-	protocatechuate	[82]
	Stenotrophomonas maltophilia KB2	*pcaH* *pcaG*	JQ364963 JQ364964	protocatechuate	[68]
	Streptomyces sp. 2065	*pcaHG*	AF109386.2	protocatechuate	[15]
Hydroxyquinol 1,2-dioxygenase	*Arthrobacter* sp. BA-5-17	*tftH*	AB016258	hydroxyquinol, catechol	[14]
	Azotobacter sp. GP1	-	-	hydroxyquinol, chlorohydroxyquinol	[41,83]
	Burkholderia cepacia AC1100	*tftH*	U19883.1	hydroxyquinol	[84]
	Burkholderia sp. AK-5	-	-	hydroxyquinol	[85]
	Nocardioides simplex 3E	*chqB*	AY822041.2	hydroxyquinol, 6-chlorohydroxyquinol, 5-chlorohydroxyquinol,	[17,86]
	Pseudomonas sp. 1-7	*pdcDE*	FJ821777.2	hydroxyquinol	[8]
	Pseudomonas sp. WBC-3	*pnpG*	EF577044.1	hydroxyquinol	[51]
	Ralstonia eutropha JMP134	*tcpC*	AF498371	6-chlorohydroxyquinol	[87]
	Ralstonia pickettii DTP0602	*hadC*	D86544	hydroxyquinol pyrogallol 3-methylcatechol 6-chlorohydroxyquinol	[88]
	Rhizobium sp. MTP-10005	*graB*	AB266211.1	hydroxyquinol	[89]
	Rhodococcus opacus SAO101	*npcC*	AB154422	hydroxyquinol	[90]

Table 1. Intradiol dioxygenases from various microorganisms

3. Molecular structure and reaction mechanism of intradiol dioxygenases

Intradiol dioxygenases belong to metalloenzymes with non-heme iron (III) that plays a crucial role for the binding and activation of dioxygen in the cleavage reaction [55,59]. Metal ion is ligated by two tyrosine and two histidine residues that are highly conserved in all investigated intradiol dioxygenases. The fifth ligand is a hydroxyl group derived from the solvent.

Two structurally different intradiol dioxygenases families can be singled out: catechol/hydroxyquinol 1,2-dioxygenases and protocatechuate 3,4-dioxygenases. Catechol/hydroxyquinol 1,2-dioxygenases are dimmers $(\alpha Fe^{3+})_2$ with identical or similar subunits while protocatechuate 3,4-dioxygenases are typically composed of two homologous subunits in oligomeric complexes $(\alpha\beta Fe^{3+})_{2\text{-}12}$ [66] (Fig. 3).

3.1. Catechol 1,2-dioxygenases characterization

Catechol 1,2-dioxygenases catalyze cleavage of catechol and its derivatives. The tertiary structure of the enzymes from this class is similar [53,59,61,64,66]. They form homodimers with two subunits connected with helical zipper motif. Each subunit has a molecular mass of 29.0-38.6 kDa.

Subunits of catechol 1,2-dioxygenases contain two molecules of phospholipids bound into a hydrophobic cavity at the dimeric interface [38,47,49,50,53,59,61,64,66,67]. On the base of electron density and the stereochemistry these phospholipids were identified as a phosphatidylcholine with two C14-15/C12-C17 hydrophobic tails [66]. It is suggested that the phospholipid chains may modulate the activity of catechol 1,2-dioxygenases by increasing the local concentration of substrate or the lipids act as an effector molecule. The binding of phospholipid to hydrophobic amino acid of the active site can lead to a conformational change at this place. During catalysis catechol is cleaved to *cis,cis-* muconic acid which is known to be toxic to the cell at low levels. Perhaps the next enzyme of intradiol pathway- *cis-cis-* muconate lactonizing enzyme, interacts with the hydrophobic tunnel and allows the proteins to pass the product from one enzyme to next. Moreover, phospholipids can alter the phospholipid bilayer in response to toxic levels of aromatic compounds [47,66].

