Conversion of 3-Chlorocatechol by Various Catechol 2,3-Dioxygenases and Sequence Analysis of the Chlorocatechol Dioxygenase Region of *Pseudomonas putida* GJ31

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*Pseudomonas putida* GJ31 contains an unusual catechol 2,3-dioxygenase that converts 3-chlorocatechol and 3-methylcatechol, which enables the organism to use both chloroaromatics and methylaromatics for growth. A 3.1-kb region of genomic DNA of strain GJ31 containing the gene for this chlorocatechol 2,3-dioxygenase (*cbzE*) was cloned and sequenced. The *cbzE* gene appeared to be plasmid localized and was found in a region that also harbors genes encoding a transposase, a ferredoxin that was homologous to XyIT, an open reading frame similar to a protein of a meta-cleavage pathway with unknown function, and a 2-hydroxymuconic semialdehyde dehydrogenase. *CbzE* was most similar to catechol 2,3-dioxygenases of the 2C subfamily of type 1 extradiol dioxygenases (L. D. Eltis and J. T. Bolin, J. Bacteriol. 178:5930–5937, 1996). The substrate range and turnover capacity with 3-chlorocatechol were determined for *CbzE* and four related catechol 2,3-dioxygenases. The results showed that *CbzE* was the only enzyme that could productively convert 3-chlorocatechol. Besides, *CbzE* was less susceptible to inactivation by methylated catechols. Hybrid enzymes that were made of *CbzE* and the catechol 2,3-dioxygenase of *P. putida* UCC2 (TdnC) showed that the resistance of *CbzE* to suicide inactivation and its substrate specificity were mainly determined by the C-terminal region of the protein.

Microbial degradation of most chlorinated aromatics occurs via chlorocatechols as intermediates. These chlorocatechols are usually further degraded via a modified ortho-cleavage pathway. This pathway involves an intradiol dioxygenase which cleaves the aromatic ring at the ortho position. Dechlorination occurs further along the pathway (43).

An alternative route for the degradation of catechol derivatives is the so-called meta-cleavage pathway. This pathway is mostly involved in the degradation of methylated aromatics. It involves an extradiol dioxygenase that cleaves the catechol ring at the 2,3 position, yielding a 2-hydroxymuconic semialdehyde derivative (47). Many extradiol dioxygenases have been cloned and sequenced, and the crystal structures of two of them were determined (11, 44). These and most other extradiol dioxygenases have a two-domain structure, in which the C-terminal domain contains the active site. The enzymes usually require Fe(II) as a cofactor (9).

When extradiol dioxygenases are confronted with 3-chlorocatechol (3CC), they usually become inactivated (e.g., see references 2, 5, 10, 13, 15, and 22). This inactivation might be caused by the strong chelating activity of 3CC, which removes the Fe(II) cofactor of the enzyme (22), or by suicide inactivation of the enzyme due to the formation of a reactive intermediate or product, such as an acetylchloride (5). Inactivation of the catechol 2,3-dioxygenases results in a low turnover capacity of the enzyme with 3CC that is insufficient for growth (50).

It was assumed for a long time that it was impossible to metabolize 3-chlorinated catechols via the meta-cleavage pathway, because they would inactivate the extradiol dioxygenase. However, we recently described a *Pseudomonas putida* strain that does degrade chlorobenzene via 3CC by this pathway (19, 25). The strain contains a novel chlorocatechol 2,3-dioxygenase (*CbzE*) that can efficiently cleave 3CC at the 2,3 position, leading to simultaneous ring cleavage and dechlorination. In this paper we describe the cloning and sequence of the *cbzE* gene. Furthermore, we determined the substrate ranges and characteristics of 3CC conversion of several catechol 2,3-dioxygenases and of constructed hybrid enzymes.

