Regular paper

Altering substrate specificity of catechol 2,3-dioxygenase from *Planococcus* sp. strain S5 by random mutagenesis

Katarzyna Hupert-Kocurek✉, Danuta Wojcieszyńska and Urszula Guzik

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

c23o gene, encoding catechol 2,3-dioxygenase from *Planococcus* sp. strain S5 was randomly mutagenized to generate variant forms of the enzyme with higher degradation activity. Additionally, the effect of introduced mutations on the enzyme structure was analyzed based on the putative 3D models the wild-type and mutant enzymes. C23OB58 and C23OB81 mutant proteins with amino acid substitutions in close proximity to the enzyme surface or at the interface and in the vicinity of the enzyme active site respectively showed the lowest activity towards all catecholic substrates. The relative activity of C23OC61 mutant towards *para*-substituted catechols was 20–30% lower of the wild-type enzyme. In this mutant all changes: F191I, C268R, Y272H, V280A and Y293D were located within the conserved regions of C-terminal domain. From these F191I seems to have significant implications for enzyme activity. The highest activity towards different catechols was found for mutant C23OB65. R296Q mutation improved the activity of C23O especially against 4-chlorocatechol. The relative activity of above-mentioned mutant detected against this substrate was almost 6-fold higher than the wild-type enzyme. These results should facilitate future engineering of the enzyme for bioremediation.

Key words: *Planococcus*, catechol 2,3-dioxygenase, substrate specificity, mutagenesis

INTRODUCTION

Chlorinated aromatic compounds are common environmental pollutants of serious concern because of their toxicity and persistence. However, ability of some microorganisms to degrade these compounds has been described (Riegert *et al.*, 1998; Mars *et al.*, 1999; Schmidt *et al.*, 2013). The microbial degradation of various chloroaromatic compounds occurs *via* chlorocatechols pathway (Mars *et al.*, 1999; Riegert *et al.*, 2001; Schmidt *et al.*, 2013). The enzymatic ring cleavage of these substrates is catalyzed mainly by intradiol dioxygenases (Schmidt *et al.*, 2013). An alternative route for degradation of chlorocatechols is extradiol pathway which involves aromatic ring cleavage enzymes — (chloro)catechol 2,3-dioxygenases (Riegert *et al.*, 1998; Mars *et al.* 1999; Hupert-Kocurek *et al.*, 2012; Schmidt *et al.*, 2013). These enzymes convert chlorocatechols by proximal cleavage to hydroxymuconate or by distal cleavage to 3-chloro-2-hydroxymuconic semialdehyde (Schmidt *et al.*, 2013). Three-dimensional

structures of (chloro)catechol 2,3-dioxygenases reveal a conserved region in the active site containing two histidine residues, one glutamate and two molecules of water as Fe^{2+} ligands (Huang *et al.*, 2010; Wojcieszyńska *et al.*, 2011). The catalytic mechanism starts with bidentate binding of the substrate as catecholate monoanion to the active-site metal with simultaneous displacing of water molecules (Costas *et al.*, 2004; Cho *et al.*, 2010). In the next step the oxygen binds to the ferrous form resulting in semiquinone-Fe^{2+}-superoxide intermediate formation. After Criegee rearrangement and O-O bond cleavage lactone intermediate and an Fe^{2+}-bound hydroxide ion is formed. Hydrolysis of the lactone leads to the reaction product (2-hydroxymuconate semialdehyde) formation (Bugg, 2003; Costas *et al.*, 2004).

Because of the structural diversity of aromatic compounds for bioremediation purposes enzymes with broad substrate specificity and high activity are required. Such enzymes may be obtained either by isolation from natural sources or by genetic engineering (Fortin *et al.*, 2005; Zielinski *et al.*, 2006). Hupert-Kocurek *et al.* (2012) isolated from *Planococcus* sp. strain S5 catechol 2,3-dioxygenase with remarkable preference for catechols with a substituent in the *para*-position, especially 4-chlorocatechol. This feature distinguishes this enzyme from other catechol 2,3-dioxygenases from Gram-positive and Gram-negative strains (Milo *et al.*, 1999; Hupert-Kocurek *et al.*, 2012; Wojcieszyńska *et al.*, 2012).

In the present study, a random mutagenesis was used to generate variant forms of catechol 2,3-dioxygenase with higher activity and improved efficiency of catechol and *para*-substituted catechols utilization. The wild-type enzyme and its mutant forms were functionally expressed in *Escherichia coli* and their activity along with substrate specificity were characterized. Additionally, a putative three-dimensional (3D) structure of the wild-type and mutant enzyme was determined to analyze the effect of introduced mutations on the structure of the enzyme. Results from this work will have important implications for the engineering of catechol 2,3-dioxygenase for removal of aromatic compounds from polluted environments.

