Rational engineering of the regioselectivity of TecA tetrachlorobenzene dioxygenase for the transformation of chlorinated toluenes

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The tetrachlorobenzene dioxygenase (TecA) of Ralstonia sp. PS12 carries out the first step in the aerobic biodegradation of chlorinated toluenes. Besides dioxygenation of the aromatic ring of 4-chloro-, 2,4-, 2,5- and 3,4-dichlorotoluene as the main reaction, it also catalyses mono-oxygenation of the methyl groups of 2,3-, 2,6-, 3,5-di- and 2,4,5-trichlorotoluene as the main reactions, channelling these compounds into dead-end pathways. Based on the crystal structure of the homologous naphthalene dioxygenase (NDO) and alignment of the α-subunits of NDO and TecA, the substrate pocket of TecA was modelled. Recently, for NDO and the homologous 2-nitrotoluene dioxygenase (2NTDO), two amino acids (Phe352 of NDO and Asn258 of 2NTDO) were identified which control the regioselectivity of these enzymes. The corresponding amino acids at Phe366 and Leu272 of TecA were substituted to change the regioselectivity and to expand the product spectrum. Position 366 was shown to control regioselectivity of the enzyme, although mutations resulted in decreased or lost activity. Amino acid substitutions at Leu272 had little or no effect on the regioselectivity of TecA, but had significant effects on the product formation rate. Substitutions at both positions changed the site of oxidation of 2,4,5-trichlorotoluene slightly. As new products, 3,4,6-trichloro-1-methyl-1,2-dihydroxy-1,2-dihydrocyclohexan-3,5-diene, 4,6-dichloro-3-methylcatechol, 3,6-dichloro-4-methylcatechol and 3,4-dichloro-6-methylcatechol were identified.

INTRODUCTION

The aerobic degradation of many natural and xenobiotic aromatic compounds is initialized by dioxygenases, which catalyse the incorporation of two oxygen atoms into the aromatic ring to form arene-cis-dihydrodiols followed by a dehydrogenation reaction catalysed by a cis-dihydrodiol dioxygenase to give catechols or substituted catechols which serve as substrates for oxygenolytic cleavage of the aromatic ring. The aromatic ring dioxygenases, such as benzoate dioxygenase, serve as substrates for oxygenolytic cleavage of the aromatic dioxygenase to give catechols or substituted catechols which determine the substrate specificity of the enzyme to carry out the substrate activation (Gibson & Parales, 2000).

The recently characterized tetrachlorobenzene dioxygenase (TecA) of the 1,2,4,5-tetrachlorobenzene-degrading strain Ralstonia sp. PS12 (Beil et al., 1997) is a broad specificity enzyme which catalyses the first step in the degradation of a wide variety of chlorinated benzenes and toluenes (Beil et al., 1997; Lehning et al., 1997; Pollmann et al., 2001; Sander et al., 1997). Like other dioxygenases acting on hydrophobic substrates, the enzyme is composed of an electron transport chain involving a reductase and a ferredoxin and the terminal dioxygenase consisting of χ- and β-subunits. The χ-subunit of TecA contains a Rieske (2Fe2S) centre and a mononuclear non-haem iron. Recent studies have shown that the χ-subunit is responsible for determining the substrate specificity of the enzyme (Beil et al., 1998). Similarly, the χ-subunits have been reported to be dominantly responsible for the substrate specificities of biphenyl, naphthalene and nitrotoluene dioxygenases (Kauppi et al., 1998; Parales et al., 1998; Tan & Cheong, 1994). Besides dioxygenation of the aromatic nucleus, TecA catalyses mono-oxygenation of the methyl group of various chlorosubstituted toluenes, with the ratio of di- versus...
mono-oxygenation dependent on the substitution pattern (Lehning et al., 1997; Pollmann et al., 2001).

Recent analyses have shown that only those chlorinated toluenes which were exclusively or predominantly subject to dioxygenation, such as 4-chlorotoluene, 2,4-dichlorotoluene, 2,5-dichlorotoluene and 3,4-dichlorotoluene, can be used as growth substrates by strain PS12 whereas mono-oxygenation channels the substrates into dead-end pathways (Lehning et al., 1997; Pollmann et al., 2002). Thus, the regioselectivity of attack as determined by TecA controls if and to what extent a substrate can be mineralized by strain PS12. It is, therefore, important to know which amino acids of the protein control regioselectivity and if the specificity of the enzyme can be changed to prevent or reduce mono-oxygenation reactions. To date the most thoroughly studied dioxygenase acting on hydrophobic aromatics is the naphthalene dioxygenase (NDO) from Pseudomonas sp. NCIB 9816-4 (Ensley & Gibson, 1983; Ensley et al., 1982; Simon et al., 1993), the z-subunit of which, on the amino acid level, is 36% identical to that of TecA. NDO catalyses the oxidation of naphthalene and a wide variety of aromatic compounds as well as mono-oxygenation (Ensley et al., 1982; Lee & Gibson, 1996; Parales et al., 2000a), desaturation, sulfoxidation and O- and N-dealkylation reactions with selected substrates. This enzyme has been crystallized and amino acids ligated to or close to the active-site iron have been identified (Carredano et al., 2000; Kauppi et al., 1998).

