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# Specificity of Catechol *ortho*-Cleavage during *para*-Toluate Degradation by *Rhodococcus opacus* 1cp

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**Abstract**—Degradation of *para*-toluate by *Rhodococcus opacus* 1cp was investigated. Activities of the key enzymes of this process, catechol 1,2-dioxygenase and muconate cycloisomerase, are detected in this microorganism. Growth on *p*-toluate was accompanied by induction of two catechol 1,2-dioxygenases. The substrate specificity and physicochemical properties of one enzyme are identical to those of chlorocatechol 1,2-dioxygenase; induction of the latter enzyme was observed during *R. opacus* 1cp growth on 4-chlorophenol. The other enzyme isolated from the biomass grown on *p*-toluate exhibited lower rate of chlorinated substrate cleavage compared to the catechol substrate. However, this enzyme is not identical to the catechol 1,2-dioxygenase cloned in this strain within the benzoate catabolism operon. This supports the hypothesis on the existence of multiple forms of dioxygenases as adaptive reactions of microorganisms in response to environmental stress.

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*Key words*: *para*-toluate, degradation, catechol 1,2-dioxygenase, muconate cycloisomerase, *Rhodococcus opacus* 1cp

Effectiveness of microbial degradation of stable aromatic compounds depends on both position of a substituent (*ortho*-, *meta*-, or *para*-) and its nature. Comparative study of degradation of aromatic compounds bearing substituents of different nature (e.g. halogens and methyl group) is especially interesting because microorganisms may transform or completely degrade stable compounds including polyaromatic hydrocarbons, but not some "simple" aromatic compounds such as 2-chlorotoluene [1] and dichlorobenzoates [2-5], which are persistent to microbial attacks. The reason for such resistance of these compounds may be attributed to insufficient activity of the two first enzymes (dioxygenase and muconate cycloisomerase) responsible for aromatic ring opening and subsequent conversion of the muconates formed. It is also possible that microorganisms are unable to degrade chloro/methyl-muconolactones formed. Microbial degradation of methyl substituted aromatic compounds usually occurs via the *meta*-pathway of methylcatechol degradation because the *ortho*-cleavage results in formation of methyl-substituted 4-carboxymethylbut-2-en-4-olides (methylmuconolactones) as dead-end products [6, 7]. Degradation of 4-methylmuconolactone is especially interesting because the presence of alkyl substituent at C-4 prevents subsequent decomposition of this compound in the classic 3-oxoadipate pathway, and most microorganisms studied so far employ the *meta*-pathway for 4-methylcatechol degradation [8]. Nevertheless, several exceptions also exist. In *Ralstonia eutropha* JMP134, *Rhodococcus* sp., and *R. rhodochrous* N75 the modified *ortho*-pathways for methylcatechol degradation have been described, and in the most studied *R. eutropha* and *R. rhodochrous* N75 differ [9-11] (Fig. 1).

Thus, there are two major problems in microbial decomposition of methyl substituted aromatic compounds: 1) the metabolic fate of methyl-catechol formed; 2) the steps of methyl muconolactone conversion (provided that it involves *ortho*-cleavage of methyl-catechol). *R. opacus* 1cp strain can decompose a wide range of substrates including aromatic compounds. This strain is unique in realization of metabolic "scenarios" for *ortho*-pathway degradation of various substrates. This microorganism degrades phenol and benzoate via the ordinary *ortho*-pathway; degradation of 4-chlorophenol involves the modified *ortho*-pathway, whereas degradation of 2-

*Abbreviations*: (C)Cat) (chloro)catechol; (C)C-1,2-DO) (chloro)catechol 1,2-dioxygenase; 4-CP) 4-chlorophenol; MCI) muconate cycloisomerase.