✉e-mail: katarzyna.hupert-kocurek@us.edu.pl
Abbreviations: BphC, 2,3-dihydroxybiphenyl-1,2-dioxygenase; CumC, 3-isopropylcatechol dioxygenase; C23O, catechol 2,3-dioxygenase; C23Owt, wild-type enzyme; *c23o*, gene encoding catechol 2,3-dioxygenase; DMSO, dimethyl sulfoxide; IPTG, isopropyl-β-D-thiogalactopyranoside; MPY, metapyrocatechase; X-gal, 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside; 2-HMS, 2-hydroxymuconic semialdehyde

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, enzymes and fine chemicals. *Escherichia coli* DH5α [F⁻ *end*A1 *gln*V44 *thi*-1 *rec*A1 *rel*A1 *gyr*A96 *deo*R *nup*G Φ80d*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F)U169, *hsd*R17(r_K⁻ m_K⁺), λ–] and *E. coli* BL21 (DE3) [*fhu*A2 *[lon] omp*T *gal [dcm]* ⊿*hsd*S] were used as hosts of the cloning and overexpression vectors, respectively. Cells of *E. coli* strain were grown in LB medium at 37°C and agitated at 125 rpm. When necessary, ampicillin and IPTG were added to the LB medium at 100 μg ml⁻¹ and 1 mM, respectively.

Cloning vector pUC19, restriction enzymes, and *Pfu*, and *Taq* DNA polymerase were obtained from MBI Fermentas (Lithuania). Oligonucleotides for the PCR were purchased from IBB PAN (Warsaw, Poland). The expression vector pET-22(b) (Novagen) was purchased from Merck (Germany). All other reagents used in this work were of the highest analytical grade and commercially available.

DNA manipulation and cloning of *c23o* gene. Plasmid DNA of *Planococcus* sp. strain S5 as well as recombinant plasmids from *E.coli* transformants were isolated with Plasmid Mini Kit purchased from A&A Biotechnology (Poland). Agarose gel electrophoresis, DNA digestion with restriction enzymes and ligation were done by standard procedures (Sambrook *et al.*, 1989). Transformation of *E.coli* with plasmid DNA was performed by the RbCl procedure (Hanahan, 1983).

The gene encoding C23O from *Planococcus* sp. strain S5 was PCR-amplified by *Pfu* DNA polymerase with primers C23OEco (5'GA*GAATTC*ATGAAAAAA-GGCGTAATG3'; *EcoRI* site underlined) and C23OHind (5'AT*AAGCTT*AGCACGGTCATGAAACG3'; *HindIII* site underlined) purchased from IBB PAN (Warsaw, Poland). Primers were designed based on the regions of the catechol 2,3-dioxygenases with high sequence homology, and allowing amplification of C23O gene from start to stop codons (Hupert-Kocurek *et al.*, 2012). PCR reaction was performed in 25 μL volumes containing approximately 0.1 μg template DNA, 0.5 μM of each primer, 1x *Pfu* buffer (MBI Fermentas), 3% (v/v) DMSO (Sigma), 0.2 mM of each dNTPs, 1U *Pfu* DNA polymerase (MBI Fermentas). PCR amplification was performed in a PTC-150 MiniCycler (MJ Research, USA) under the following conditions: initial denaturing 5 min at 94°C; 10 cycles of 1 min at 94°C, 30 s at 59°C, 1 min at 72°C; 10 cycles of 1 min at 94°C, 30 s at 57°C, 1 min at 72°C; 15 cycles of 1 min at 94°C, 30 s at 55°C 1 min at 72°C plus an additional 5 min cycle at 72°C. The resulting purified PCR product (about 950 bp) was digested by *Eco*RI and *Hin*dIII and inserted into the *Eco*RI and *Hin*dIII endonuclease sites of pET-22(b) (Novagen) with T4 DNA ligase (Lithuania). The ligation mixture was then transformed into competent *E. coli* DH5α and plated on LB medium supplemented with ampicillin (100 μg ml⁻¹). Recombinant plasmids were isolated and these which contained the correct insert were identified by restriction enzyme analysis, verified by DNA sequencing, and then transformed into *E. coli* BL21 cells for expression.