Recent studies have identified that the amino acid Phe352 plays a major role in controlling the regioselectivity of attack on bi- or tricyclic aromatics and the stereochemistry of the products (Parales et al., 2000a, b). 2-Nitrotoluene dioxygenase (2NTDO) is a related enzyme, which catalyses dioxygenation of the aromatic nucleus of 2-nitrotoluene (Parales et al., 1996). Substitution in 2NTDO of Asn258 for Val was claimed to result in an enzyme that no longer oxidized the aromatic ring but formed the mono-oxygenation product 2-nitrobenzyl alcohol, whereas substitution of the corresponding Val260 for Asn in NDO was claimed not to change the specificity (Parales et al., 2000a), although no detailed data were reported. Based on the sequence alignments of the x-subunits of NDO (Simon et al., 1993), 2NTDO (Parales et al., 1996), toluene dioxygenase (Zylstra & Gibson, 1989) and TecA (Beil et al., 1997), the amino acids Phe and Leu in positions 366 and 272 of the TecA x-subunit could be identified as corresponding to the amino acids Phe352 and Val260 of NDO, respectively, and thus are promising candidates for the introduction of single mutations to change the direction of the attack.

In this study, five TecA derivatives with amino acid substitutions at Phe366 and Leu272 were generated and characterized.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used were Escherichia coli DH5z (Clonetech) as host for cloning; pBluescript II KS(+) (Stratagene); pSTE44, carrying the genes tecA1A2A3 encoding tetrachlorobenzene dioxygenase (TecA) and dihydrodiol dehydrogenase (TecB) (Pollmann et al., 2001); pKPM1 was obtained by cloning a 3976 bp EcoRI fragment comprising the complete tecA1A2A3 genes from a digest of pSTE44 into the EcoRI site of pBluescript II KS(+); pKPM2, pKPM3, pKPM4, pKPM5 and pKPM6 were obtained by cloning the 1164 bp RsI1- and MluI-digested 1240 bp PCR products obtained from splicing by overlap extension (SOE)-PCR carrying mutations in F366W (pKPM2), F366Y (pKPM3), L272W (pKPM4), L272F (pKPM5) and F366L (pKPM6), respectively, into RsI1- and MluI-digested pKPM1; pKPM7, pKPM9, pKPM10, pKPM11 and pKPM12 were obtained by cloning a 1922 bp Eco47III fragment comprising the complete mutated tecA1 gene from pKPM2, pKPM3, pKPM4, pKPM5 and pKPM6 into the Eco47III site of pSTE44. Thus, pKPM7, pKPM9, pKPM10, pKPM11 and pKPM12 differ from pSTE44 only by the respective mutation. Strains were routinely grown in Luria–Bertani broth (LB) containing 100 μg ampicillin ml−1 and incubated at 30°C on a rotary shaker operated at 130 r.p.m.

DNA manipulations. Standard procedures were performed as described previously (Sambrook et al., 1989). Plasmid DNA was extracted with a Plasmid Midi Kit (Qiagen). Restriction enzymes were purchased from New England Biolabs and MBI Fermentas. IPTG and X-Gal were obtained from Carl Roth (Karlsruhe, Germany). Oligonucleotides were purchased from Invitrogen. The oligonucleotides used in this study are listed in Table 1. Tag polymerase was obtained from Qiagen. Elution of DNA from 1% agarose gels was performed with a QIAquick gel extraction kit (Qiagen). PCR products were purified with a QIAquick PCR Purification Kit (Qiagen). Sequencing reactions on both strands were performed with an Applied Biosystems model 3100 DNA sequencer according to the protocols of the manufacturer (Applied Biosystems) for Tag cycle-sequencing with fluorescent-dye-labelled dideoxynucleotides. Site-specific mutations were introduced using SOE-PCR (Horton et al., 1989). Sequences of all de novo synthesized DNA molecules and correct insertion of fragments in the plasmids were confirmed by sequencing.

Oligonucleotides and site-directed mutagenesis. To obtain PCR fragments carrying the mutations, three PCRs for each mutation were performed. In the first reaction, a PCR fragment was amplified from pSTE44 using the 1970forw primer and a reverse mutagenic primer. In a second reaction, a PCR fragment was amplified from pSTE44 using the 3187rev primer and a mutagenic

Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
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<tbody>
<tr>
<td>1970forw</td>
<td>CATCCCCTGCAACCTGGAAAACCT</td>
</tr>
<tr>
<td>3187Rev</td>
<td>GCTGGAATCTCTGACTGCGGCCCTC</td>
</tr>
<tr>
<td>L272Wforw</td>
<td>AGAAACCGATTTGGCGTCTGGCCAT</td>
</tr>
<tr>
<td>L272Wrev</td>
<td>TGGGCTGAGCCACAATCGGTTTTC</td>
</tr>
<tr>
<td>L272Fforw</td>
<td>AGAAACCGATTCTCGTCTGGCCAT</td>
</tr>
<tr>
<td>L272Frev</td>
<td>ATGGGACAGAGAAATCGGGTTTCT</td>
</tr>
<tr>
<td>F366Yforw</td>
<td>TGCCGACACTTACCTCGGTCG</td>
</tr>
<tr>
<td>F366Yrev</td>
<td>GCCACCGGAGATAGTGGCCGA</td>
</tr>
<tr>
<td>F366Wforw</td>
<td>TGGCAGCCTTGGCTCGGCG</td>
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<tr>
<td>F366Wrev</td>
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<tr>
<td>F366Lforw</td>
<td>TGCGCACTCTTCCGTCGCG</td>
</tr>
<tr>
<td>F366Lrev</td>
<td>GCCAGCGGAGAGGTCGCG</td>
</tr>
</tbody>
</table>