Unique helical N-terminal domain was first described by Vetting and Ohlendorf [47] for catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus* ADP1. Sequence aligments indicate that this helical domain is conserved among all members of catechol 1,2-dioxygenase class.

Catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus* ADP1 is an elongated molecule, which is composed of helices and β sheets. The overall shape of this enzyme is similar to boomerang and it is divided into three domains: the two catalytic domains at each end of the molecule, and one central linker domain which is composed of several helices. The catalytic domain consists of two mixed topology β sheets which form a β sheets sandwich [47]. The similar structure is also observed in another catechol 1,2-dioxygenases [38,50].

The linker domain of catechol 1,2-dioxygenases contains 12 α helices. Five of them belong to the N-terminal chain of each monomer and one extending from each catalytic domain. The

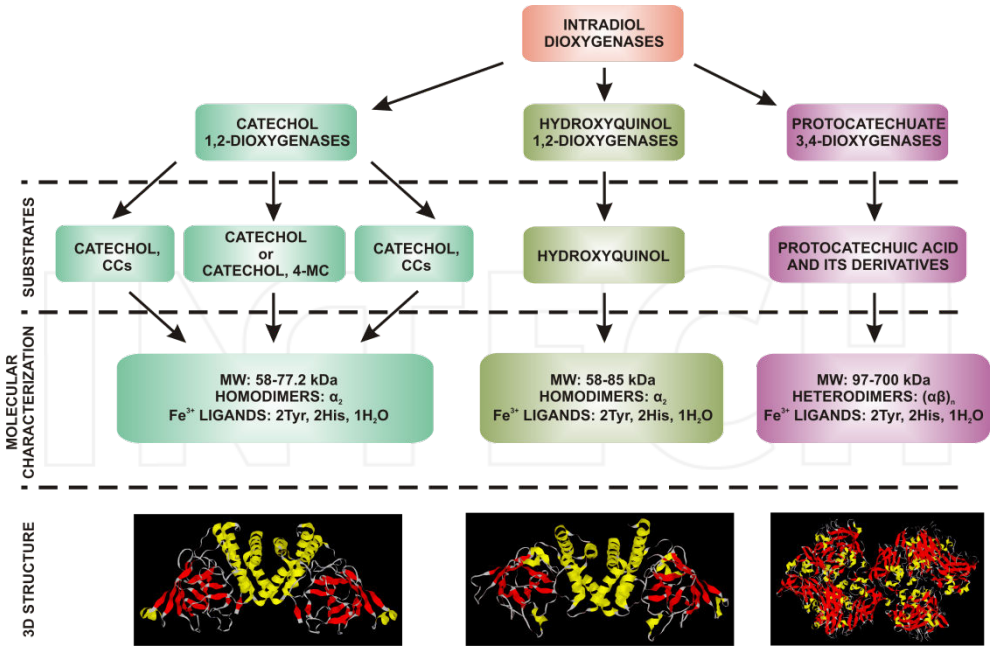

Figure 2. Characterization of intradiol dioxygenases

active site with ferric ion is located between β sheets sandwich and the linker domain [47]. Matera et. al. [50] compared structure of catechol 1,2-dioxygenase from Gram- positive strain *Rhodococcus opacus* 1CP with the enzyme from *Acinetobacter calcoaceticus* described above and observed that the differences in the structure are localized at the N-terminus. In this terminal chain helix 5 is shorter, strand 1 and helix 7 are missing and a new short helix is present between position 124 and 128. Moreover the C-terminal chain of catechol 1,2-dioxygenase from Gram-positive strain is shorter than in the other catechol 1,2-dioxygenases from Gram-negative bacteria.