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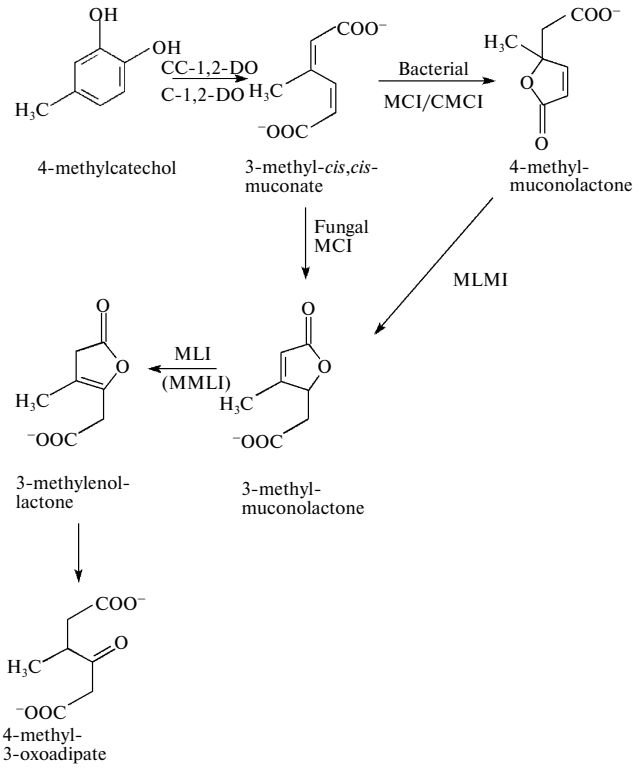


**Fig. 1.** The *ortho*-pathway for 4-methylcatechol degradation (from [12]). C-1,2-DO, catechol 1,2-dioxygenase; CC-1,2-DO, chlorocatechol 1,2-dioxygenase; MCI, muconate cycloisomerase; CMCI, chloromuconate cycloisomerase; MLMI, 4-methylmuconolactone methylisomerase; MLI (MMLI), muconolactone isomerase (methylmuconolactone isomerase modified for metabolism of methylmuconolactones); ELH (MELH), 3-oxoadipatenol-lactone hydrolase (isozyme modified for metabolism of methyl-substituted analogs).

chlorophenol occurs via a novel modified *ortho*-pathway. Such possibility for use of parallel pathways for degradation of various substrates is determined by enzymes exhibiting various substrate specificities. *R. opacus* 1cp uses modified *ortho*-pathways for degradation of 2- and 4-chlorophenols; this results in formation of 3-chloro- and 4-chlorocatechols, respectively. Subsequent conversion of chlorocatechols catalyzed by chlorocatechol 1,2-dioxygenases (CC-1,2-DOs) is accompanied by aromatic ring opening followed by formation of corresponding chloromuconates. Studies in this field have revealed that CC-1,2-DOs differ in substrate specificity; these enzymes are adapted to effective cleavage of the substrate that has been formed as a key intermediate [13, 14]. The 3-CC- and 4-CC-1,2-DOs from *R. opacus* 1cp have been crystallized [15, 16]. X-Ray analysis and comparison of subunit structure of these dioxygenases revealed the molecular basis underlying differences in substrate specificity of these enzymes. The following features were shown to be responsible for these differences: 1) amino acid substitutions in the active site structure of 3-CC-1,2-DO/4-CC-1,2-DO; 2) structure of a "plane" in that region of the active site of 4-CC-1,2-DO that is responsible for positioning of substrate C-4 carbon atom; this provides correct localization of 4-chlorocatechol in the active site required for effective substrate cleavage.

In this study we have searched for isozymes synthesized by *R. opacus* 1cp during growth on different substrates undergoing degradation via formation of substituted catechols. *p*-Toluate is one such substrate. We have also investigated the initial steps of *p*-toluate degradation by *R. opacus* 1cp.

## MATERIALS AND METHODS

**Microorganism and methods for its cultivation.** The strain *R. opacus* 1cp capable of utilizing phenol, 4-chlorophenol, and 2,4-dichlorophenol was isolated from the accumulating culture maintained for several months in the presence of 2,4-dichlorophenol [17]. The long-term adaptation yielded a microbial variant utilizing 2-chlorophenol as the only source of carbon and energy [14].

The strain was cultivated in mineral medium containing (g/liter): $Na_2HPO_4$, 0.73; $KH_2PO_4$, 0.35; $MgSO_4 \cdot 7H_2O$, 0.1; $NaHCO_3$, 0.25; $MnSO_4$, 0.002; $NH_4NO_3$, 0.75; $FeSO_4 \cdot 7H_2O$, 0.02; distilled water up to 1 liter [17]. For preparation of biomass grown on *p*-toluate, the strain was cultivated at 28°C, partial oxygen pressure corresponding to 70% of saturation using a 10-liter bioreactor containing 7 liters of the medium and rate of stirring of 400 rpm. *p*-Toluate was added in portions of 0.15 mM up to the final concentration of 5 mM. Growth of the culture was monitored by the decrease in oxygen consumption, change in pH, and absorbance at 545 nm; pH of the growth medium was maintained at the level of 7.0-7.2 by periodic additions of 0.1 M NaOH. The final optical density (OD) value was 2.36 at 545 nm. Cells were sedimented by centrifugation (7000 rpm, 10 min), washed twice in 50 mM Tris-HCl buffer (pH 7.2), and kept at −20°C.