Mutagenesis and construction of the expression vectors coding for mutant C23Os. Mutagenesis of *c23o* gene was carried out by an error-prone PCR amplification with *Taq* DNA polymerase (Fermentas), plasmid DNA of *Planococcus* sp. strain S5 as the DNA template, and the primers C23OEco and C23OHind used for the construction of pUC19*c23o*mut. To obtain mutations during the PCR process, the reaction mixtures were supplemented with the excess of MgCl₂. In the final volume of 25 μl, the reaction mixtures contained: 100 ng of template DNA, 50 pmol of each primer, 1x *Taq* buffer, 3% (v/v) DMSO (Sigma), 0.2 mM of each dNTPs, 2U *Taq* DNA polymerase and 2.5 mM of MgCl₂. PCR amplification was performed in a PTC-150 MiniCycler (MJ Research, USA) under the conditions described above. The DNA carrying mutated *c23o* gene was digested with *Eco*RI and *Hin*dIII endonuclease and ligated into the *Eco*RI and *Hin*dIII endonuclease sites of pUC19 with T4 DNA ligase (Fermentas). The ligation mixture was then transformed into competent *E. coli* DH5α and plated on LB medium supplemented with ampicillin (100 μg ml⁻¹), IPTG (79.2 μg ml⁻¹) and X-gal (32 μg ml⁻¹). After transformation ampicillin-resistant white colonies on IPTG plates were screened for the presence of C23O activity by spraying the plates with a solution of 100 mM catechol (Junca & Pieper, 2004). Colonies of cells that express active C23O turn yellow due to the formation of 2-hydroxymuconic semialdehyde. From such transformants plasmid DNA was isolated and those containing the correct insert were identified by restriction enzyme analysis. Mutations were verified by DNA sequencing and the mutated *c23o* gene was subcloned into pET-22(b) expression vector by digestion with the restriction enzymes *Eco*RI and *Hin*dIII and transformed into *E. coli* BL21 cells for expression.

DNA sequencing and analysis. DNA sequencing was performed at the Department of Molecular Biology, Institute of Oncology DNA Sequencing Facility by using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystem) and AbiPrism 3100 Genetic Analyzer. Assembly and analysis of DNA sequences was performed with Chromas LITE software (Technelysium Pty, Tewantin, Australia).

Catechol 2,3-dioxygenase sequence analysis and modelling. Amino acid sequence of the wild type and mutated form of catechol 2,3-dioxygenase was deduced using the CLC Free Workbench 4.0.1 software. The prediction of secondary structure, structural disorder, protein solvation and tertiary fold recognition were carried out *via* the GeneSilico metaserver (http://genesilico.pl/meta2/) (Kurowski & Bujnicki, 2003).

Homology modelling of the wild type and mutant proteins was performed using the 'FRankenstein's monster' approach (Kosinski *et al.*, 2003). Models were built with Swiss-MODEL (Arnold *et al.*, 2006; Biasini *et al.*,2014; Guex *et al.*, 2009) based on the sequence alignment between target and template. Final models were evaluated using MetaMQAP method (Pawlowski *et al.*, 2008) and ProQ server (Cristobal *et al.*, 2001). The quality of models was assessed using the ProQ method: Lgscore >1.5, fairly good model; Lgscore >2.5, very good model; Lgscore >4, extremely good model; MaxSub >0.1, fairly good model; MaxSub >0.5, very good model; MaxSub >0.8, extremely good model. Figures were prepared in PyMol.

Preparation of cell-free extract. For overexpression of C23O, *E. coli* BL21 harboring either the wild type gene *(c23o)* or its mutated forms harboring nucleotide exchanges were cultivated in 50 ml of LB medium supplemented with ampicillin (100 μg ml⁻¹) overnight at 37°C, and 5 ml cells was then subcultured into 250 ml of the same medium and cultured under the same conditions. After the cell OD₆₀₀ reached 0.4–0.6, IPTG was added in the final concentration of 1mM. After further incubation for 4 h, cells were centrifuged at 4500 × *g* for 15 min at 4°C. Next, the cells were washed with 50 mM phosphate buffer, pH 7.5, resuspended in the same buffer at concentration equivalent to an OD₆₀₀ of 1.0 and ruptured by

pulsed sonication (Vibra Cell TM) 6 times for 15 s. The disrupted cells suspensions were centrifuged at $9\,000 \times g$ for 30 min at 4°C to remove cell debris. The clear supernatant was used as a crude extract for enzyme assays.

Enzyme assay. In order to determine catechol 2,3-dioxygenase activity, the formation of 2-hydroxymuconic semialdehyde was measured at 375 nm ($\varepsilon_{2\text{-HMS}}$ at 375 nm $=36\,000$ M^{-1} cm^{-1}) in a reaction mixture containing 20 µl of catechol (50 mM), 960 µl of phosphate buffer pH 7.5 (50 mM) and 20 µl of crude extract in a total volume of 1 ml (Feist & Hegeman, 1969). Control reactions (without crude extract) were performed for each assay. Protein concentrations of the crude extract from cultured bacteria were determined by the Bradford method (Bradford, 1976). One unit of C23O activity was defined as the enzyme amount required to generate 1 µmol of product per minute. The specific activity of the enzyme is defined in mU per milligram of protein.

Substrate specificity of catechol 2,3-dioxygenase. The substrate specificity of the catechol 2,3-dioxygenase was examined with 3-methylcatechol, 4-methylcatechol and 4-chlorocatechol. The aromatic ring cleavage was determined spectrophotometrically by measuring the increase in the absorbance at the corresponding wavelength of each *meta*-cleavage product formed. Activity of C23O was assayed under the reaction conditions described above, using tested aromatic compounds instead of catechol as a substrate. The molar extinction coefficient used for the product from 3-methylcatechol was 13 800 $M^{-1} \times cm^{-1}$ (at 388 nm) (Bayly *et al.*, 1966) from 4-methylcatechol was 28,100 $M^{-1} \times cm^{-1}$ (at 382 nm) (Bayly *et al.*, 1966) and from 4-chlorocatechol 40 000 $M^{-1} \times cm^{-1}$ (at 379 nm) (Bae *et al.*, 1996).