Nucleotides that were changed using SOE-PCR are indicated in bold type.
forward primer corresponding to the first reaction. The PCR products obtained from both reactions were purified, mixed and used as template in a third PCR with 1970forw and 3187rev as primers. Each PCR contained 5 μl of 10× PCR buffer containing 15 mM MgCl₂, 200–500 ng template DNA, 1 pmol forward primer, 1 pmol reverse primer, 200 μM of each dNTP and 2-5 U Taq DNA polymerase in a volume of 50 μl. PCR conditions were as follows: 5 min at 94°C; 30 cycles with 1 min at 94°C, 1 min at 60°C and 1 min at 72°C; 10 min at 72°C; 4°C.

Resting cell assays. E. coli strains were pre-cultured in LB containing 1 mM IPTG and 0-1 mg ampicillin ml⁻¹ at 30°C on a rotary shaker at 130 r.p.m. Two hundred to one thousand millilitres of the same medium were inoculated with 1 ml of the pre-culture. The cells were harvested at an OD₅₅₀ value of 2-7, pelleted (10 min, 20°C, 5900 g) and washed twice with assay buffer (10 mM glucose +0-1 × M9 minimal medium) (Sambrook et al., 1989). Pre-warmed assay buffer (10–100 ml), containing 0-5 mM of 2-chlorotoluene, 3-chlorotoluene, 4-chlorotoluene, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-, 3,5-dichlorotoluene or 2,4,5-trichlorotoluene (from a 100 mM chlorotoluene, 3-chlorotoluene, 4-chlorotoluene, 2,3-, 2,4-, 2,5-, 2,6-, etc. solution) were mixed with the resting cells (100 ml) of E. coli strain pKPM10 (L272W) and incubated at 30 °C in an Aquatron shaker (130 r.p.m.).

Quantification of proteins. To quantify the expression of TecA mutants, E. coli producing the wild-type TecA, the mutated TecA and as a control, E. coli carrying pBlueScript II KS(+) were grown in LB supplemented with 1 mM IPTG to an OD₅₅₀ value of 2-7. Harvested cells were resuspended in assay buffer and disrupted using a French press (Aminco). Cell debris was removed by centrifugation at 100 000 g for 40 min at 4°C. Protein concentrations in the extracts were determined using the Bradford method (Bradford, 1976) using BSA as the standard. Proteins of the cell extracts (corresponding to 10 μg) were separated by SDS-PAGE, essentially as described by Laemmli (1970). For quantification of TecA, gels were stained using the fluorescent dye Sypro Ruby (MoBiTec). Gels were scanned using a Fujifilm LAS-1000 CCD camera. The relative amounts of the soluble TecA z-subunit protein band with a size of 50–51 kDa and the TecB dihydrodihydroxide dioxygenase band with a size of 31–32 kDa were determined using the AIDA 2.1 software package (Raytest Isotopenmessgeräte, Straubenhardt, Germany).

Analysis of transformation products by HPLC. To monitor product formation, 200 μl aliquots were removed from resting cell assays at regular time intervals between 0 and 120 min and after 16 h incubation and shock-frozen in liquid nitrogen. The samples were stored at −20°C for subsequent HPLC analyses. Use of the same bacterial host and assay conditions allowed a direct comparison of transformation rates of the different dioxygenase variants. Product formation was analysed with a Shimadzu HPLC system (LC-10AD liquid chromatograph, DGU-3A degasser, SPD-M10A diode array detector, FCV-10AL solvent mixer) equipped with an autosampler and a sample cooler operating at 4°C on a SC125/Lichrospher 5-µm (Bischoff, Leonberg, Germany) column. The aqueous solvent system (flow rate 1 ml min⁻¹) contained 0-1% (v/v) H₃PO₄ (87%) and 50 or 60% (v/v) methanol. Portions of the samples (10 μl) were injected after removing cells by centrifugation (20°C, 10 min, 10 000 g). The alcohol products were identified and quantified by comparison with authentic standards. Concentrations of dichloromethylcatechols and 2,4,5-trichlorobenzylalcohol were determined by HPLC analyses as described previously (Pollmann et al., 2001). Substrate transformation rates were expressed as the amount of product formed per time as determined by HPLC.