Specific growth rate μ was determined by the time-dependence of natural logarithm of cultural liquid $OD_{545}$ using the formula:

$$\mu = (\ln x_t - \ln x_0)/(t - t_0),$$

where $x$ and $t$ are OD and time, respectively. The time required for cell duplication $t_d$ was calculated by the formula $t_d = \ln 2/\mu$. The economic coefficient $y$ was determined as ratio of biomass augmentation ($\Delta x$) to substrate consumption ($\Delta s$) during a certain time interval.

**Cell free extract was obtained** as described earlier [14].

**Enzyme activities were determined** by spectrophotometric assay at 25°C using a Shimadzu UV-160

(Shimadzu Co, Japan) spectrophotometer and quartz cuvettes with optical pathlength of 1 cm.

Activity of CC-1,2-DO was assayed using the modified method of Hayaishi et al. [18]. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.2), 0.25 mM catechol or substituted catechol, 1.3 mM EDTA, and the enzyme preparation. The reaction was started by enzyme addition. The enzyme activity was calculated by the rate of product formation (*cis*,*cis*-muconic acid, or substituted muconates) registered at 260 nm.

Activity of muconate cycloisomerase was determined as described earlier [19]. The reaction mixture contained 50 mM Tris-HCl, pH 7.2, 2 mM $MgCl_2$, 0.1 mM *cis*,*cis*-muconic acid or 2-chloromuconate, and the enzyme preparation. The reaction was started by enzyme addition. The reaction rate was determined by substrate disappearance registered at 260 nm.

Enzyme activities were calculated using molar extinction coefficients determined by Dorn and Knackmuss [20]: 16,800 $M^{-1}\cdot cm^{-1}$ for catechol; 17,100 $M^{-1}\cdot cm^{-1}$ for 3-chlorocatechol; 12,400 $M^{-1}\cdot cm^{-1}$ for 4-chlorocatechol; 12,000 $M^{-1}\cdot cm^{-1}$ for 3,5-dichlorocatechol; 18,000 $M^{-1}\cdot cm^{-1}$ for 3-methylcatechol; 13,900 $M^{-1}\cdot cm^{-1}$ for 4-methylcatechol.

One unit of enzyme activity was defined as the amount of enzyme catalyzing conversion of 1 μmol of substrate (or 1 μmol of product) per minute.

**Purification of enzymes from *R. opacus* 1cp biomass grown on *p*-toluate.** *Purification of catechol-1,2-dioxygenase I.* The two first purification steps (chromatography on Q-Sepharose and phenyl-Sepharose) were carried out in a cold room (at 4°C) using Pharmacia (Sweden) columns. Subsequent purification steps employing an FPLC system (Pharmacia) were carried out at room temperature. The cell free extract was applied to Q-Sepharose column (26 × 20 cm, volume 80 ml) equilibrated with 50 mM Tris-HCl buffer, pH 7.2, containing 2 mM $MnSO_4$ (buffer A). The column was washed with one volume of the same buffer, and the protein was eluted with a linear gradient of NaCl (0-0.5 M) in 1200 ml of buffer A at a flow rate of 1.2 ml/min. The volume of each collected fraction was 10 ml. Fractions containing enzyme activity were pooled, concentrated using an ultrafiltration cell with UM-30 membrane (Amicon, USA), and the resulting volume was mixed with an equal volume of 2.4 M ammonium sulfate. After centrifugation at 12,000 rpm for 20 min, the preparation was applied to a phenyl-Sepharose column (26 × 40 cm, volume of 133 ml) equilibrated with 50 mM Tris-HCl buffer, pH 7.2 (buffer B), containing 1.2 M ammonium sulfate. The protein was eluted with a linear gradient of $(NH_4)_2SO_4$ (1.2-0 M) in 2 liters of buffer B at flow rate of 1.85 ml/min. Fractions of 10 ml with maximal enzyme activity were pooled and concentrated to 2 ml by ultrafiltration using a UM-30 membrane. The resulting preparation was applied onto a Superdex 200 column (16 × 70 cm, volume of 120 ml) equilibrated with buffer B containing 0.1 M NaCl; the protein was eluted with the same solution at flow rate of 1 ml/min. Fractions of 1.5 ml exhibiting enzymatic activity were pooled, desalted, and applied to Resource Q (volume of 6 ml) equilibrated with buffer B. The protein was eluted with a linear gradient of NaCl (0-0.35 M) at flow rate of 2.5 ml/min. Fractions of 1.5 ml exhibiting enzymatic activity were pooled, desalted, concentrated by ultrafiltration, and applied to a Mono P column (volume of 1 ml) equilibrated with buffer B. The protein was eluted with a linear gradient of NaCl (0-0.5 M) at flow rate of 0.4 ml/min. Fractions containing the peak of catecholase activity were pooled, desalted, and concentrated using the same cell and membrane and repeated twice chromatography on the Mono P column under the same conditions. The resulting desalted preparation of C-1,2-DO was used for enzyme characterization. In all the above steps, the enzyme activity was assayed using catechol as substrate.