RESULTS AND DISCUSSION

Extradiol dioxygenases play a key role in degradation of aromatic compounds and their substrate specificity determine the range of compounds that can be used by microorganisms as growth substrates (Williams & Sayers, 1994; Vaillancourt *et al.*, 2006; George *et al.*, 2011). Based on their amino acid sequence and structural folds similarity they can be divided into three types with the majority of the enzymes belonging to type I that consist of five families and a number of subfamilies (Andujar & Santero, 2003; Vaillancourt *et al.*, 2006). Each of the known enzymes in the specified subfamily displays restricted substrate specificity (Ninnekar, 1992; Cerdan *et al.*, 1994; Siani *et al.*, 2006). C23Os are the enzymes that belong to subfamily 1 which generally catalyze catechol more efficiently than substituted catechols as they are rather unstable and become inactivated during the catalysis of methyl- or chloro-derivatives (Okuta *et al.*, 2004).

However, there are some C23Os able to cleave catechols with small substituents at positions 3 and 4 at the bond adjacent to the diol and proximal to the substituent (Cerdan *et al.*, 1994; Viggiani *et al.*, 2004). As aromatic compounds occur in the environment primarily as complex mixtures, alteration of catabolic potential of C23Os by widening the number of substrates they can utilize represents a valuable tool for bioremediation strategies.

In the present study, to determine substrate specificity of mutated forms of C23O from *Planococcus* sp. S5 their catalytic activities with catechol, 3-methylcatechol, 4-methylcatechol and 4-chlorocatechol were measured. Additionally, to localize introduced mutations that influence on the enzyme activity homology models of catechol 2,3-dioxygenase and its mutant forms were built. 1MPY from *Pseudomonas putida* MT-2, the closest relative with the known 3D structure (Kita *et al.*, 1999) was selected as the best template for homology modeling with the following scores: pdbblast — 4.62e-79, hhsearch — 100 and mgenthreader — 8e-17. C23O model was assessed with ProQ method where LGscore (5.070) and MaxSub (0.568) showing that 3D model was of very good quality.

The substrate specificity of all examined mutants of C23O for tested catecholic compounds is shown in Table 1. The lowest activity towards catechol and catechols with substituent in *para*-position (4-chlorocatechol, 4-methylcatechol) was found for mutant C23OB58 (Table 1). In this mutant three amino acid changes were localized: H24R, F168S, Q275R. Based on the structural predictions, mutations H24R and F168S were located in close proximity to the enzyme's surface while the residue at position 275 was at the interface (Fig. 2AB). Similar results were obtained for C23OB81 (Table 1). In this form of C23O two mutations were identified: A23T and F212S localized close to the enzyme surface and in the vicinity of the enzyme active site, respectively (Fig. 1AC). Studies of Viggiani *et al.* (2004) on catechol 2,3-dioxygenase from *Pseudomonas stutzeri* OX1 showed that replacement of one amino acid residue within the active site resulted in complete inactivation of the enzyme. Therefore, basing on these results we assume that replacement of hydrophobic phenylalanine by serine could have the greatest impact on the enzyme activity.

In C23OC61 all changes (F191I, C268R, Y272H, V280A and Y293D) were located within conserved regions of C-terminal domain (Fig. 2AB). As it is shown in Fig. 2AC introduced mutations caused the changes in a spatial orientation of residues involved in interaction with substrate. Such reorganization of the active site could lead to decreased catalytic efficiency of the enzyme. From these mutations F191I seems to have significant implications for enzyme activity. F191 (number-

Table 1. Activity of the wild-type (wt) and mutant C23O dioxygenases using different catechols as the substrate

Enzyme	Specific activity[a], mU mg^{-1} (relative activity, %)[b]			
	CA	3-MCA	4-MCA	4-CCA
C23Owt	19.66 ± 0.95 (100)	1.18 ± 0.78 (100)	20.62 ± 3.99 (100)	9.63 ± 3.51 (100)
C23OB58	5.72 ±0.45 (26.48)	1.08 ±0.46 (91.53)	5.47 ±0.68 (26.53)	2.98 ± 0.35 (30.94)
C23OB65	27.75 ±3.52 (128.47)	1.38 ± 0.56 (116.95)	22.23 ± 2.06 (107.81)	56.05 ±7.43 (582.04)
C23OB81	8.78 ±0.57 (40.65)	0.73 ± 0.58 (61.86)	8.06 ± 0.91 (39.09)	2.72 ± 1.06 (28.25)
C23OC61	8.27 ±1.07 (38.29)	0.90 ± 0.57 (76.27)	14.87 ±5.34 (72.11)	8.11 ± 1.33 (84.22)