**MATERIALS AND METHODS**

Bacterial strains and plasmids. *P. putida* GJ31 was isolated on chlorobenzene; its characteristics were described previously (25). *Burkholderia cepacia* G4 (32) contains a catechol 2,3-dioxygenase (TomB) that is involved in the conversion of catechol and 3-methylcatechol (3MC) (36, 45). *Escherichia coli* NM522 (pWW15.3201) contains a 2.1-kb EcoRI fragment carrying the gene for C23OII of *P. putida* MT15 (20) in pUC18 and was a gift from P. A. Williams (University of Wales, Bangor, Wales, United Kingdom). Plasmid pTDN1-1018 contains a 2.1-kb SmaI-HindIII fragment from *P. putida* UCC2 in pHG327 on which *tdnC* is localized (39) and was a gift from N. C. McClure (Flinders University of South Australia, Adelaide, Australia). pAW31 was derived from pEMBL9 and contains *xylTE* of *P.putida* mt-2 (3, 7).

* E. coli JM101 (53) was used for cloning and construction of hybrid dioxygenases, and *E. coli* BL21(DE3) (48) was used to express catechol 2,3-dioxygenases that were cloned behind a T7 promoter. pBluescript SK(+) (Stratagene, La Jolla, Calif.) was used as a cloning vector, and pGEM(+) (made from pGELAF(+) by deleting blpl) (42)] was used to make translational fusions of (hybrid) catechol 2,3-dioxygenase genes behind a T7 promoter.

DNA isolation and hybridization and cloning of the chlorocatechol 2,3-dioxygenase gene. Total DNA from *P. putida* GJ31 was isolated by the method of Ausubel et al. (4). Plasmid DNA was isolated from *P. putida* GJ31 by a modified method of Kado and Liu (18) as described by Du et al. (8). Southern hybridization and chemiluminescent detection of plasmid DNA or genomic DNA that was digested with *PstI* were done with, as the probe, a digoxigenin-labeled DNA fragment that was generated by PCR according to the instructions of the manufacturer of the kit (Boehringer, Mannheim, Germany). This fragment was obtained by amplification of a DNA segment of *P. putida* GJ31 with degenerated primers that were designed against the N-terminal amino acid sequence (SIM

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the experiments, the crude catechol 2,3-dioxygenase activities in crude cell extract were measured spectrophotometrically as described previously (25) with different catechols at 0.1 mM. The conversion of catechol, 3MC, 4MC, 4-me-hydroxyacetophenone, and 2-hydroxymuconic semialdehyde derivative. The concentration of 3CC was measured over time by reversed-phase high-performance liquid chromatography, with an absorbance of 280 nm, respectively, with extinction coefficients of 36,000, 16,800, 31,500, and 15,000, respectively (40, 41). The 3CC depletion curves by fitting to the equation with the program Scientist for Windows 2.0 (Micromath Scientific Software, Salt Lake City, Utah). The turnover capacity was calculated by 

\[
V_{\text{max}} = \frac{S_{p}}{S_{i} + S_{p}K_{m}} = \frac{V_{\text{cat}}}{K_{m}}
\]

The concentration of 3CC was converted to molar concentration, taking the amount of 3CC that can be converted per amount of enzyme (nanomoles per unit of activity with catechol). This was measured by adding a defined amount of crude cell extract to an assay mixture, which contained 115 to 140 μM 3CC in phosphate buffer (25), unless indicated otherwise. The turnover capacity with 3CC was measured by reverse phase performance liquid chromatography. For this, a Merck-Hitachi L-6200 pump (Merck-Hitachi, Darmstadt, Germany) was used that was connected to a LiChrosorb RP18 column (Chrompack, Bergen op Zoom, The Netherlands). The samples were injected directly from the incubation mixture with a Marathons Basic autosampler (Spark-Holland, Emmen, The Netherlands) and eluted with water containing 25% (vol/vol) acetonitrile and 0.2% (wt/vol) H3PO4. 3CC was detected at 210 nm with a Merck-Hitachi L-4200 UV-visible light detector, while acquisition of the data was done with PC integration package software (Kontron Instruments, Milan, Italy).

For most enzymes, the catechol 2,3-dioxygenase was rapidly inactivated within the first one to two minutes of the incubations, and substrate-independent loss of enzyme activity was negligible during this short period. For these catechols, 2,3-dioxygenases, the number of data points that could be obtained was insufficient to be fitted to a mathematical description of the depletion of 3CC, and the turnover capacity with 3CC was calculated by dividing the amount of 3CC that was converted by the amount of enzyme that was added via the crude cell extract. The latter value was determined as the amount of activity with catechol that was present in the extract.