Preparation of metabolites for GC-MS and ¹H-NMR analysis. Resting cells (100 ml) of E. coli pPKM10 (L272W) were incubated with 2,4,5-trichlorotoluene or 4-chlorotoluene (0-5 mM) for 10 h, and resting cells of E. coli (pSTE44) were incubated with 2-chlorotoluene or 3-chlorotoluene (0-5 mM) for 4 h. After centrifugation (20°C, 10 min, 5900 g) the acidified supernatants (pH 6) were extracted twice with equal volumes of ethyl acetate. The organic phases were dried over MgSO₄ and evaporated under vacuum on a rotary evaporator.

Characterization of transformation products by GC-MS. Metabolites formed from 2,4,5-trichlorotoluene were dissolved in 1 ml dry acetone. Fifty microlitres of the solution were transferred to an autosampler vial and dried under a stream of nitrogen, and 20 μl of the borating reagent [50 mg C₄H₄B(OH)₂ (butyrboronic acid) ml⁻¹ in acetone] was added and incubated for 10 min at 50°C (Kirsch & Stan, 1994). After evaporation under a stream of nitrogen, the intermediates were redissolved in 30 μl n-hexane. Products formed from 2,4,5-dichlorotoluene were analysed by GC-MS. Aliquots of derivatized samples (1 μl) were injected and analysed with a Shimadzu GC-17A gas chromatograph equipped with an XTI-5 column (30 m × 0.25 mm; film thickness, 0-5 μm; Restek) and coupled to a QP-5000 quadrupole mass spectrometer. The mass spectrometer was operated in the electron impact mode at 70 eV with an ion source temperature of 320°C. Helium was used as carrier gas with a flow rate of 1 ml min⁻¹. The oven temperature was maintained at 35°C for 5 min and then increased to 100°C at a rate of 5°C min⁻¹, followed by an increase to 320°C at a rate of 10°C min⁻¹. GC-MS was operated in the splitless mode with an injection temperature of 270°C.

Characterization of chloromethylcatechols by ¹H-NMR. Extracted metabolites were dissolved in 0-7 ml d₄-acetone (deuterated acetone). To prepare a defined standard of 3-chloro-4-methylcatechol (from 4-chlorotoluene), the metabolite solution was spiked with a defined concentration of 3-chlorobenzylic alcohol. ¹H-NMR spectra were recorded on a Bruker DPX 300 (Bruker) apparatus using tetramethysilane as the internal standard; the concentration of 3-chloro-4-methylcatechol in the mixed sample was quantified by comparison of the resonance lines at δ=4-61 p.p.m. (protons of the alcohol residue of 3-chlorobenzylic alcohol) and δ=6-59 p.p.m. (H-2 proton of 3-chloro-4-methylcatechol). The composition and identity of the product mixtures formed from 2- and 3-chlorotoluene were analysed using one-dimensional (¹H and nuclear Overhauser enhancement difference spectra with 10 s delays between pulses) and two-dimensional (COrelated SpectroscopY, COSY) NMR spectra recorded on a Bruker ARX 400 NMR spectrometer.

Chemicals. 3,5-Dichlorotoluene and 2,4,5-trichlorotoluene were synthesized and kindly provided by W. Reineke (Bergische Universität – Gesamthochschule Wuppertal, Chemische Mikrobiologie, Gausstr. 20, D-42097 Wuppertal, Germany) and S. Kaschabek (Interdisziplinäres Ökologisches Zentrum, TU Bergakademie Freiberg, Leipziger Str. 29, D-09599 Freiberg, Germany). C₄H₉B(OH)₂ was obtained from Acros organics and 2,3-dichlorobenzyl alcohol from TCI (Chemos). All other chemicals were purchased from Sigma-Aldrich or Merck.

Enzyme modelling. The structure model of TecA was generated using MODELLER 6 version 1 (Sali & Blundell, 1993) based on the NDO structures (pdb-ID: 1NDO and 1EG9) and a sequence alignment (Fig. 1) generated with CLUSTALX (Thompson et al., 1997). In order to stay as close as possible to the template structure, no additional molecular dynamics calculations were attempted. Coordinates for the F366L, L272F and L272W models were derived by manual substitution of the corresponding side chain. Models of enzyme–substrate complexes were generated by hand based on the structure of the indole complex of the NDO (pdb-ID: 1EG9) and by setting manually orientations obtained with AUTODOCK 3.0 (Morris et al., 1998). In both cases orientations were selected that placed those atoms in the vicinity of the active iron atom that allowed formation of the main product. SVYLL 6.8 (Tripos, St Louis, MO, USA) was used for generation of co-ordinates for the substrate.
molecules and for generation of proton positions and charges (Kollman-all-atom for the protein and Gasteiger for the ligand) of protein and ligand in the preparation for AUTODOCK. AUTODOCK calculations were carried out on a box of 64 Å side length, rasterized with 0.5 Å and centred at the centre of gravity of the protein, and consisting of 10 runs per ligand according to the genetic algorithm with a population size of 50 and maximally 1,000,000 energy calculations or 27,000 generations.