[a]One unit of C23O activity was defined as the amount of enzyme that required generating 1 µmol of product per minute. The specific activity is defined in mU per milligram of protein. [b]Expressed as a percentage of the C23Owt specific activity which is set as 100%. All data shown were expressed as mean ± standard deviation (n=4)
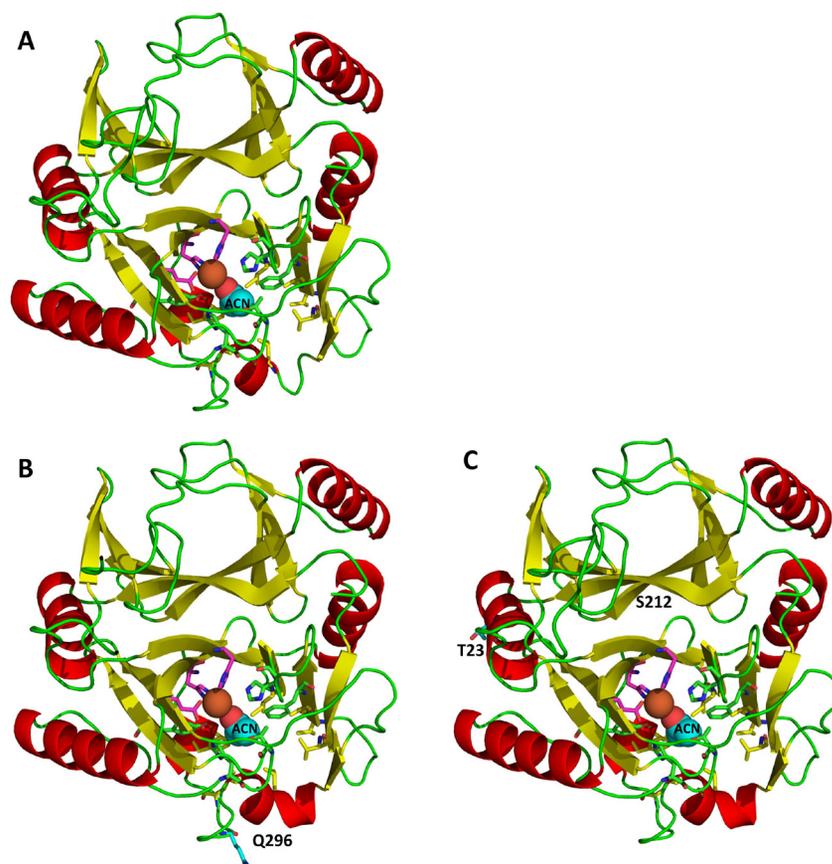
Figure 1. The deduced 3-D structure of the wild type (A) and mutant forms: C23OB65 (B) and C23OB81 (C) of C23O from *Planococcus* sp. S5.
Amino acid residues forming the outer opening of the substrate channel are shown in yellow, amino acid residues involved in substrate binding, in green. At the active site, Fe atoms as brown spheres coordinated by three residues (in magenta) and acetone molecules (ACN) are shown. The residues corresponding to the replaced amino acids are shown in blue and labeled.

ing of MPC_mt2 from *Pseudomonas putida* mt-2) is highly conserved in many extradiol dioxygenases as for example BphC from LB400, BphC from IPO1 or CumC (Hofer *et al.*, 1993; Aoki *et al.*, 1996; Kita *et al.*, 1999). Phenylalanine at this position interacts with the hydroxylated ring of the substrate during enzyme-substrate complex formation. The interaction involves a Cε proton of phenylalanine aromatic ring and the π electron cloud of the substrate (Senda *et al.*, 1996). According to Siani *et al.* (2006) the contacts between the edge of the dihydroxylated substrate ring and the active site pocket are probably involved in determination of substrate specificity. We assume that substitution of F191 with isoleucine impairs formation of such interactions. It may cause either inappropriate positioning of the substrate or destabilization of the enzyme–substrate complex resulting in slowing down the catalytic reaction and finally in a low activity of mutant protein towards different catechols, compared with the wild-type enzyme (Table 1). Despite lower activity, C23OC61 showed higher affinity to catechol and was more sensitive to substrate concentration than nonmutated enzyme (results not presented). These results further indicate that substitutions within C-terminal domain of C23OC61 influence enzyme–substrate complex formation rather than substrate binding.