As it was mentioned earlier the metal ion is ligated by four amino acid residues and a hydroxyl group from water in trigonal bipyramidal geometry. One molecule of tyrosine and one histidine are the axial ligands, whereas the second Tyr, His and a solvent molecule make up the equatorial plane. [38,47,50,53]. Since the two Fe^{3+} ions from catalytic domains are separated by more than 40 Å Vetting and Ohlendorf [47] suggested that they act structurally and catalytically independently. It explains the lack of allosteric effect [25,46,57,59,61,70,80]. However, catechol 1,2-dioxygenase from *Acinetobacter radioresistens* exhibits a sigmoidal kinetics indicating cooperation of subunits [59].

The substrate specificity of catechol 1,2-dioxygenases depends on electronic factors, which can play a significant role in determining the efficiency of conversion of catechol derivatives. Currently, there is little information about the influence of steric factors on catalyt-

ic properties of catechol 1,2-dioxygenases [50]. Sauret-Ignazi et al.[61] revealed that very high inhibition constant is connected with steric hindrance, for example by methoxyl group. However another studies have indicated that a steric hindrances are not determining the rate-limiting step but there is a strong correlation between substituent electronegativity and k_{cat} [25,50,61].

The catalytic cycle of catechol 1,2-dioxygenases involve binding of the catechol or its derivatives as a dianion with simultaneous dissociation of one tyrosine and water molecule (a-e). One hydroxyl group of substrate binds in an axial position *trans* to His and the other binds in an equatorial position *trans* to Tyr. The ligand position *trans* to equatorial His is unoccupied and is postulated to be the oxygen-binding pocket [28,47,50,61]. The active site geometry is converted into a square-pyramidal geometry [91]. The substrate chelates asymmetrically to the iron(III) center. The long Fe-O bonds is *trans* to a tyrosinate ligand and the short Fe-O bond is *trans* to a neutral histidine ligand [40,91]. Binding of the distal oxygen leads to species with a peroxide bridge between the ferric ion and catechol substrate [92]. At the initial stage dioxygen molecule is one-electron reduced by the catecholate ligand and in this way it formes superoxide radical anion that interacts with iron(III) (f,g). In the next step superoxide and catecholate radicals recombine forming peroxo species (h). The last can easily change its conformation because of the hydroxyl group that becomes a ketone one, which is a much weaker ligand. Arginine 457 is supposed to stabilize the carboanion produced by the C3-O bond ketonization [92]. In consequences a coordination site of the axial tyrosine is open and it allows returning of the tyrosine to the coordination sphere of iron. The tyrosine binds to iron (III) as a phenolate (i), whereas the proton either neutralizes the peroxide ligand or it is released from the active site [40,91,92].

In the first case, proton remains in the active site (i) and the peroxide oxygen atom is protonated. It prevents oxidation of iron to a high-valent Fe(IV)=O state during the O-O bond cleavage. As a result peroxide ligand undergoes a Criegee rearrangement with O-O bond heterolysis and migration of the acyl group to the peroxide oxygen (k). It is also possible that the peroxide ligand converts to muconic anhydrate product via O-O bond cleavage made easier by the equatorial tyrosine, which is oxidized to a tyrozyl radical (j). The reduction of tyrozyl radical to tyrosinate is connected with the oxyl radical attack on the carbonyl carbon [40].

In the second case, proton is released from the active site (n) and peroxide bridge is cleaved to reactive oxoferryl group and an alkoxyl radical (o). Alkoxyl radical attacks the adjacent carbonyl carbon, followed by the ring expansion and protonation of the oxo ligand leads to the cyclic anhydride (k). Hydrolysis of the anhydride leads to the final reaction product – muconic acid (l,m) (Fig. 3) [40,92].

The electrophilic attack of molecular oxygen on iron (III)-bound catecholate is judged to be an essential step in the catalytic reaction of catechol 1,2-dioxygenase [50]. For this type of chemical reaction the σ-complex is assumed to be next to the transition state. The formation of σ-complex requires the highest free energy of activation and must consequently be rate-limiting [25].