*Purification of catechol-1,2-dioxygenase II.* Since the two dioxygenases were separated only at the stage of hydrophobic chromatography, conditions for chromatography on Q-Sepharose and phenyl-Sepharose are identical to those that have just been described above. Fractions exhibiting the enzymatic activity were pooled and concentrated by ultrafiltration to 2 ml. The resulting preparation was applied to a Superdex 200 column (16 × 70 cm, volume of 120 ml) equilibrated with buffer B containing 0.1 M NaCl and eluted with the same solution at the flow rate of 1 ml/min. Fractions of 1.5 ml exhibiting the enzymatic activity were pooled, desalted, concentrated, and applied to a Resource Q column (volume of 6 ml) equilibrated with buffer B. The protein was eluted with a linear gradient of NaCl (0-0.3 M) at the flow rate of 2 ml/min. Fractions of 1.5 ml containing the enzymatic activity were pooled, and after addition of ammonium sulfate to the final concentration of 1.6 M centrifuged at 16,000 rpm for 25 min. The resulting preparation was applied to a Resource Iso column (volume of 1 ml) equilibrated with buffer B containing 1.6 M ammonium sulfate and eluted with a decreasing linear gradient (1.6-0 M) of ammonium sulfate in buffer B at the flow rate of 2 ml/min. Fractions with maximal enzymatic activity were pooled, desalted, and used for enzyme characterization. In all the above steps, the enzyme activity was assayed using catechol as substrate.

*Purification of muconate cycloisomerase.* Chromatography on Q-Sepharose was carried out as described above. The volume of each collected fraction was 10 ml. Fractions containing the enzyme activity were pooled, heated on a water bath to 60°C, incubated for 20 min, and centrifuged at 16,000 rpm for 25 min. The supernatant was concentrated by ultrafiltration and applied to a Superdex 200 column (16 × 70 cm, volume of 120 ml) equilibrated with buffer A containing 0.1 M NaCl. The protein was eluted with the same solution at the flow rate of 2 ml/min. Fractions exhibiting cycloisomerase activity

were pooled, and after addition of ammonium sulfate to final concentration of 0.8 M centrifuged at 16,000 rpm for 25 min at 4°C. The resulting preparation was applied to a phenyl-Sepharose column (16 × 23 cm, volume of 27 ml) equilibrated with buffer A containing 0.8 M $(NH_4)_2SO_4$. The protein was eluted with a linear gradient of ammonium sulfate (0.8-0 M) in buffer A at the flow rate of 1.5 ml/min. Fractions exhibiting maximal cycloisomerase activity were pooled, desalted, and kept at 4°C. In all purification steps the enzyme activity was assayed using *cis*,*cis*-muconate as substrate.

**Characterization of physicochemical properties of the isolated enzymes.** Purity of the isolated enzyme preparations and molecular masses of subunits were analyzed by SDS-PAGE by a modification of the method of Laemmli [21] using a mini VE Complete (Hoefer Pharmacia Biotech, USA) and plates (10 × 10.5 × 0.1 cm) with 4% stacking and 12% separating gels. Gels were stained with Coomassie G-250 [22]. Molecular masses of protein subunits were evaluated using SDS-LMW protein molecular weight markers (Sigma, USA).

Amino-terminal amino acid sequences were determined after SDS-PAGE and protein electrotransfer to the Immobilon P membrane (Millipore, USA) as described earlier [23].

**Determination of kinetic characteristics.** Michaelis constants ($K_m$) and $V_{max}$ values were determined using double reciprocal plots. For determination of $K_m$ and $V_{max}$ of C-1,2-DO, the following ranges of substrate concentrations were used: 0.5-20 μM catechol; 0.5-35 μM 3-methylcatechol and 4-methylcatechol.

