Modern research methods for determining structures of intradiol dioxygenases

Danuta WOJCIESZYŃSKA, Katarzyna HUPERT-KOCUREK, Małgorzata SITNIK, Urszula GUZIK – Department of Biotechnology, Faculty of Biology and Environmental Protection, University of Silesia in Katowice

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Introduction

In the era of a large interest in biodegradation processes of aromatic compounds, considerable attention has been directed to the studies on degrading enzymes, among which aromatic ring-cleavage dioxygenases have a key meaning [1,2]. Such enzymes catalyse the opening of a ring as a result of attaching two atoms of molecular oxygen and they are classified into two following groups depending on the regiospecificity of the ring cleavage: intradiol and extradiol [3]. The ring cleavage with the participation of intradiol dioxygenases does not only provide the degradation of uneasily degradable aromatic compounds such as: chlorophenols and nitrophenols, biphenyls and polycyclic aromatic hydrocarbons, but it also results in obtaining valuable intermediates for organic syntheses (*cis, cis*-muconic acid and 3-carboxy-*cis, cis*-muconic acid).

Intradiol dioxygenases – enzymes involved in decomposition of aromatic compounds

Intradiol dioxygenases catalyse the opening of the aromatic ring between two hydroxylated carbon atoms of the aromatic system, initiating the decomposition of aromatic compounds in the *ortho* pathway. It is a small family of enzymes with nonheme iron (III). They probably originate from the same evolutionary line. The analysis of spatial structure and amino acid sequence formed the basis for dividing dioxygenases from this group into the following: catechol 1,2-dioxygenases composed of α subunits, protocatechuate 3,4-dioxygenases composed of different numbers of $\alpha\beta$ subunits and hydroxyquinol 1,2-dioxygenases composed of α subunits, similarly to catechol dioxygenases. Despite different subunit composition, catalytic domains of intradiol dioxygenases are formed similarly.

In the active site of intradiol dioxygenases, there is iron in trigonal bipyramidal coordination geometry bound with four endogenous protein ligands. In the trigonal bipyramidal formation, the central atom is linked to 5 molecules at the tops of both pyramids [2]. Tyrosine 408, Histidine 460 and hydroxy group are attached to Fe (III) in the equatorial plane, whereas Tyrosine 447 and Histidine 462 in the axial plane. The composition of the active site is closely related to its function. The tests on the structure of enzyme-substrate complex of protocatechuate 3,4-dioxygenase showed that the attachment of substrate was associated with the separation of hydroxy group and Tyrosine 447, and the attached substrate gave its two protons to the detached ligands [4, 5].

Protocatechuate 3,4-dioxygenase is the enzyme that catalyses the transformation of protocatechuic acid into 3-carboxy-*cis, cis*-muconic acid [1,6,7]. This enzyme is characterised by the oligomer structure and is composed of two different types of subunits – α and β forming the $(\alpha\beta Fe)_n$ structure. β subunits combine protomers forming the oligomer structure and they are arranged along the axis of tetrahedron symmetry making the hollow sheath having the diameter of 50Å. α subunits are arranged in proximity to the peaks of two axes; they can be also found in the corners of opposite walls. Between α and β subunits, near the peak of the symmetry axis, there is an active site. The subunits are homologous towards each other. β subunit is composed of ca. 200 amino acid residues, while α subunit is composed of 230 amino acid residues. The secondary structure has the conformation of β-barrel constituted of β-sheet structure that is composed of 8 chains, twisted and coiled into the closed structure. It is similar to a piece of paper when folded in half [2, 5, 6].

Catechol 1,2-dioxygenases belong to the widely described class of enzymes [EC 1.13.11.1]. These enzymes cleave the catechol aromatic ring into *cis,cis*-muconic acid. Regarding the catalysed substrate, two subclasses of these enzymes are distinguished. They are:

I – catechol dioxygenases catalysing the decomposition of catechol and methylcatechol, and less often chlorocatechol

II – chlorocatechol dioxygenases decomposing catechol as well as its chlorinated and methylated forms [7÷10].

Catechol dioxygenases are homodimers of two identical α subunits, both of which contain Fe(III) cofactor. Each subunit is composed of ca. 300 amino acids and coiled into two domains: the catalytic domain whose structure is similar to the core of protocatechuate dioxygenases, and the terminal domain involved in dimerisation. N-terminal domain consists of ca. 100 amino acid residues making 5 helices. Dioxygenases of this class differ from other intradiol dioxygenases regarding both the subunit structure and the helical connector present at the subunit boundaries, to which phospholipid is being attached. The role of phospholipids has not been closely investigated so far [5, 6, 9].