RESULTS

Expression of mutant TecA α-subunits

After electrophoretic separation and staining of proteins from cell extracts of E. coli cells expressing dihydrodiol dehydrogenase (TecB) and wild-type TecA or mutant TecA, distinct bands with a size of 50–51 kDa, which can be related to the TecA α-subunit (Beil et al., 1998), and a size of 31–32 kDa, which can be related to TecB (Pollmann et al., 2001), were present in all extracts except those of the control not producing TecAB. The expression of the proteins varied to some extent (relative intensities wild-type TecA : F366W : F366Y : F366L : L272W : L272F 100 : 90 : 98 : 72 : 59). Since all variants showed significant expression of TecA and TecB, the inability of the variants F366W and F366Y to transform chlorinated toluenes (see below) was not caused by the absence of the proteins.

Transformation of chlorinated toluenes by TecA variants and TecB

The potential of the TecA variants to transform various chlorinated toluenes was characterized by HPLC analysis of supernatants of resting E. coli cells expressing the different enzyme variants and incubated with the respective substrates. Strains producing enzymes carrying the mutations F366L, L272F and L272W were able to transform all tested substrates, i.e. 2-, 3- and 4-chlorotoluene, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-dichloro- and 2,4,5-trichlorotoluene (either quantitatively or in part) into the corresponding benzyalcohols, and 2-, 3-, 4-chloro-, 2,4-, 2,5-, 2,6- and 3,4-dichlorotoluene (either quantitatively or in part) into the corresponding catechols. Qualitative differences were observed during 2,4,5-trichlorotoluene turnover. Whereas wild-type TecAB formed exclusively 2,4,5-trichlorobenzylalcohol by monooxygenolytic attack, four additional products were formed by the variants F366L, L272F and L272W. Three of these products could be identified by HPLC analysis as 4,6-dichloro-3-methylcatechol (net RV = 14.8 ml, λmax = 205 nm, using 50% methanol as eluent), 3,6-dichloro-4-methylcatechol (net RV = 8.2 ml, λmax = 204 nm, 50% methanol) and 3,4-dichloro-6-methylcatechol (net RV = 10.4 ml, λmax = 204 nm, 50% methanol), respectively (Pollmann et al., 2001). The fourth product (net RV = 3.7 ml, 50% methanol) showed an UV spectrum with λmax = 284 nm indicative of the formation of a dihydrodiol (Pollmann et al., 2001). Confirmation of the identity of this intermediate as a dihydrodiol was obtained by GC-MS analysis of the boronated derivatives. Besides three signals showing molecular ion masses of m/z 258, 260 and 262, originating from 4,6-dichloro-3-methylcatechol, 3,6-dichloro-4-methylcatechol and 3,4-dichloro-6-methylcatechol (Pollmann et al., 2001), one signal showing a molecular ion mass of m/z 294, 296, 298 (relative intensities, 100:94:31), indicative of a trichlorinated compound, was observed. The fragmentation pattern showed the typical characteristics described for boronated dihydrodiols formed from substituted benzenes (Beil et al., 1998; Pollmann et al., 2001), i.e. loss of C4H9 (M+ −57), loss of Cl–C4H8 (M+ −91) and loss of one or two chlorines (Fig. 2). As dioxygenolytic attack on 1,2,4-trichlorotoluene involving a chlorosubstituted and an unsubstituted carbon atom, as shown above (and as previously described for 1,2,4,5-tetrachlorobenzene transformation; Beil et al., 1998), in all three possible cases resulted in a spontaneous chloride elimination and concomitant formation of a catechol, the observed dihydrodiol should have been formed by a distinct dioxygenolytic attack. Thus, the product 3,4,6-trichloro-1-methyl-1,2-dihydroxy-1,2-dihydroxybenzenec-3,5-diene results from dioxygenolytic attack on a methyl substituted carbon and unsubstituted carbon atom. Such a product cannot be transformed into a catechol derivative.
Identification of 4-chloro- and 5-chloro-3-methylcatechol as the dominant dioxygenation products formed from 2- and 3-chlorotoluene, respectively

From 2-chlorotoluene, two different catechols ($\lambda_{\text{max}} = 201$ nm, RV = 4·1 ml and 6·2 ml, 50 % methanol) were formed apart from 2-chlorobenzylalcohol. Previous studies have shown the formation of 3-methyl-4-chloro-cis-1,2-dihydroxy-1,2-dihydrocyclohexa-3,5-diene and 4-methyl-3-chloro-cis-1,2-dihydroxy-1,2-dihydrocyclohexa-3,5-diene by TecA-mediated dioxygenation (Lehning et al., 1997). Thus, the formation of 3-chloro-4-methylcatechol and 4-chloro-3-methylcatechol from the dihydrodiols by TecB or from 2-chlorotoluene by the activity of the TecAB enzyme system may be assumed (Fig. 3). Assuming similar extinction coefficients at 210 nm, the two catechols (RV = 4·1 ml and RV = 6·2 ml) were formed in a 1:4 ratio. However, with the current knowledge, it cannot be deduced which of the above-mentioned catechols is formed as the dominant product and which one only in minor amounts. Similarly, two products ($\lambda_{\text{max}} = 201$ nm, RV = 4·1 ml and 6·2 ml, 50 % methanol) were formed from 3-chlorotoluene apart from 3-chlorobenzylalcohol, and these products may be assumed to be 3-chloro-5-methylcatechol and 5-chloro-3-methylcatechol (Fig. 3). The two catechols (RV = 4·1 ml and RV = 6·2 ml) were formed in a 1:10 ratio.