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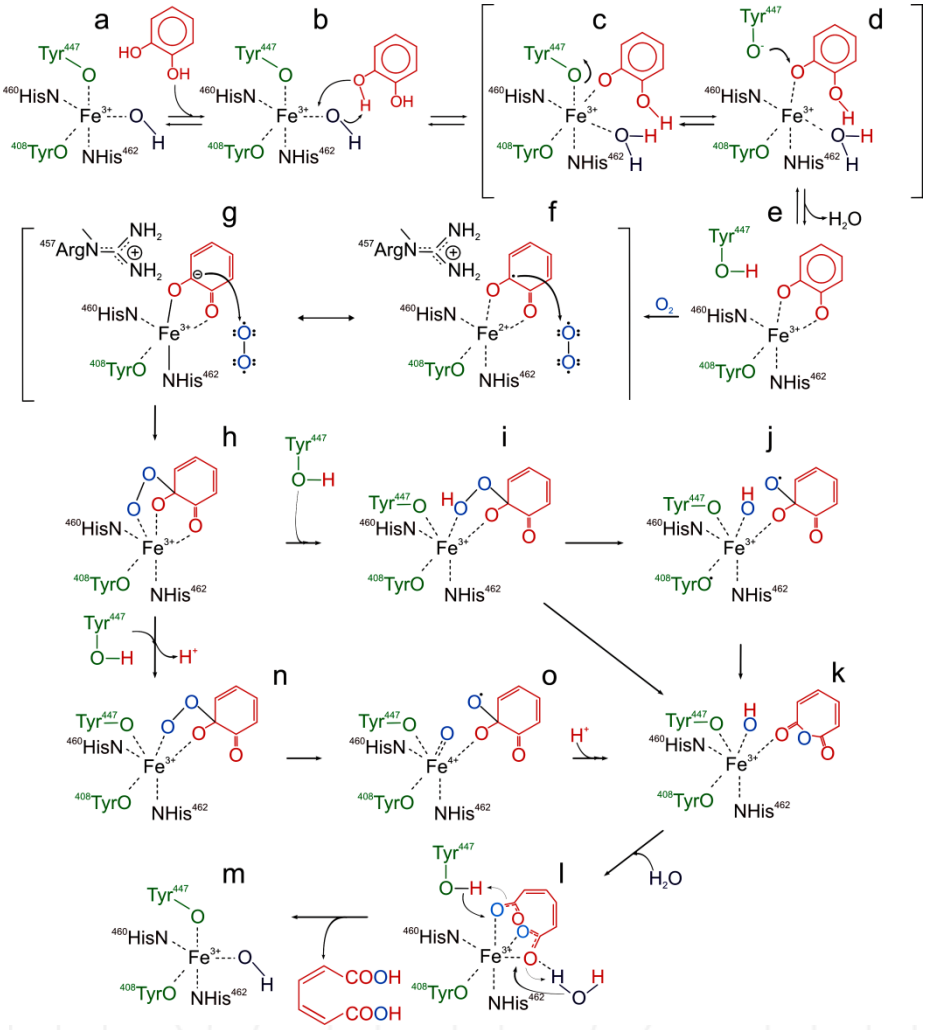

Figure 3. The catalytic cycle of the intradiol dioxygenases [92].

Sauret-Ignazi et al. [61] observed that monophenolic compounds are also bound by enzyme and they suggested that only one deprotonated hydroxyl may be sufficient for binding of the substrate to the active site.

Matera et al. [50] calculated parameter for nucleophilic reactivity for catechol, 3-chloro-, 4-chloro-, 3-methyl-, 4-methylcatechols, and pyrogallol. They revealed that the interaction between the frontier orbitals, the highest occupied molecular orbital (HOMO) of nucleophiles (catechols) and the lowest unoccupied molecular orbital (LUMO) of electrophile (the active site iron ion), is essential and lowers the activation barrier. Since the nuclephilic attack of the

aromatic π electrons of the iron(III)-bound catecholate on the oxygen molecule is an important step in the cleavage reaction, the HOMO of the catecholate is assumed to be the relevant frontier orbital. The natural logarithm of the k_{cat} is proportional to the activation energy of the rate-limiting step and is correlated to the energy of HOMO [50].