Hydroxyquinol 1,2-dioxygenases belong to 3rd group of intradiol dioxygenases [EC 1.13.11.37]. They catalyse hydroxyquinol transformation into 3-hydroxy-*cis, cis*-muconate. The model of spatial structure was described for hydroxyquinol dioxygenase of *Nocardioides simplex* strain 3E. It is a homodimer having the dimensions of 110x50x50 Å. Its structure and subunit composition (α_2) are similar to catechol dioxygenase, which is related to their close affinity. Despite that close affinity with catechol dioxygenases, hydroxyquinol dioxygenases have specific amino acid residues in their structure (Leu80, Asp83, Val107, Phe108, Gly109, Pro110, Phe111,Ile199, Pro200, Arg218, Val251) and big openings for attaching the substrate in the upper part of the active site [5, 8].

The intradiol mechanism of ring cleavage has not been fully investigated. The mechanism of intradiol aromatic ring cleavage was suggested on the basis of the analysis of the spatial structure of protocatechuate 3,4-dioxygenase and catechol 1,2-dioxygenase and enzyme-substrate complexes as well as on the basis of biochemical and spectroscopic properties.

The process of ring cleavage began from substrate attachment to the active site. The substrate gave two protons – one to hydroxy group, and the second to tyrosine in the axial position, which caused the deprotonation of both hydroxy groups of the substrate. Simultaneously, the dissociation of ligands coordinated with iron from the active site and the attachment of the dianonic substrate were observed. This led to the

formation of chelate ring activating the electrophilic attack of oxygen molecule. The process outcome was the formation of peroxy bond between iron and substrate. Tyrosine in the equatorial arrangement facilitated ketonization between C3-O, which resulted in arginine-stabilised carbanion. Arginine along with tyrosine controlled the direct attack of oxygen molecule. Consequently, this resulted in opening of the ring structure and releasing the product – *cis, cis*-muconic acid or its derivative [2, 11, 12].

Research methods used to determine structures of intradiol dioxygenases

The research works on intradiol dioxygenase properties, including their structures, have been a subject of interest for a few years. Nowadays, the studies on the enzymes widely apply the methods related to two following fields: crystallography (X-ray crystallography) and spectroscopy (mass spectrometry, electron paramagnetic resonance (EPR) spectroscopy and X-ray absorption (XAS) spectroscopy).

At first, crystallography was related to mineralogy and materials science. At present, it is applied in many branches of industry and science, including biology, since it allows the ordered structure of a molecule, such as e.g. proteins, to be presented [13]. The X-ray crystallography has been applied for determining the dimensions and geometry of crystalline lattice formed by a unit cell. The method performance is based on recording diffraction spectra of X-rays created by radiation interaction with electron clouds in molecules displaying the ordered structure. The application of X-ray crystallography enabled us to learn about the three-dimensional structure of catechol 1,2-dioxygenase of *Acinetobacter radioresistens* strain LGM S13 (Ar-1,2-CTD) and about two groups of its mutants: A72G and L69A. As the result of conducted analyses, the enzymes were proved to belong to the C2 symmetry group. They have one monomer in an asymmetric part, which creates a functional model of homodimer. The structure of Ar-1,2-CTD was shown to be very similar to the structure of most catechol 1,2-dioxygenases. Ar-1,2-CTD was also observed to be folded into C-terminal β-sheet structure forming random coils due to which the whole reflected the catalytic domain. It was connected with N-terminal domain composed of 4 α-helices. The strongly hydrophobic space with a clearly visible phospholipid was also observed. The active site structure was the same as the structure of the whole family of intradiol enzymes. Fe(III) was coordinately connected with 5 ligands in the trigonal bipyramidal geometry, Tyr161, Tyr195, His219, and His221 respectively. One of the most noticeable differences was the hollow in the enzyme-substrate complex, which turned out to be larger in mutants than in the parent enzyme. The mutants also demonstrated stronger preference for 4-chlorocatechol [9].

The analysis of hydroxyquinol 1,2-dioxygenase of N*ocardioides simplex* strain 3E was another example of applying the method of X-ray crystallography. Copper (I) ion, which is probably responsible for stabilising enzyme by its presence in the helical connector, was found in the region of the active site [8].

Mass spectrometry (MS) is the technique for analysing ionised forms of molecules in the gas phase. This technique is used for determining molecular mass, structures of unknown compounds, for confirming the structures of synthesised compounds or for determining the qualitative and quantitative composition of mixtures [14].

Using MALDI-TOF mass spectrometry in order to detect phospholipid molecules in the helical connector of catechol 1,2-dioxygenase of *A. radioresistens* strain LGM S13 exemplifies the application of MS technique. For this purpose, MALDI-TOF spectrometer equipped with nitrogen laser emitting the wavelength of 337 nm was used. Thus, it was possible to observe the regions of increased electron density responsible for the presence of hydrophobic phospholipid chains, whereas the regions characterised by a weaker signal could suggest the presence of hydrophilic part of phospholipid. The signals obtained for the standard phospholipids were compared in order to determine the types of phospholipids present in the helical connector of the tested enzyme. It was the basis for identifying two following phospholipids: glycerophosphoinositol and glycerophosphoinositol monophosphate. However, an attempt to determine unambiguously which of them was present in the connector failed due to too weak signal in the regions of hydrophilic part of the tested phospholipid [9].