The $^1$H-NMR spectrum of the product mixture formed from 3-chlorotoluene showed the presence, in addition to 3-chlorobenzylalcohol, of one major catechol derivative with two aromatic protons resonating at 6·64 and 6·71 p.p.m. (Fig. 3). The coupling constant of 2·3 Hz indicates these are located in meta-position to one another. The methyl group of the chloromethylcatechol resonates at 2·18 p.p.m. and upon low power irradiation affords a nuclear Overhauser enhancement (nOe) only to the signal at 6·64 p.p.m. This is unambiguous evidence that only this proton is located in an ortho-position with respect to the methyl substituent and thus the product is 5-chloro-3-methylcatechol.

The major catechol formed from 2-chlorotoluene exhibited the signals of a methyl group at 2·24 p.p.m. and two ortho aromatic protons at 6·69 and 6·72 p.p.m. with coupling constants of 8·6 Hz (Fig. 3). These signals obviously overlap with those of one signal of a further aromatic system of a second catechol derivative present in the mixture. The second aromatic proton of this compound resonates at 6·64 p.p.m. Low power irradiation of the methyl signal at 2·24 p.p.m. affords an nOe only to the small signal at 6·64 p.p.m. Thus, the major catechol derivative formed from 2-chlorotoluene is 4-chloro-3-methylcatechol, and the derivative formed in minor amounts is 3-chloro-4-methylcatechol.

Fig. 2. Mass spectrum of the boronated product formed from 2,4,5-trichlorotoluene by TecA L272W, L272F and F366L variants.

Fig. 3. $^1$H-NMR data of chloromethylcatechols formed from 2- and 3-chlorotoluene. Chemical shifts (in p.p.m.) are indicated as well as coupling constants (in Hz), with the respective coupling protons. Signal assignments were evident from the $^1$H nuclear Overhauser enhancement difference data and from the detectable long-range couplings of ortho and para protons to their respective methyl groups.

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Regioselectivity of chlorotoluene transformation by modified TecA proteins

Products formed from chlorotoluenes, dichlorotoluenes and 2,4,5-trichlorotoluene were quantified by comparison with authentic standards in the case of mono- and dichlorobenzylalcohols or as previously described (Pollmann et al., 2001) in the case of dichloromethylecatechols or 2,4,5-trichlorobenzylalcohol. For quantification of chloromethylcatechols formed from 2- and 3-chlorotoluene, a standard of 3-chloro-6-methylcatechol was prepared and the concentrations of the differently substituted chloromethylcatechols were determined, assuming similar absorption at \( \lambda = 210 \text{ nm} \) for all the monochlorinated methylcatechols.

Wild-type TecAB oxidized 2,4-, 2,5- and 2,6-dichlorotoluene to 94 : 6, 81 : 19 and 11 : 89 mixtures of the corresponding substituted catechols and benzylalcohols, respectively (Fig. 4). In the case of 2-chlorotoluene, 2-chlorobenzylalcohol was the main product (64 : 5%), and in addition 3-chloro-4-methylcatechol and 4-chloro-3-methylcatechol were produced in a 7 : 29 ratio. The mixture produced from 3-chlorotoluene consisted of 3-chlorobenzylalcohol as the main product (80%) and by-products of 3-chloro-5-methylcatechol and 5-chloro-3-methylcatechol in a 2 : 18 ratio. For 2,4,5-trichlorotoluene, mono-oxygenation of the methyl group produced 2,4,5-trichlorobenzylalcohol exclusively (Fig. 4).

Whereas two out of the three mutants carrying substitutions in position F366, i.e. F366W and F366Y, did not form detectable products from any tested substrate, the F366L mutant was active with all substrates and showed significant changes in regioselectivity. In contrast to wild-type TecA, 2,5-dichlorobenzylalcohol was the major product (54%) formed from 2,5-dichlorotoluene. Similarly, in the case of the other chloro- and dichlorotoluenes tested, the direction of attack usually shifted significantly towards monooxygenation to form the corresponding benzyl alcohols (Fig. 4). In contrast, for 2,4,5-trichlorotoluene significant amounts of dioxygenation products (comprising about 14% of the products) were formed.

TecAB mutant enzymes with changes at position 272 differed only slightly from the wild-type enzyme with regard to the regioselectivity of attack (Fig. 4). However, similar to the F366L variant, significant amounts of products resulting from a dioxygenolytic attack were formed from 2,4,5-trichlorotoluene.

Relative activities of the mutant TecA enzymes

Whereas the F366W and F366Y variants were not active with any of the substrates, the F366L variant converted the substrates, specifically the monochlorinated toluenes, at significantly reduced rates compared to those of wild-type TecA (Table 2). Thus, all enzymes with substitutions at position 366 were severely defective in catalysing oxygenation of the chlorinated toluenes. However, the F366L mutant retained a significant activity with 3,4-dichlorotoluene of 25% that of wild-type TecA.