The main interaction of catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus* ADP1 with the substrate involves residues Leu73, Pro76, Ile105, Pro108, Leu109, Arg221, Phe253 and Ala254. Vetting and Ohlendorf [47] postulate that Arg221 may play an important role in proper positioning of the substrate's hydroxyl groups to the iron by van der Waals interactions with the aromatic ring. These interactions prevent ring rotation into two equatorial positions [47]. Matera et al. [50] observed that Arg217, Gln236 which play role in the deprotonation of substrate, together with Gly104, Pro105 and Tyr106 are required for the proper placing of aromatic compounds into the active site of catechol 1,2-dioxygenase from Gram- positive strain 1CP. Moreover residue Tyr106 forms a hydrogen bond with Tyr196 during its dissociation from the iron after the binding of catechol.

Catechol derivatives with the substituents on the C3 position can be bound in two different orientations in the enzyme active site from ADP1 strain. The substituent may be located in a pocket created by residues Ile105, Pro108, His226, Gln240 and Arg221 or its positioning may lead to a steric conflict with Tyr200. However, rearrangement in the active site makes possible the interaction of substituent on C3 with the π electrons of Tyr200 [47]. According to Matera et al. [50] the lower affinity of catechol 1,2-dioxygenase from 1CP strain for 3-substituents results from the lack of Tyr106. Tyr 196 is not connected then and it can cause the more open conformation of the space to the 3-substituent binding. It influences a certain degree of disorder in substrates binding to the iron and/or a not suitable coordination for productive conversion.

Ferraroni et al. [66] showed that preference of chlorocatechol dioxygenases for 3-chlorocatechol is connected with Val53 and Tyr78 residues whereas 4-chlorocatechols are cleaved by dioxygenases with Leu and Ile at 49 and 74 position respectively. Moreover, cysteine residues are important for interacting with chlorine substituents.

Presence of large substituents makes impossible active binding of the aromatic substrates to the active site because they interact unfavorably with Pro76, Leu73 and Ile105. Vetting and Ohlendorf [47] suggest that catechol derivatives are ligated the iron through only one of the hydroxyl group or they are trapped in an initial binding state.

3.2. Hydroxyquinol 1,2-dioxygenases characterization

Hydroxyquinol 1,2-dioxygenases have very similar structure and reaction mechanism to catechol 1,2-dioxygenases. They form homodimers with molecular weight of 58-85 kDa. The iron (III) is coordinated by two molecules of tyrosine, two molecules of histidine and one molecule of water [17,41,84-86,90].

However, Ferraroni et al. [17] revealed that His42 from H2 helix of the linker domain is connected with the copper ion in an oxidation state of +1. Probably it plays a role in stabilization of the enzyme quaternary structure. Moreover, a few different amino acid residues of hydrox-

yquinol 1,2-dioxygenases responsible for substrate selection were selected: Leu80, Asp83, Val107, Phe108, Gly109, Pro110, Phe111, Ile199, Pro200, Arg218, and Val251 [17].

One of the most characteristic feature of hydroxyquinol 1,2-dioxygenases is the extensive opening of the upper part of catalytic cavity which is responsible for favorable binding of hydroxyquinol [17,83]. Zaborina et al. [83] connected this fact with lower activation energy. Catalytic process of *ortho* cleavage proceeds through semiquinone radical. This intermediate can be stabilized by mesomeric delocalization of the unpaired electron in the 2-, 4-, or 6-position of semiquinone ring. With a hydroxyquinol as a substrate additional mesomeric delocalization of the unpaired electron is possible in position 1. The increase in the semiquinone character leads to a reduction of activation energy [83].