The pH dependence of enzymatic reactions was studied using phosphate buffer (pH 6.0-7.2) or Tris-HCl buffer (pH 7.2-8.7) and catechol as substrate. Temperature dependence of catecholase reactions was studied in the temperature range from 5 to 60°C using a Shimadzu (Japan) temperature controller.

Protein content was determined using a modification of the Bradford method [24] with bovine serum albumin as standard.

**Reagents** used for preparation of mineral media were of analytic grade from Reakhim (Russia). Biochemical reagents were obtained from Sigma (USA) and Serva (Germany). 3-Methylcatechol was obtained from Koch-Light (England), and 4-methylcatechol was obtained from Fluka (Switzerland). Sorbents for column chromatography and the columns Resource Q, Resource Iso, and Mono P were purchased from Pharmacia (Sweden). Reagents for SDS-PAGE were from Bio-Rad (USA).

## RESULTS AND DISCUSSION

**Growth of *R. opacus* 1cp on *p*-toluate.** *R. opacus* 1cp was grown in medium containing *p*-toluate as the only source of carbon and energy. Figure 2 shows the time-course of its growth. The lag-period lasted several hours, and the strain rapidly reached exponential growth phase and substrate additions became more frequent. The cultivation was stopped at optical density of cell suspension of 2.36.

Using semilogarithmic plots of time dependence of the optical density of the cultivation liquid, we calculated specific growth rate of 0.12 $h^{-1}$; the duplication time $t_d$ was 5.8 h. This value was lower than the corresponding parameter obtained during growth on 4-chlorophenol [25]. This may be attributed to lower toxicity of 4-methylbenzoate. The economic coefficient *y* of bacterial growth was 0.013.

**Enzymes of the *ortho*-pathway of catechol degradation by *R. opacus* 1cp strain.** Determination of enzyme activity in the cell-free extract revealed the presence of C-1,2-DO and muconate cycloisomerase (MCI) in it. C-1,2-DO exhibited unusual substrate specificity: the relative rate of 4-chlorocatechol cleavage was 29% of the enzyme activity with catechol as substrate, whereas the rate of 4-methylcatechol cleavage was 137% of the enzyme activity with catechol as substrate. This suggests the presence of one dioxygenase in the extract, which was very active with chlorinated catechol as substrate; this was demonstrated earlier for *R. rhodnii* 135 and *R. rhodochrous* 89 [26]. It was also possible that bacterial growth on *p*-toluate was accompanied by induction of two (or even more) enzymes: one was active with 4-methylcatechol, whereas another was active with 4-chlorocatechol. Figure 3 shows the elution profile of cell-free extract after ion-exchange chromatography. There was one protein peak exhibiting dioxygenase activity; depending on the slope of the salt gradient, this peak could be subdivided into two peaks or it could just have a "shoulder". Such behavior suggested the presence of at least two catechol-cleaving enzymes. However, the first purification step did not result in their complete separation. There was also one peak exhibiting MCI activity. No MCI activity was detected with 2-chloromuconate as substrate. This suggests lack of CMCI during bacterial growth using *p*-toluate as substrate.

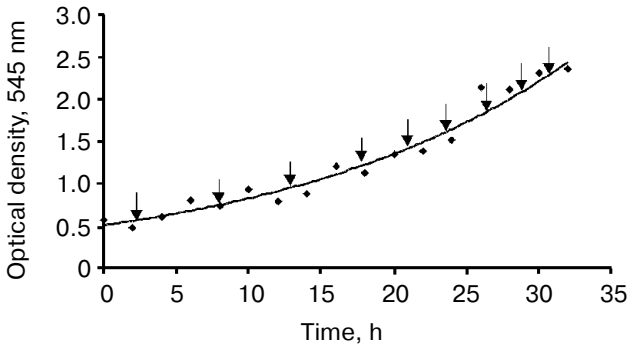


**Fig. 2.** Growth of *R. opacus* 1cp on *p*-toluate. Arrows show substrate additions at certain time intervals.

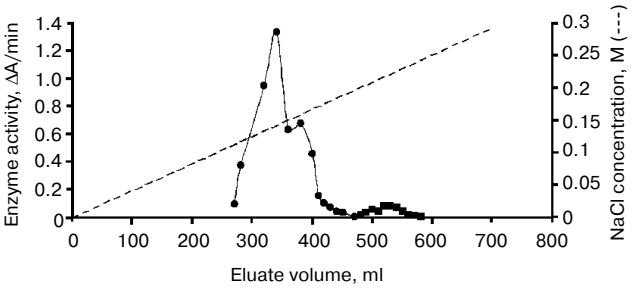


**Fig. 3.** Profile of *R. opacus* 1cp enzyme elution from Q-Sepharose. Designations: circles, catechol 1,2-dioxygenase; squares, muconate cycloisomerase; dashed line, NaCl linear gradient.