From all mutant proteins C23OB65 shoved the highest activity towards all tested substrates (Table 1). The enzyme activity detected against 4-methylcatechol was comparable to its activity against catechol while only marginal activity of the enzyme against 3-methylcatechol

was detected (4.97% of that against catechol) (Table 1). Above all, it is worth noting that C23OB65 showed remarkably high activity towards 4-chlorocatechol (203% of that against catechol). The relative activity detected against this substrate was almost 6 fold higher than that of the wild-type enzyme. The increased activity of catechol 2,3-dioxygenase from *Pseudomonas putida* strain with 4-ethylcatechol was observed by Cerdan *et al.* (1994) as a result of L226S and T253I substitutions. A point mutation in *xylE*, the structural gene for this enzyme, which produced the substitution V291I was responsible for the decreased affinity of the mutant protein for 3-chlorocatechol and increased affinity for 3-methylcatechol compared to the wild-type enzyme (Wasserfallen *et al.*, 1991). None of the mutations described above were localized in the substrate binding pocket (Junca & Pieper, 2004). Similarly, in C23OB65 only one amino acid substitution (R296Q) was found. It was localized at the interface, in the C-terminal domain of the enzyme (Fig. 1AB). Therefore, no direct influence of this amino acid on the active site can be envisaged. However, as it is known, in catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 C-terminal end region of the C-terminal domain is involved in formation of the lid over the channel through which the substrates enter the active site (Kita *et al.*, 1999). Though R296 is not directly involved in the formation of the substrate binding pocket, its replacement with glutamine residue which is smaller than arginine may somehow facilitate the access of the substrate to the active site of the enzyme. It is also known, that in MPC_mt2
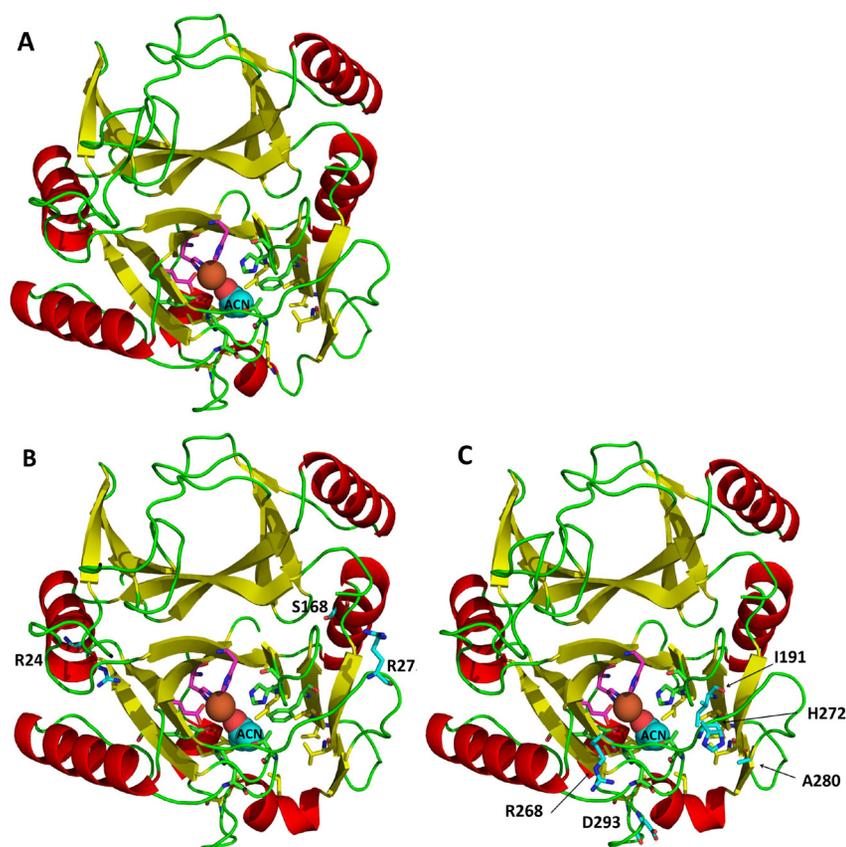
Figure 2. The deduced 3-D structure of the wild type (A) and the mutant forms: C23OB58 (B) and C23OC61 (C) of C23O from *Planococcus* sp. S5.
Amino acid residues forming the outer opening of the substrate channel are shown in yellow, amino acid residues involved in substrate binding, in green. At the active site, Fe atoms as brown spheres coordinated by three residues (in magenta) and acetone molecules (ACN) are shown. The residues corresponding to the replaced amino acids are shown in blue and labeled.

residue R296 forms a salt bridge with D236 from the adjacent subunit (Hatta *et al.,* 2003). Therefore, mutation R296 could loosen the subunit/subunit interactions, thus increasing the plasticity of the C-terminal domain containing the active site and allowing for significant movement of this segment during binding of substrate.

In summary, our studies confirmed that some mutations do not alter the mean structure of the protein but exert an effect on the enzyme activity. Moreover, data obtained for C23OB58 and C23OB65 show that amino acid residues located outside the active site pocket can affect substrate specificity and activity of catechol 2,3-dioxygenase. Additionally, the results obtained in this work suggest that replacement of the conserved residue R296 responsible for subunit/subunit interactions influences the binding-induced changes required to place substrates into appropriate position and orientation by increasing the mobility of the C-terminal domain. Based on the results mutant C23OB65 with increased activity towards *para*-substituted catechols may serve as an attractive tool for removal of man-made pollutants from the environment.