3.3. Protocatechuate 3,4-dioxygenases characterization

Protocatechuate 3,4-dioxygenases catalyze cleavage of protocatechuic acid and its derivatives. The mechanism of this reaction is the same as intradiol fission of catechol ring [13,92]. During substrate binding as a dianion to the active site of the enzyme the axial tyrosine is dissociated from the iron(III). It causes conformational changes that yields the chelated iron(III)-protocatechuate complex. Vetting et al. [16] suggested that the function of the structural rearrangements would be to facilitate the reaction of oxygen with the substrate. Electron transfer from the substrate to dioxygen aids a binding of dioxygen to the iron(III). The activation of substrate for an electrophilic attack by dioxygen leads to the formation of a peroxo bridge between the iron and C4 of substrate. O-O bond cleavage occurs after acyl migration to the peroxo oxygen. It leads to the cyclic anhydride formation and its hydrolysis to the final acyclic product [16,80,92,93].

Although protocatechuate 3,4-dioxygenases are heterodimers with molecular mass of 97.0-700.0 kDa [12,18,72,75,82] theirs tertiary structure is similar to the enzymes from catechol 1,2-dioxygenase family. Protocatechuate 3,4-dioxygenases belong to the non-heme iron dioxygenases and are composed of equimolar amounts of two different α and β subunits [11, 68,78,82,93]. These enzymes have a tetrahedral shape with α subunit forming the apex of the tetrahedron. The central cavity is accessible to solvent through triangular channels. A number of basic residues (mainly arginine and lysine) line the entrance to the active site of the enzyme producing regions of positive electrostatic potential. It is possible that these residues funnel the negatively charged substrate into the active site [16].

α and β subunits have similar folds. The core of each subunit contains two four-strand β-sheets that form a β sandwich. One β sheet consists of antiparallel strands, whereas the other has a mixed topology. The core structure is surrounded by series of small helices and large irregular loops. Two β sheets form a part of the interface between the subunits, with surrounding loops providing the remaining contacts. The amino terminus of the α subunit passes completely through a gap between the core β sandwich, supports structure of the β subunit and makes up one wall of the active-site cavity [16].

Aligments of the structure and sequences of α- and β-chain of protocatechuate 3,4-dioxygenases from more than 26 bacterial strains revealed that the β-chain show higher sequence

identities than the α-chain. It may be connected with the fact that β-chain provides most of the active site [19]. However, amino acids from α-subunit may interact with substrate and in this way stabilize it in the active site. Harnett et al. [69] observed that Arg-133 from α-subunit participates in forming ionic bond with carboxyl group of protocatechuic acid.

Protocatechuate 3,4-dioxygenases from different bacterial strains are consist of variable number of $\alpha\beta$ protomers (2 – 12) and each protomer has a cylindrical shape [12,16,78]. The minimal catalytic unit of all protocatechuate 3,4-dioxygenases is an $\alpha\beta Fe^{3+}$ structure, but $(\alpha\beta)_2Fe^{3+}$ structure has been also reported [11,72]. The crystal structure of these enzymes shows that the high-spin iron(III) is bound to the active site in a distorted trigonal bipyramidal coordination geometry with two inequivalent tyrosine ligands, two histidines, and exogenous hydroxide ion [68,69,73,77,79,92,93]. Kurahashi et al. [93] proposed that the electronic effect of the water ligand in the active site of protocatechuate 3,4-dioxygenase controls not only the distorted trigonal-bipyramidal structure but also plays a role in the oxidation of substrate. The two tyrosine ligands are thought to stabilize the ferric ion and give protocatechuate 3,4-dioxygenase its characteristic burgundy-red color via tyrosinate ligand–to-metal charge transfer [13,79].