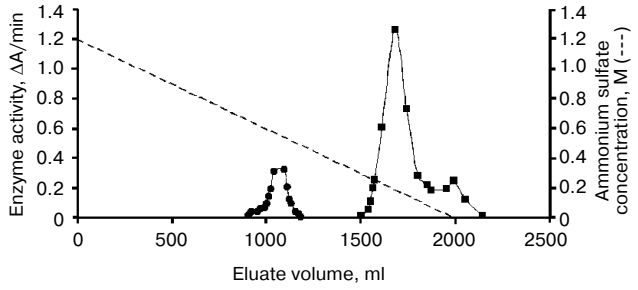


**Fig. 4.** Profile of elution from phenyl-Sepharose of enzymes exhibiting dioxygenase activity. Designations: circles, catechol 1,2-dioxygenase I; squares, catechol 1,2-dioxygenase II; dashed line, linear gradient of ammonium sulfate.

**Catechol-1,2-dioxygenase I.** *Purification*. C-1,2-DO was purified in five steps. After the second step (hydrophobic chromatography) the enzyme exhibiting catechol-cleaving activity was separated into two peaks; one peak was eluted at 0.82 M $(NH_4)_2SO_4$, whereas the second was eluted at 0.3 M $(NH_4)_2SO_4$ (Fig. 4). Table 1 shows relative catalytic activity of the two peaks assayed with 4-chlorocatechol and 4-methylcatechol. Based on these assays, these enzyme activities were defined as C-1,2-DO I and C-1,2-DO II (methylcatecholdioxygenase), respectively. C-1,2-DO I was eluted from Q-Sepharose at 0.11-0.18 M NaCl (volume 266 ml; total protein 452 mg, specific activity 1.85 U/mg). This enzyme was eluted from phenyl-Sepharose at 0.82-0.79 M $(NH_4)_2SO_4$ (volume 234 ml, total protein 21 mg, specific activity 5.16 U/mg), from the Resource Q column at 0.22 M NaCl (volume 25 ml, total protein 3.3 mg, specific activity 14.3 U/mg), and from the Mono P column at 0.27 M NaCl (volume 2.6 ml, total protein 1.7 mg, specific activity of 7.5 U/mg). The resulting preparation of C-1,2-DO I had specific activity 7.5 U/mg, its yield being 1.53%.

*Physicochemical properties of C-1,2-DO I.* SDS-PAGE revealed the presence of two closely positioned bands stained with the same intensity. They corresponded to molecular masses of 33 and 35 kD. Comparison of C-1,2-DO elution from the Superdex 200 column with that of previously characterized (chloro)catechol dioxygenases from the same strain revealed molecular mass of the native protein of about 70 kD. It is possible that C-1,2-DO I is a heterodimer or it represents a mixture of two homodimeric proteins.

Purification of C-1,2-DO from biomass grown on benzoate also yielded the enzyme preparation demonstrating on SDS-PAGE two bands of the same molecular masses (33 and 35 kD) [27]. However, genetic study of the operon encoding enzymes involved in the ordinary *ortho*-pathway revealed only one open reading frame encoding a protein of molecular mass of 35 kD in *R. opacus* 1cp [28, 29]. This suggests that the cell response to a growth substrate involves induction of genes encoding C-1,2-DO located within different operons. However, this suggestion needs further experimental investigation.

C-1,2-DO I had temperature optimum at 40-50°C. Increasing the temperature to 60°C was accompanied by sharp enzyme inactivation. The pH optimum for this enzyme was at 7.2-7.4.

*Catalytic properties of C-1,2-DO I.* The enzyme catalyzed conversion of several compounds. Table 2 shows results of relative oxidation rate of the substrates used at the same concentration. The highest activity was detected with catechol and 3- and 4-methylcatechols. Catalytic activity with 4-chlorocatechol was only 2.1% of the maximal activity; no enzymatic activity was found with dichloro-substituted catechols.

Using the three best substrates of this enzyme, we determined $K_m$ and specificity constant ($k_{cat}/K_m$) values (Table 3). For calculations we used the subunit mass of 34 kD as the mean of the two molecular masses of subunits determined by SDS-PAGE.