Acknowledgements

REFERENCES

Andujar E, Santero E (2003) Site-directed mutagenesis of an extradiol dioxygenase involved in tetralin biodegradation identifies residues important for activity or substrate specificity. *Microbiology* **149**: 1559–1567.

Aoki H, Kimura T, Habe H, Yamane H, Kodama T, Omori T (1996) Cloning, nucleotide sequence, and characterization of the genes encoding enzymes involved in the degradation of cumene to 2-hydroxy-6-oxo-methylocta-2,4-dienoic acid in *Pseudomonas fluoresens* IPO1. *J Ferment Bioeng* **81**: 187–196.

Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. *Bioinformatics* **22**: 195–201.

Bae HS, Lee M, Kim JB, Lee ST (1996) Biodegradation of the mixtures of 4-chlorophenol and phenol by *Comamonas testosteroni* CPW301. *Biodegradation* **7**: 463–469.

Bayly RC, Dagley S, Gibson DT (1966) The metabolism of cresols by species of *Pseudomonas*. *Biochem J* **101**: 293–301.

Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Gallo Cassarino T, Bertoni M, Bordoli L, Schwede T (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* **12**: 252–258.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–258.

Bugg TDH (2003) Dioxygenase enzymes: catalytic mechanisms and chemical models. *Tetrahedron* **59**: 7075–7101.

Cerdan P, Wasserfallen A, Rekik M, Timmis KN, Harayama S (1994) Substrate specificity of catechol 2,3-dioxygenase encoded by TOL plasmid pWW0 of *Pseudomonas putida* and its relationship to cell growth. *J Bacteriol* **176**: 6074–6081.

Cho HJ, Kim K, Sohn SY, Cho HY, Kim KJ, Kim MH, Kim D, Kim E, Kang BS (2010) Substrate binding mechanism of a type I extradiol dioxygenase. *J Biol Chem* **285**: 34643–34652.

Costas M, Mehn MP, Jensen MP, Que L (2004) Dioxygen activation at mononuclear nonheme iron active sites: enzymes, models, and intermediates. *Chem Rev* **104**: 939–986.

Cristobal S, Zemla A, Fischer D, Rychlewski L, Elofsson A (2001) A study of quality measures for protein threading models. *BMC Bioinformatics* **2**: 5.

Feist CF, Hegeman GD (1969) Phenol and benzoate metabolism by *Pseudomonas putida*: regulation of tangential pathways. *J Bacteriol* **100**: 869–877.

Fortin P, Macpherson I, Neau D, Bolin J, Eltis L (2005) Directed evolution of a ring-cleaving dioxygenase for polychlorinated biphenyl degradation. *J Biol Chem* **280**: 42307–42314.

George KW, Kagle J, Junker L, Risen A, Hay AG (2011) Growth of *Pseudomonas putida* F1 on styrene requires increased catechol-2,3-dioxygenase activity, not a new hydrolase. *Microbiology* **157**: 89–98.

Guex N., Peitsch MC, Schwede T (2009) Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: A historical perspective. *Electrophoresis* **30**: 162–173.

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**: 557–580.

Hatta T, Mukerjee-Dhar G, Damborsky J, Kiyohara H, Kimbara K (2003) Characterization of a novel thermostable Mn(II)-dependent 2,3-dihydroxybiphenyl 1,2-dioxygenase from a polychlorinated biphenyl- and naphthalene-degrading *Bacillus* sp. JF8. *J Biol Chem* **278**: 21483–21492.

Hofer B, Eltis LD, Dowling DN, Timmis KN (1993) Genetic analysis of a *Pseudomonas* locus encoding a pathway for biphenyl/polychlorinated biphenyl degradation. *Gene* **130**: 47–55.

Huang SL, Hsu YCh, Wu ChM, Lynn JW, Li WH (2010) Thermal effects on the activity and structural conformation of catechol 2,3-dioxygenase from *Pseudomonas putida* SH1. *J Phys Chem B* **114**: 987–992.

Hupert-Kocurek K, Guzik U, Wojcieszyńska D (2012) Characterization of catechol 2,3-dioxygenase from *Planococcus* sp. strain S5 induced by high phenol concentration. *Acta Biochim Pol* **59**: 1–7.

Junca H, Pieper DH (2004) Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting comparative deversity assesment against bacterial isolates and PCR-DNAclone libraries. *Environ Microbiol* **6**: 95–110.

Kita A, Kita S, Fujisawa I, Inaka K, Ishida T, Horiike K, Nozaki M, Miki K (1999) An archetypical extradiol-cleaving catecholic dioxygenase: the crystal structure of catechol 2,3-dioxygenase (metapyrocatechase) from *Pseudomonas putida* mt- 2. *Structure* **7**: 25–34.