Orville et al. [94] demonstrated that at least two iron binding sites can be occupied by exogenous ligand such as substrates, analog of substrate, and small molecules. It leads to the dynamic changes in the iron coordination sphere or the orientation of the aromatic substrate and influences the catalytic cycle. An external ligand with a higher position in the spectrochemical series ($Cl « RO < H_2O$) induces a larger degree of distortion [80,93].

The protocatechuate 3,4-dioxygenases show a high similarity not only for the ligands of the active site but also for the whole pocket in which the iron complex is located [77].

Orville et al. [94] studied cleavage mechanism in the presence of inhibitors. They proposed three different orientations of inhibitors in the active site. First, in which inhibitor protrudes out of the active site cavity but still coordinates the iron to yield trigonal bipyramidal coordination geometry. Second, in which inhibitor binds within the active site that is sufficient to dissociate the active site solvent molecules but without direct coordination to the iron. Third, in which inhibitor is completely bound within the active site, directly coordinates the iron, and yields octahedral geometry.

Many protocatechuate dioxygenases have narrow substrate specificities and regioselectivities [9,10,12]. Hou et al. [10] suggested that free sulfhydryl groups located at or near the active center are responsible for substrate specificity of the enzyme. It was showed that protocatechuic acid derivatives are cleaved much slower than protocatechuic acid. Transient kinetic results suggest that slower fission is probably connected with the reduced rates for substrate association and product release rather than the ring opening reaction [13].

4. Application of intradiol dioxygenases in bioremediation

Bioremediation is an effective treatment process that use organisms through their enzymatic activities. However, slow growth of microorganisms or difficulties in the control and maintain

the optimal conditions for the microbial growth are disadvantages of biodegradation processes. The direct application of enzymes in the environmental treatment processes has been quite limited due to the loss of enzyme activity [95] and therefore, novel methods of enzyme stabilization are developed. One of them is immobilization which has been used as a tool to improve many of enzyme properties such as operational stability, inhibitor resistance and performance in organic solvents [95,96]. Kalogeris et al. [96] showed that immobilization of catechol 1,2-dioxygenase improved its performance at higher temperatures. It extended catechol 1,2-dioxygenase storage stability and retained its activity after operation cycles - properties which are fundamental for continuous bioprocesses during removal of toxic pollutants. Catechol 1,2-dioxygenase from *Acinetobacter radioresistens* S13 after immobilization on nanosponges showed the ability to convert the substrate, after 50 reaction cycles. Moreover, this enzyme was more thermostable [60].

Immobilized intradiol dioxygenases can also be used as biosensors because of increased interest in the detection of aromatic compounds. Zucolotto et al. [97] successfully fabricated biosensor for catechol detection. Chlorocatechol 1,2-dioxygenase was immobilized in nanostructured films in conjuction with poly(amidoamine) dendrimer in a layer-by-layer fashion. Chlorocatechol 1,2-dioxygenase remained active on these films for longer than three weeks. Moreover, this biosensor was able to detect catechol at concentration even 10^{-10} M [97].

Polychlorinated phenols, amino- and nitrophenols have become widespread environmental pollutants because of their resistance to microbial degradation and their broad toxicity. During their degradation hydroxyquinol as a key intermediate is frequently formed. Therefore, it is believed that hydroxyquinol-metabolized enzymes play an important role in the microbial degradation of various aromatic xenobiotics [51,85,88]. Immobilization of hydroxyquinol 1,2-dioxygenase from *Arthrobacter chlorophenolicus* A6 onto single-walled carbon nanotubes increased its resistance capacity to the variable environmental factors [95].

The important properties of degradation enzymes is a resistance to inhibitors such as metal ions, alcohols, chelators, hydrocarbons, phenols and other [58,59,61]. Metal ions can lead to the conformational changes in the enzymes such as a reduction in α-helices and β sheets, which can result in the loss of enzymatic activity [98] or replacement of the original metal ion at the active site of the enzymes with other metal. It may modulate their activities in accordance with the Irving-Williams studies on the stability of the metal complexes [99]. Moreover, the thiol groups of enzyme structure may bind transition metals that can deactivate the enzyme.