The enzyme exhibited the highest affinity towards catechol, whereas the highest $V_{max}$ value was obtained with 3-methylcatechol as substrate. However, in spite of these differences, specificity constants for these substrates were very close; this suggests wide substrate specificity of this enzyme with respect to methylated catechols. The literature data indicate that C-1,2-DOs are enzymes with narrow substrate specificity [30]. This does not contradict our results because in most cases analysis of substrate specificity is based on determination of oxidation rates of catechol and halogen-substituted substrates with which the described enzyme exhibits low catalytic activity (Table 3).

**C-1,2-DO II (methyldioxygenase).** *Enzyme purification*. This enzyme was separated from the fraction of total catecholase activity at the stage of phenyl-Sepharose chromatography (Fig. 4). After this stage, the enzyme was purified separately. C-1,2-DO II was eluted from the phenyl-Sepharose column at 0.3 M $(NH_4)_2SO_4$ (volume 595 ml, total protein 298 mg, specific activity 2.04 U/mg). This enzyme was eluted from the Resource Q col-

umn at 0.15-0.25 M NaCl (volume 43 ml, total protein 57 mg, specific activity 6.32 U/mg) and from the Resource Iso column at 1.12-0.8 M $(NH_4)_2SO_4$ (volume 27 ml, total protein 42.5 mg, specific activity 9.74 U/mg). This purification protocol gave homogenous enzyme preparation with yield of 37.5%.

*Physicochemical properties of C-1,2-DO II.* The molecular mass of subunits of this enzyme was 26-27 kD. This is consistent with molecular masses of *R. opacus* 1cp CC-1,2-DO induced during growth on 4-chlorophenol [28]. The purified enzyme had the following $NH_2$-terminal sequence (33 residues): AlaAsnThrArgValIleGlyLeuPheAspGluPheThrAspLeuIleArgAspPheIleValArgHisGlyIleThrThrProGluIleGluThrIle. This completely corresponds to the $NH_2$-terminal sequence of *R. opacus* 1cp CC-1,2-DO induced during growth on 4-chlorophenol [28]. These data suggest that growth of this microbial strain on *p*-toluate is accompanied by induction of the enzyme identical or very similar to the enzyme induced during growth the microbial growth on 4-chlorophenol.

*Catalytic properties of C-1,2-DO II.* This enzyme catalyzed oxidation of a wide range of substituted catechols. Maximal activity of C-1,2-DO II was found with methyl-substituted catechols as substrates; with 4-chlorocatechol the enzyme activity was 113% of the activity with catechol as substrate (Table 2). The rate of cleavage of dichloro-substituted catechol was also comparable with the rate of catechol cleavage and was 22.5% of the catechol cleavage. These results are similar to the data obtained for CC-1,2-DO involved in degradation of 4-chlorophenol [13]. Thus, study of catalytic properties and $NH_2$-terminal amino acid sequence of the methylcatechol dioxygenase isolated from the biomass of *R. opacus* 1cp grown on *p*-toluate indicates the existence of high similarity of the purified enzyme with CC-1,2-DO induced during the microbial growth on 4-chlorophenol. Thus, *R. opacus* 1cp possible employs the enzyme, which functions in the modified *ortho*-pathway, for decomposition of 4-chlorocatechol and utilization of 4-methyl-substituted catechols.

**Muconate cycloisomerase.** *Enzyme purification.* MCI was purified in four steps from biomass cultivated on *p*-toluate. The steps included ion-exchange and hydrophobic chromatography, heating at 60°C for 15 min (volume 141 ml, total protein 45 mg, specific activity 0.38 U/mg), and gel filtration (volume 20 ml, total protein 28.4 mg, specific activity 0.5 U/mg). The enzyme was eluted from Q-Sepharose at 0.19-0.21 M NaCl (volume 145 ml, total protein 98 mg, specific activity 0.15 U/mg) and from phenyl-Sepharose at 0.25-0.19 M $(NH_4)_2SO_4$ (volume 43 ml, total protein 3.3 mg, specific activity 1.9 U/mg). During purification, the specific activity of the MCI increased from 0.02 to 1.92 U/mg; this corresponds to 96-fold purification. The yield of activity was 37%.

*Molecular mass.* The molecular mass of the MCI subunit was 40 kD. Similar results were also obtained for MCI and CMCI isolated from *R. opacus* 1cp [14, 28, 31, 32] and other bacteria.