Kosinski J, Cymerman IA, Feder M, Kurowski MA, Sasin JM, Bujnicki JM (2003) A 'Frankenstein's monster' approach to comparative modeling: merging the finest fragments of fold-recognition models and iterative model refinement aided by 3D structure evaluation. *Proteins* **6**: 369–379.

Kurowski MA, Bujnicki JM (2003) GeneSilico protein structure prediction meta-server. *Nucleic Acids Res* **31**: 3305–3307.

Mars AE, Kingma J, Kaschabek SR, Reineke W, Janssen DB (1999) Conversion of 3-chlorocatechol by various catechol 2,3-dioxygenases and sequence analysis of the chlorocatechol dioxygenase region of *Pseudomonas putida* GJ31. *J Bacteriol* **181**: 1309–1318.

Milo RE, Duffner FM, Muller R (1999) Catechol 2,3-dioxygenase from the thermophilic, phenol-degrading *Bacillus thermoleovorans* strain A2 has unexpected low thermal stability. *Extremophiles* **3**: 185–190.

Ninnekar HZ (1992) Purification and properties of 2,3-dihydroxy-p-cumate-3,4-dioxygenase from *Bacillus* species. *Biochem Int* **28**: 97–103.

Okuta A, Ohnishi K, Harayama S (2004) Construction of chimeric catechol 2,3-dioxygenase exhibiting improved activity against the suicide inhibitor 4-methylcatechol. *Appl Environ Microbiol* **70**: 1804–1810.

Pawlowski M, Gajda MJ, Matlak R, Bujnicki JM (2008) MetaMQAP: a meta-server for the quality assessment of protein models. *BMC Bioinformatics* **9**: 403–420.

Riegert U, Bürger S, Stolz A (2001) Altering catalytic properties of 3-chlorocatechol-oxidizing extradiol dioxygenases from *Sphingomonas xenophaga* BN6 by random mutagenesis. *J Bacteriol* **183**: 2322–2330.

Riegert U, Heiss G, Fischer P, Stolz A (1998) Distal cleavage of 3-chlorocatechol by an extradiol dioxygenase to 3-chloro-2-hydroxy-muconic semialdehyde. *J Bacteriol* **180**: 2849–2853.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schmidt E, Mandt Ch, Janssen DB, Pieper DH, Reineke W (2013) Degradation of chloroaromatics: structure and catalytic activities of wild-type chlorocatechol 2,3-dioxygenases and modified ones. *Environ Microbiol* **15**: 183–190.

Senda T, Sugiyama K, Narita H, Yamamoto T, Kimbara K, Fukuda M, Sato M, Yano K, Mitsui Y (1996) Three-dimensional structures of free form and two substrate complexes of an extradiol ring-cleavage type dioxygenase, the BphC enzyme from *Pseudomonas* sp. strain KKS102. *J Mol Biol* **255**: 735–752.

Siani L, Viggiani A, Notomista E, Pezzella A, Di Donato A (2006) The role of residue Thr249 in modulating the catalytic efficiency and substrate specificity of catechol-2,3-dioxygenase from *Pseudomonas stutzeri* OX1. *FEBS J* **273**: 2963–2976.

Vaillancourt FH, Bolin JT, Eltis LD (2006) The ins and outs of ring-cleaving dioxygenases. *Crit Rev Biochem Mol Biol* **41**: 241–67.

Viggiani A, Siani L, Notomista E, Birolo L, Pucci P, Di Donato A (2004) The role of the conserved residues His-246, His-199, and Tyr-255 in the catalysis of catechol 2,3-dioxygenase from *Pseudomonas stutzeri* OX1. *J Biol Chem* **279**: 48630–48639.

Wasserfallen A, Rekik M, Harayama S (1991) A *Pseudomonas putida* strain able to degrade *m*-toluate in the presence of 3-chlorocatechol. *Nat Biotechnol* **9**: 296–298.

Williams PA, Sayers JR (1994) The evolution of pathways of aromatic hydrocarbon oxidation in *Pseudomonas*. *Biodegradation* **5**: 195–217.

Wojcieszyńska D, Hupert-Kocurek K, Greń I, Guzik U (2011) High activity catechol 2,3-dioxygenase from the cresols-degrading *Stenotrophomonas maltophilia* strain KB2. *Int Biodeter Biodegrad* **65**: 853–858.

Wojcieszyńska D, Hupert-Kocurek K, Jankowska A, Guzik U (2012) Properties of catechol 2,3-dioxygenase from crude extract of *Stenotrophomonas maltophilia* strain KB2 immobilized in calcium alginate hydrogels. *Biochem Eng J* **66**: 1–7.

Zielinski M, Kahl S, Standfuss-Gabisch Ch, Camara B, Seeger M, Hofer B (2006) Generation of novel-substrate-accepting biphenyl dioxygenases through segmental random mutagenesis and identification of residues involved in enzyme specificity. *Appl Environ Microbiol* **72**: 2191–2199.