When the conversion of 3CC continued for a longer period, such as with CbzE and TdnC, while they remain visible in the protein profiles obtained with whole cells. Insoluble protein aggregates (inclusion bodies) are absent from crude cell extracts because they are removed by centrifugation (30 min, 10,000 × g). A fresh Fe(II) (NH4)2SO4 solution was added to a concentration of 0.1 mM to ensure high fidelity of the PCR product. The Expand High Fidelity PCR system was used according to the manufacturer (Boehringer, Mannheim, Germany). For the construction of the C-terminal primers that encoded the desired genes were transformed to E. coli BL21(DE3) and E. coli JM101 cells. Transformants were plated on Luria agar plates containing 100 μg of ampicillin ml−1. The spontaneous loss of enzyme activity was described with the equation

\[
V_{\text{cat}} = \frac{S_{p}}{S_{i} + S_{p}K_{m}}
\]

The concentration of 3CC at time and zero (minutes), respectively; 

\[
S_{p} = \frac{V_{\text{cat}}}{K_{m}} - \frac{V_{\text{cat}}S_{i}}{S_{p}K_{m}}
\]

For these catechol 2,3-dioxygenases (19). This sequence was used together with a conserved C-terminal sequence (YFFDP) from homologous catechol 2,3-dioxygenases to determine the rate constant for substrate-independent inactivation, an amount of extract that was equal to the amount used in the turnover measurements was incubated in phosphate buffer without 3CC. The remaining catechol 2,3-dioxygenase activity was measured over time with catechol, and k was obtained from the slope of the logarithmic plot of the catechol 2,3-dioxygenase activity versus time.

The values for V_{\text{cat}} and p were estimated from the 3CC depletion curves by fitting to the equation with the program Scientist for Windows 2.0 (Micromath Scientific Software, Salt Lake City, Utah). The turnover capacity was calculated by using an inactivated enzyme instead of a serum. The NeoBam sites were cloned behind the T7 promoter of pGEF+.

Protein expression and construction of hybrid catechol 2,3-dioxygenases. The genes encoding CbzE and TdnC were amplified with PCR by using primers against the start and end of the genes. The N-terminal primers contained an NcoI site (P1, 5′-GCGGTCGCTAGCTCGTTACGATGCGAGTTGGC-3′ for cbzE; 5′-GCGGTCGCTAGCTCGTTACGATGCGAGTTGGC-3′ for tdnC). The C-terminal primers contained a BamHI site (P3, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′ for cbzE; P4, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′ for tdnC). BamHI sites underlined, stop codons shown in bold). To ensure high fidelity of the PCR product, Pso DNA polymerase or the Expand High Fidelity PCR system was used according to instructions of the manufacturer (Boehringer, Mannheim, Germany). Due to the introduction of an NcoI site in cbzE, the second codon of cbzE was changed to encode a glycine instead of a serine. The NeoBam sites were cloned between the NcoI and BamHI sites of pGEF+.

Protein expression was done in E. coli BL21(DE3). For this, constructs of pGEF+ containing the desired genes were transformed to E. coli BL21(DE3) and E. coli JM101 cells. Transformants were plated on Luria agar plates containing 100 μl of LB-Amp agar plates at 30°C. The next day, the transformants were collected from the plates and used to inoculate 100 ml of LB-Amp. The cultures were grown at 15°C for 2 days, after which the cells were harvested and used to prepare crude cell extracts as described above. The amounts of soluble and insoluble catechol 2,3-dioxygenases that were produced by E. coli BL21(DE3) were estimated by comparing the protein profile of crude cell extract with the protein profile of whole cells on a 12.5% polyacrylamide gel containing sodium dodecyl sulfate (SDS) as described by Schanstra et al. (42). Insoluble protein aggregates (inclusion bodies) are absent from crude cell extracts because they are removed by centrifugation (30 min, 10,000 × g, 4°C), while they remain visible in the protein profiles obtained with whole cells. Hybrid catechol 2,3-dioxygenases were made of CbzE and TdnC by using PCR fusion reactions (54). For this, fragments of cbzE and tdnC were amplified with PCR by using primers that generated overlapping regions. The fragments were fused together by PCR using P1 or P2 as the forward primer and P3 or P4 as the reverse primer. The sequences of the other oligonucleotides were as follows: P14, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′; P12, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′; P10, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′; P11, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′; P9, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′; P8, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′; P7, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′; P5, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′; P3, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′; P1, 5′-GACGCTGCGACGTTCTATGTTACATCAC-3′. The nucleotides of the primers that are complementary to tdnC are shown in italics, and the nucleotides that are complementary to cbzE are underlined. This primer set was used to amplify the target genes. The PCR fusion products were cloned behind the T7 promoter of pGEF+, and the hybrid enzymes were expressed in E. coli BL21(DE3). The hybrid genes that were made with PCR fusions were checked with restriction analyses and cycle sequencing.