**Table 1.** Dioxygenase activity of the enzymes separated by phenyl-Sepharose chromatography

| Substrate | Specific activity, U/mg | |
|---|---|---|
| | C-1,2-DO I | C-1,2-DO II |
| Catechol | 9.6 | 5.7 |
| 4-Chlorocatechol | 0.23 | 5.6 |
| 4-Methylcatechol | 6.6 | 11.7 |

**Table 2.** Substrate specificity of C-1,2-DO I

| Substrate | Relative activity, % | |
|---|---|---|
| | C-1,2-DO I | C-1,2-DO II |
| Catechol | 100 | 100 |
| 4-Chlorocatechol | 2.1 | 113 |
| 3-Methylcatechol | 73 | 191 |
| 4-Methylcatechol | 89 | 253 |
| 4,5-Dichlorocatechol | 0 | not determined |
| 3,5-Dichlorocatechol | 0 | 22.5 |

**Table 3.** Kinetic characteristics of C-1,2-DO I

| Substrate | $K_m$, μM | $k_{cat}$, $min^{-1}$ | $k_{cat}/K_m$, $\mu M^{-1} \cdot min^{-1}$ |
|---|---|---|---|
| Catechol | 6.5 | 309 | 47.9 |
| 3-Methylcatechol | 20 | 850 | 42.5 |
| 4-Methylcatechol | 9.1 | 340 | 37.4 |

*Catalytic properties.* The MCI catalyzed conversion of substituted muconates (Table 4). Table 4 also shows data for MCI isolated from the biomass of the same microbial strain grown on benzoate. The MCI exhibited the highest activity with *cis*,*cis*-muconate; the rate of cycloisomerization was much lower. Similar ratio of catalytic activities was also typical for MCI involved in the traditional *ortho*-pathway induced during growth on benzoate. The $K_m$ value of the MCI for *cis*,*cis*-muconate was 81 μM.

Thus, growth of *R. opacus* 1cp on *p*-toluate revealed the presence of two dioxygenases; one shows similarity to

**Table 4.** Relative activity of MCI induced during microorganism growth on various substrates

| Substrate | Relative activity of the inducible enzymes, % (specific activity, U/mg) | |
|---|---|---|
| | growth on *p*-toluate | growth on benzoate* |
| *cis*,*cis*-Muconate | 100 (19.6) | 100 (36.9) |
| 3-Chloro-*cis*,*cis*-muconate | 14.2 (2.8) | 35.8 (13.2) |
| 2-Methyl-*cis*,*cis*-muconate | 5.8 (1.1) | 8.1 (3.0) |
| 3-Methyl-*cis*,*cis*-muconate | 24.0 (4.7) | 13.0 (4.8) |

* [23].

catecholase of the ordinary *ortho*-pathway, whereas properties of the other are similar to CC-1,2-DO from the same strain grown on 4-chlorophenol. Such mode of enzyme induction is rather unusual for this strain because the presence of two dioxygenases was not previously recognized during cultivation of this microorganism on any substrate. The presence of only one MCI similar to the enzyme involved in the ordinary *ortho*-pathway is quite "reasonable" because CMCI induced during the microbial growth on 4-chlorophenol is characterized by low value of the specificity constant for 3-methylmuconate (0.73 $\mu M^{-1} \cdot min^{-1}$) compared with 3-chloromuconate (21 $\mu M^{-1} \cdot min^{-1}$). However, the specificity constant of the MCI of the ordinary *ortho*-pathway for muconate was just 4.5 times higher than for 3-methylmuconate [31]. Taking into consideration the narrow substrate specificity of MCI, such difference is rather insignificant.

The set of enzymes involved in various modified pathways induced for utilization of *p*-toluate is interesting. It is known that benzoate and muconate can act as inductors of dioxygenases and muconate isomerases. Induction of C-1,2-DO and also MCI similar to that of the MCI typical for the ordinary *ortho*-pathway induced during growth on benzoate is biologically relevant. However, induction of additional dioxygenase, probably CC-1,2-DO of the 4-chlorophenol pathway and lack of parallel induction of CMCI of this pathway suggest more complex microbial strain response to a new substrate. On one hand, this helps the microbial strain to effectively decompose a given compound and to avoid non-productive induction of enzyme exhibiting low effectiveness with respect to forming 3-methylmuconate. On the other hand, it represents an example illustrating the appearance of possible new metabolic pathways increasing the metabolic potential of microorganisms.

In the future, we plan to investigate the second key step of *p*-toluate decomposition, the reaction of 4-methyl-muconolactone conversion.

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