Enzymes with substitutions in position 272 showed high activity with all tested substrates (Table 2). Whereas transformation rates with monochlorinated toluenes were, at first glance, significantly reduced compared to wild-type TecA (32–64%), transformation rates of dichlorotoluenes were mostly elevated. However, taking into account the lower expression level of the L272F and L272W enzyme variants (60–70% of wild-type TecA, see above), these mutants showed approximately the same activity with chlorotoluenes as the wild-type enzyme. In particular, the variant L272W showed increased transformation rates with 2,3-, 2,5-, 2,6-, 3,4- and 3,5-dichlorotoluene (200–380% of wild-type TecA).

Enzyme modelling

The model generated by MODELLER (Sali & Blundell, 1993) showed 87-9% of the 387 residues in the most favoured region of a Ramachandran plot calculated with PROCHECK (Laskowski et al., 1993), in good agreement with the structural template (88-5% for the 1.6 Å resolution structure pdb-ID 1eq9). The root mean square deviation of 413 equivalent Cα-atoms is 0·7 and 0·3 Å for the 252 equivalent Cα-atoms of the residues 160 to 436 containing the active site pocket. AUTODOCK runs using the generated models, and co-ordinates for 2,4,5-trichlorotoluene, 2,5-dichlorotoluene and 2,6-dichlorotoluene showed in all cases, regardless of wild-type or modified protein model, several orientations of the ligand in the active site with similar docking energies. Manual placement of the ligands into the active site pocket using O (Jones et al., 1991) also showed that several different orientations of the ligands were possible given the size of the active site pocket and the lack of specific interaction sites, either hydrophilic or electrostatic, on the ligands and the protein.

DISCUSSION

General docking of substrates and mechanism of action

Based on the crystal structure of the related NDO, it was possible to model the substrate pocket and the active site of TecA (Fig. 5A). This model gave information about the docking of the chlorinated toluenes and the mechanism of action.

As described for the NDO (Carredano et al., 2000), the surface of the substrate pocket of TecA is mainly hydrophobic with the exception of the iron centre and a polar region located on the bottom of the pocket. On one side of the iron, an elongation of the cavity is formed by the side chains from several residues, among them Phe366 (Fig. 5A). The distribution of products and product yields indicate a preferred orientation of substrates such that at least one chloride points preferentially not towards Phe366 but...
**Fig. 4.** Products and relative product distributions (%) formed by wild-type and mutant TecA enzymes with different chlorinated toluenes as substrates. (A) 2-Chlorotoluene; (B) 3-chlorotoluene; (C) 2,4-dichlorotoluene; (D) 2,5-dichlorotoluene; (E) 2,6-dichlorotoluene; (F) 2,4,5-trichlorotoluene. The data shown are means from three independent experiments ± SD.
Table 2. Rates of transformation of chlorinated toluenes catalysed by wild-type and mutant TecA enzymes

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Product formation (μM min⁻¹)†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>TecA†</td>
</tr>
<tr>
<td>2-CT</td>
<td>5·5 (100)</td>
</tr>
<tr>
<td>3-CT</td>
<td>7·4 (100)</td>
</tr>
<tr>
<td>4-CT</td>
<td>5·7 (100)</td>
</tr>
<tr>
<td>2,3-DCT</td>
<td>0·6 (100)</td>
</tr>
<tr>
<td>2,4-DCT</td>
<td>1·0 (100)</td>
</tr>
<tr>
<td>2,5-DCT</td>
<td>1·0 (100)</td>
</tr>
<tr>
<td>2,6-DCT</td>
<td>0·7 (100)</td>
</tr>
<tr>
<td>3,4-DCT</td>
<td>1·3 (100)</td>
</tr>
<tr>
<td>3,5-DCT</td>
<td>0·6 (100)</td>
</tr>
<tr>
<td>2,4,5-TCT</td>
<td>1·9 (100)</td>
</tr>
</tbody>
</table>

*CT, chlorotoluene; DCT, dichlorotoluene; TCT, trichlorotoluene.
†Numbers in parentheses refer to the transformation rate (%) relative to that of TecA.
‡The data for the DCTs and 2,4,5-TCT are from Pollmann et al. (2001). All rates were determined at a cell density producing an OD₆₀₀ value of 1·8.

towards the opposite side of this elongation and might interact with the side chains there. In this position, the space between substrate and iron is suitable for a dioxygen bridge, initializing the transformation of the substrate. The methyl groups of the substrates 2-, 3-chloro-, 2,3-, 2,6-, 3,5-dichloro- and 2,4,5-trichlorotoluene can be assumed to be orientated preferentially towards the elongation of the cavity, thus having suitable space for interaction with the side chain of Phe₃⁶⁶ (Fig. 5B, D). In this position the methyl group is close to the iron of the active site, favouring monooxygenolytic attack. In contrast, 4-chloro-, 2,4-dichloro-, 2,5-dichloro- and 3,4-dichlorotoluene are obviously in a reverse orientation that affords preferential dihydroxylation of the aromatic ring with the methyl group pointing towards the opposite side of the active site (Fig. 5C).