X-ray absorption (XAS) spectroscopy is a widely applied technique belonging to atomic absorption spectrometry. It employs the action of X-ray radiation on a tested object. XAS was used to test the active sites of hydroxyquinol 1,2-dioxygenase of *N. Simplex* strain 3E and of chlorocatechol 1,2-dioxygenase of *R. erythropolis* strain 1CP. Native enzymes and their complexes with substrates were compared. The obtained spectra were typical for the metal combined with oxygen and nitrogen ligands. The performed calculations suggested the presence of Fe(III) as chromophore coordinated with 5 ligands. The noticeable weaker signal and interference during the formation of enzyme-substrate complex were also important. They indicated the significant change in the active site resulting from the direct interaction of substrate with metal ion in the enzyme active site. On the basis of the observation, it was assumed that the substrates of intradiol dioxygenases characterised by two hydroxy groups in the *ortho* position were attached to the active site as catechol dianions. The spectra of enzyme-substrate complexes for hydroxyquinol 1,2-dioxygenase and chlorocatechol 1,2-dioxygenase, obtained through X-ray spectroscopy using the absorption edge method, exhibited their energy – 10.8×10^{-2}eV and 10.3×10^{-2}eV respectively. It indicated that the coordination number of this ion did not change during the exchange of amino acid ligands of Fe(III) by the substrate [12].

Electron paramagnetic resonance (EPR) spectroscopy belongs to the techniques employed in analysing the structures with at least one unpaired electron (organic and inorganic free radicals, complexes with ions of transition metals having incomplete *d* shell). This method is based on testing electron spins. The EPR method was used in the comparative research on the formation mechanism of catechol 1,2-dioxygenase complex of *P. putida* strain DSM 437 along with the substrate in organic solvents and the aquatic environment. For that purpose, EPR at temperatures of liquid helium and two following types of samples were used: lyophilised enzyme suspended in the buffer of pH 8.5 and lyophilised enzyme suspended in hexane under anaerobic conditions. The tested spectrum was changing at the moment of attaching the substrate to the active site. The signals indicated the axial bonding of iron ion and considerable heterogeneity in the active site. This was typical for protocatechuate 3,4-dioxygenases. The resulting state could be explained by a number of emerging intermediates causing the formation of an important transition state. Two fundamental values of resonance were observed, and the signals for hexane had a wider range than in case of the aquatic environment. The spectra analysis of enzyme in the aquatic environment showed the homogeneity of the iron centre environment. The centre heterogeneity was observed in the lyophilisate with hexane, which could signify a different conformation of enzyme depending on the environment nature. Moreover, the changes in EPR spectrum were observed immediately after the administration when the substrate was introduced into the aquatic solution of enzyme, while for the enzyme present in the hydrophobic environment, the changes in EPR spectrum were occurring gradually. It indicated the significant slowdown of catechol attachment reaction in the organic environment in comparison to the aquatic one. Catechol-iron intermediates were probably immobilised later in the hydrophobic environment [15].

Summary

The application of modern research methods for testing the structures of intradiol dioxygenases creates the possibility for not only understanding their composition, but also for describing the complex cleavage mechanisms and distinguishing the differences between parent and mutant enzymes. Consequently, the knowledge acquired in such a way creates an opportunity for improving the methods of genetic engineering for degradation enzymes which are more and more often becoming the fundamental preparation applied in bioremediation processes.

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Danuta WOJCIESZYŃSKA – Ph.D., is an Assistant Professor in the Faculty of Biochemistry at the University of Silesia in Katowice. In 1999, she defended her doctoral thesis at the Department of Biology and Environmental Protection at the University of Silesia in Katowice. For many years, she has been engaged in the problems related to the biodegradation of aromatic compounds with a particular focus on enzymes involved in their decomposition.

e-mail: danuta.wojcieszynska@us.edu.pl; phone: +48 32 200 95 76

Katarzyna HUPERT-KOCUREK – Ph.D., is an Assistant Professor in the Faculty of Biochemistry at the University of Silesia in Katowice. In 2000, she defended her doctoral thesis at the Department of Biology and Environmental Protection at the University of Silesia in Katowice. For many years, she has been engaged in studies on genetic regulation of aromatic compound degradation.

e-mail: khupert@us.edu.pl; phone: +48 32 200 94 62

Małgorzata SITNIK is writing her master's thesis at the Faculty of Biochemistry at the University of Silesia in Katowice. In her master's thesis, she presents the biochemical description of two intradiol dioxygenases: catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase of *Stenotrophomonas maltophilia* strain KB2.

e-mail: malgorzata.sitnik@gmail.com; phone: +48 32 200 95 76

Urszula GUZIK – Ph.D., is an Assistant Professor in the Faculty of Biochemistry at the University of Silesia in Katowice. In 2007, she defended her doctoral thesis at the Department of Biology and Environmental Protection at the University of Silesia in Katowice. For many years, she has been engaged in studies on enzymatic and genetic bases for microbiological degradation of arenes.

e-mail: urszula.guzik@us.edu.pl; phone: +48 32 200 95 76