Nucleotide sequence accession number. The sequence obtained in this study has been submitted to GenBank and is available under accession no. AF109307.

RESULTS AND DISCUSSION

Cloning and sequencing of a genomic DNA fragment encoding a chlorocatechol 2,3-dioxygenase. The N-terminal amino acid sequence of the chlorocatechol 2,3-dioxygenase (CbzE) of P. putida GJ31 was determined previously and showed homology with other catechol 2,3-dioxygenases (19). This sequence was used together with a conserved C-terminal sequence (YFFDP) from homologous catechol 2,3-dioxygenases to determine...
sign degenerate oligonucleotide primers. A 761-bp PCR product was obtained, the nucleotide sequence of which corresponded with the N-terminal amino acid sequence of CbzE. This PCR product was used as a probe in Southern hybridization experiments, and a 3.1-kb PstI fragment of genomic DNA of P. putida GJ31 was found to hybridize with the probe. This fragment was cloned in pBluescript SK+ to give pBScbzE.

The nucleotide sequence of the 3.1-kb fragment was determined (GenBank accession no. AF109307). A schematic representation of the open reading frames (ORFs) that were identified is given in Fig. 1. The cbzE gene starts at position 1319 and encodes a polypeptide of 314 amino acids with a calculated molecular mass of 34,951 Da, which corresponds to the experimentally determined mass of 33.4 kDa of CbzE (19). The N-terminal amino acid sequence that was deduced from cbzE was identical to the N-terminal sequence of 43 amino acids that was determined with the purified CbzE protein (19), except for the last amino acid. In front of cbzE is a putative ribosome binding site (AGGAG).

Downstream of cbzE, a fragment that encodes an ORF of 154 amino acids was found, which was preceded by a Shine-Dalgarno-like sequence, GGA. This ORF (CbzX) was 41% identical to CmpX of Sphingomonas sp. strain HV3 (GenBank accession no. Z84817). The cmpX gene is part of an operon encoding a meta-cleavage pathway for catechol. Its function is unknown (55).

Further downstream, the beginning of an ORF (CbzG) was found that is similar to many 2-hydroxyxymonocemic semialdehyde dehydrogenases, of which DmpC of P. putida CF600 (GenBank accession no. P19059) (35) was one of the most similar enzymes, having 56% identity with CbzG. These enzymes are responsible for oxidation of the carbonyl group that is present in the meta-cleavage products that are formed when catechols are converted by catechol 2,3-dioxygenases. CbzG most likely has the same function. The corresponding genes are found in operons that encode meta-cleavage pathways (e.g., see references 23, 52, and 55). The cbzG gene continues until the PstI cloning site and is preceded by a putative ribosome binding site (GAGAG) (Fig. 1).

Upstream of cbzE, an ORF of 119 amino acids is localized (CbzT), which is also behind a putative ribosome binding site (AAGG) (Fig. 1). The derived amino acid sequence shows up to 40% identity with chloroplast-type ferredoxins that are known to play a role in maintaining the iron ion of catechol 2,3-dioxygenases in the reduced (Fe^{2+}) state (16, 37). The genes that encode these ferredoxins are found in front of the genes that encode catechol 2,3-dioxygenases (e.g., see references 12, 23, and 52), and cbzT probably encodes such a protein as well.