Effect of position 272

Substitutions of valine for Asn₂⁵₈ in 2NTDO have indicated that this residue controls regioselectivity of the enzyme. Therefore, it was thought that replacement of the corresponding Leu₂⁷₂ in TecA by larger hydrophobic amino acids such as phenylalanine and tryptophan would change the orientation of the aromatic ring of the chlorinated toluenes towards the active site, thus favouring dioxygenolytic attack. However, experiments showed only a slight effect on the regioselectivity for some substrates, although the mutants catalysed transformation of dichlorotoluenes more efficiently. The side chains Phe and Trp do not project into the hydrophobic cavity as expected (Fig. 5A) but seem to interact with adjacent side chains such that the methyl groups of the substrates still have enough space to orientate towards the iron of the active site. The increased activity of the mutants with various dichlorotoluenes, especially that of mutant L272W, can be explained by a more stable binding of these substrates or an improved orientation towards the active site facilitating the formation of a dioxygen bridge.

Relative transformation rates indicate a relatively faster transformation of dichlorotoluenes than the monochlorinated compounds compared to the wild-type enzyme. Thus, mutations at position 272 result in enzymes with an increased tendency to oxidize highly substituted toluenes, probably due to a better binding of the substrate into the active site.
Fig. 5. (A) Model of TecA indicating the potential influence of the mutations on the active site pocket. The substitutions in positions 272 and 366 are shown with grey bonds; the surface was calculated for the model of the wild-type enzyme in the absence of the Fe–O group. The substitutions in position 272, L272F and L272W, shown with rotamers similar to the wild-type enzyme without steric conflicts with neighbouring residues, barely touch the surface, indicating that these substitutions potentially have only a small influence on the shape of the substrate binding pocket. In contrast, substitutions in position 366, F366Y and F366W, have only rotamers not in steric conflict with neighbouring residues that penetrate the surface (red oxygen atom of the tyrosine and brown carbon atom of a tryptophan carbon atom), indicating that for these substitutions a potentially larger effect on the shape of the active site pocket can be expected. (B–F) Putative interaction of chlorinated toluenes in the active site of TecA. The preferential binding according to the determined product profiles of (B) 2,4,5-trichlorotoluene, (C) 2,5-dichlorotoluene, (D) 2,6-dichlorotoluene into the substrate pocket of wild-type TecA, and of (E) 2,4,5-trichlorotoluene, (F) 2,5-dichlorotoluene and (G) 2,6-dichlorotoluene into the active site of the F366L variant are shown. The orientation of the methyl group towards the iron (the ligation with two His residues and an oxygen is drawn) is preferred in case (A) and (C), forming an oxygen bridge with the iron, whereas 2,5-dichlorotoluene in the wild-type (B) is orientated towards the iron preferentially with its aromatic ring in the wild-type. In the mutant, the product profile indicates that shifts of the orientations occur (E–G). For 2,4,5-trichlorotoluene (E), only one of the multiple orientations is shown. In all cases, the ligand is shown in modelled orientations that should allow formation of the preferred product. The molecular surface was calculated with GRASP (Nicholls et al., 1991). Carbon atoms are coloured light-brown, nitrogen atoms are blue, oxygen atoms are red, hydrogen atoms are white and chlorine atoms are magenta. [Prepared with MOLSCRIPT (Kraulis, 1991) and rendered with the Persistence of Vision Raytrace (POV-Ray) (http://www.povray.org)].
Rational engineering of TecA

Recently, a number of strategies have been used to expand the substrate spectra of a variety of dioxygenases. Mutants of toluene dioxygenase were generated by random and saturation mutagenesis that exhibited higher activity towards toluene and 4-picoline (Sakamoto et al., 2001). Mutants of biphenyl dioxygenases with enhanced substrate specificity and changed regioselectivity were obtained by random and site-directed mutagenesis and gene shuffling (Brühlmann & Chen, 1999; Erickson & Mondello, 1993; Suenga et al., 2001). In this study, rational design was used in order to change the product spectra of TecA. For NDO, several amino acids of the active site were identified, out of which F352 was shown to control regioselectivity (Parales et al., 2000a). Based on the crystal structure of NDO (Carredano et al., 2000) and alignments, we could reconstruct the substrate pocket of TecA. With this information it was possible to identify amino acids that may contribute to regioselectivity and activity. Among these, the residue Phe_{366} was shown to control regioselectivity. However, mutations at Phe_{366} resulted in a loss or decrease of activity. In contrast, changes at Leu_{372} resulted in increased efficiency but only slight changes of regioselectivity. Even when much information is known about an enzyme, it is difficult to predict the effects of substitutions. Additionally, the limited range of possible substitutions hinders the rational design of improved enzymes. Thus, many attempts to alter specifically the properties of enzymes by rational design of new enzymes have failed because small changes in amino acid composition had unexpected effects on the structure and function of the target enzyme (Shao & Arnold, 1996).

Under the assumption that a chlorine of the chlorinated toluenes interacts with side chains of residues on the opposite site of the F366 pocket, mutations in this region to be responsible for dechlorination of 1,2,4,5-tetrachlorobenzene and thus enabling Ralstonia sp. PS12 to mineralize this substrate. However, further investigations are necessary.

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REFERENCES


Engineering of tetrachlorobenzene dioxygenase