Gou et al. [67] observed that activity of catechol 1,2-dioxygenase from *Sphingomonas xenophaga* QYY was increased by Pb^{2+}, Mg^{2+}, K^{+}, Fe^{3+} and Ca^{2+}, whereas the addition of Mn^{2+}, Hg^{2+}, Ni^{2+} and Co^{2+} slow down the enzyme reaction. Only Cu^{2+}, Zn^{2+} and Fe^{2+} ions strongly inhibited this enzyme. Catechol 1,2-dioxygenase from *Pseudomonas aeruginosa* was not sensitive to the presence of Mg^{2+}, Fe^{2+}, and Ca^{2+}. It was inhibited by Mn^{2+}, Cu^{2+}, and Ag^{+} [64]. Ag^{+} and Hg^{2+} showed the highest inhibitory effect on catechol 1,2-dioxygenase isozymes from *Arthrobacter* sp. BA-5-17. Catechol 1,2-dioxygenase from *Rhodococcus* sp. NCIM 2891 was inhibited completely in the presence of Fe^{3+}, Cu^{2+} and Hg^{2+} [20,62]. In contrary, catechol 1,2-dioxygenase from *Gordonia polyisoprenivorans* was resistant to these cations [21]. Decreased sensitivity of both catechol 1,2-dioxygenase and protocatechuate

3,4-dioxygenase from *Stenotrophomonas maltophilia* KB2 to metal ions was observed by Guzik et al. [68]. The presence of Mn^{2+}, Ni^{2+}, Cd^{2+} caused only 20-40% reduction of protocatechuate 3,4-dioxygenase activity, whereas, catechol 1,2-dioxygenase was even activated by Cd^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+} [68]. A high resistance of the intradiol dioxygenases to the metal ions is especially important because these ions are often found together with aromatic xenobiotics in the environment.

Some catechol 1,2-dioxgenases can be inhibited by chelating agent, especially by tiron – ferric ion chelating agent [58]. However, catechol 1,2-dioxygenase from *Arthrobacter* sp. BA-5-17 is not sensitive to chelating and sulfhydryl agents [62].

The wide substrate specificity and inhibitor resistance of some intradiol dioxygenases makes them an attractive target in the design of enzyme systems for bioremediation.

5. Conclusion

Intradiol dioxygenases are responsible for aromatic ring cleavage and for that reason are directly involved in biogeochemical cycles, and can be very useful in the development of bioremediation technology. These enzymes are the members of the aromatic-ring-cleavage dioxygenase superfamily. Based on their substrate specificity intradiol dioxygenases could be divided into three classes: catechol 1,2-dioxygenases, protocatechuate 3,4-dioxygenases, and hydroxyquinol 1,2-dioxygenase. The mechanism of intradiol fisson is common for enzymes from all clasess. In this mechanism the high-spin iron(III) is ligated by two tyrosines, two histidines, and a water ligand in trigonal bipyramidal geometry. The catalytic cycle of the intradiol dioxygenases involve binding of the catechol as a dianion and dioxygen to the metal. It leads to the formation of a peroxo bridge between iron and C4 of substrate. In the next step the Criegee rearrangement and O-O bond cleavage occurs, leading to the cyclic anhydride formation. Hydrolysis of the anhydride leads to the formation of the final acyclic product. Structural analysis of many of intradiol dioxygenases revealed only small differences in their active site structure. However, these differences significantly influence the substrate specificity and the resistance of the enzymes to the changing environmental factors.

Author details

Urszula Guzik*, Katarzyna Hupert-Kocurek and Danuta Wojcieszyńska

*Address all correspondence to: urszula.guzik@us.edu.pl

University of Silesia in Katowice, Faculty of Biology and Environmental Protection, Department of Biochemistry, Katowice, Poland

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