At the beginning of the cloned fragment is a segment of an ORF (ORF1) of 126 amino acids which has 61% identity with the C-terminal part of a transposase of Pseudomonas pseudoalcaligenes JS45 (GenBank accession no. AF028594) (unpublished data). This strain grows on nitrobenzene and uses a meta-cleavage dioxygenase for the conversion of the intermediate 2-aminophenol to 2-aminoacemic acid semialdehyde (24). ORF1 continues until the PstI cloning site. Presumably, the complete transposase gene is present in P. putida GJ31, but only the last part was cloned.

In between ORF1 and cbzT are two (incomplete) ORFs that are homologous to components of toluene and phenol monooxygenases. In front of cbzT is a fragment that starts at position 720 and encodes a sequence (ORF3) of 91 amino acids which is up to 50% identical to the C-terminal part of reductase components of these monooxygenases like TbmF of Pseudomonas sp. strain JS150 (GenBank accession no. L40033) (17). TbmF itself consists of 355 amino acids. This suggests that the N-terminal part of this gene was lost in P. putida GJ31, also, because no suitable start codon or putative ribosome binding site was found. Immediately upstream of ORF3 is an ORF (ORF2) the C-terminal part of which resembles another component of toluene and phenol monooxygenases. TbmA of the toluene/benzene-2-monooxygenase of Pseudomonas sp. strain JS150 (GenBank accession no. L40033) (17) is most similar to this part of ORF2, and it has a region of 23 amino acids (Pro-43 to Val-65) that contains 16 amino acids that are identical to the corresponding region in ORF2. The region between Pro-39 and Val-61 of DmpK of the phenol monooxygenase complex of Pseudomonas sp. strain CF600 (GenBank accession no. M37764) (34) is 57% identical to this region in ORF2. The ORF continues into the region that encodes ORF1. The N-terminal part of ORF2 lacks any homology with TbmA or with any other protein in the database, and it is unclear whether there is a real start codon for ORF2. A GTG is present at position 385, which is immediately behind the stop codon of ORF1 at position 381, but no putative ribosome binding site was found. Alternatively, an ATG is present at position 328, which is located inside the fragment that encodes ORF1.

The components of the phenol and toluene monooxygenases are normally encoded by six genes that are located in an operon the last gene of which encodes the reductase component. In front of this reductase gene are the four genes that encode the three subunits of the hydroxylase component, and a small subunit that increases the oxidation rate of the aromatic substrate in vitro. The first gene of these operons encodes the TbmA-like component, to which the C-terminal end of ORF2 has similarity. The function of this component is unknown (17, 33, 34). The presence of two ORFs (ORF2 and ORF3) that are similar to parts of TbmA and TbmF, which are encoded by the first and the last gene of the toluene monooxygenase operon in Pseudomonas sp. strain JS150 (17), suggests that recombinations have occurred in this region of the genome of P. putida GJ31. Strain GJ31 presumably does not contain an intact tolouene monooxygenase since it most likely uses a dioxygenase for growth on aromatic compounds (25).

The results show that the cbzTEXG genes are clustered. The genes that encode the enzymes of meta-cleavage pathways are usually located in operons (e.g., see references 38 and 52), and
the other enzymes that belong to the meta-cleavage pathway of \textit{P. putida} GJ31 might also be located near \textit{cbzTEXG}, but they were not cloned with the 3.1-kb fragment. The presence of a part of a transposase-like ORF (ORF1) on the border of the cloned fragment could indicate that the meta-cleavage pathway genes of \textit{P. putida} GJ31 are located on some kind of transposable element, which has been observed with other meta-cleavage pathway operons as well (52).

\textbf{Plasmid localization of \textit{cbzE}}. Plasmid isolations revealed that \textit{P. putida} GJ31 contains a large plasmid which hybridized with the probe against \textit{cbzE}. When \textit{P. putida} GJ31 was grown on benzoate, mutants arose that could no longer grow on chlorobenzene and toluene but that were still able to grow on benzoate and benzene by an ortho-cleavage pathway (25). The plasmid of the mutant (\textit{P. putida} GJ31M1) was slightly smaller than the wild-type plasmid and no longer hybridized with the probe against \textit{cbzE} (results not shown). These observations indicate that the meta-cleavage pathway genes are plasmid encoded.

\textbf{Similarity of \textit{cbzE} sequence to those of other catechol 2,3-dioxygenases}. ClustalW amino acid sequence alignments showed that \textit{cbzE} was most similar to the two-domain, iron-containing extradiol dioxygenases that cleave monocyclic diols (9). On the basis of phylogenetic analysis, Eltis and Bolin (9) proposed that this family consisted of five subfamilies, and \textit{cbzE} was most homologous to the members of subfamily C.

The identity of \textit{cbzE} with the other members of the subfamily ranged from 72\% for TdnC of \textit{P. putida} UCC2 (GenBank accession no. X59790) (unpublished data) and a catechol 2,3-dioxygenase of \textit{B. cepacia} AA1 (GenBank accession no. U47111) (unpublished data) to 51\% for \textit{TomB} of \textit{Ralstonia pickettii} PKO1 (GenBank accession no. U20258) (23).

The characterization of most extradiol dioxygenases was not sufficient to attribute any biochemical or functional properties to the various subfamilies (9), although it was suggested that subfamily C might represent a group that has an increased affinity for catechol and molecular oxygen (9, 23). However, this now seems unlikely since the recent determination of the sequence of the catechol 2,3-dioxygenase gene of \textit{B. cepacia} G4 (\textit{tomB}) (36) revealed that the \textit{TomB} enzyme also belongs to the 1.2.C subfamily, while previous measurements showed that this enzyme does not have increased affinities for oxygen and catechols (23).

An alignment of \textit{cbzE} with the other members of subfamily C, as well as \textit{XylE} (GenBank accession no. V01161) (31) and \textit{BphC} (GenBank accession no. X66122) (14), is given in Fig. 2. \textit{XylE} and \textit{BphC} are 38 and 15\% identical to \textit{cbzE}, respectively. The crystal structure of the latter enzyme is known (11), and the alignment of Fig. 2 is based on the structure-validated alignment that was made by Eltis and Bolin (9). The histidine and glutamate residues that are known to be involved in the binding of Fe$$^{2+}$$ in extradiol dioxygenases are strictly conserved in all of the enzymes.

\textbf{Substrate range of several catechol 2,3-dioxygenases}. \textit{CbzE} is the only catechol 2,3-dioxygenase described so far that is able to productively cleave 3CC at the 2,3 position. However, none of the enzymes that belong to the same subfamily as \textit{CbzE} has been tested for the ability to convert 3CC. To find out whether this capacity was restricted to \textit{CbzE} or whether it was a feature which was shared with the other members of the 1.2.C subfamily, we determined the substrate ranges of some other members of this group. For this, the initial rates of product formation were measured with various catechols for each catechol 2,3-dioxygenase by using cell extracts. \textit{CbzE} and \textit{XylE} were included as enzymes with good and very poor conversion of 3CC, respectively.

Large differences existed in the relative initial activities of the various enzymes (Table 1). TdnC and C23OII had the highest activities with 3MC, which corresponds with the data of McClure and Venables (27) and Keil et al. (20). \textit{TomB} and \textit{XylE} preferred unsubstituted catechol, although both enzymes occur in pathways that have methylcatechols as intermediates. Only \textit{CbzE} was able to convert 3CC at a high relative rate and could sustain the conversion of 3CC much longer than the other enzymes. The relative activities of the recombinant enzyme correspond rather well with the relative activities that were measured with \textit{CbzE} that was purified from \textit{P. putida} GJ31 (19). Significant conversion of 3CC with C23OII and \textit{XylE} was observed only when much higher amounts of enzyme were added. TdnC, \textit{TomB}, and C23OII became completely inactivated within 20 s, while \textit{XylE} was inactivated more slowly than the other catechol 2,3-dioxygenases of the 1.2.C subfamily (Fig. 3). The plots of the amounts of product formed in time yielded straight lines when catechol, 3MC, 4MC, or 4CC was converted by \textit{CbzE}, while a gradual decrease in the product formation rate was observed with the other enzymes (Fig. 4).

The measurements were done at catechol concentrations (100 \textmu M) well above the Michaelis-Menten constant (\textit{K}_m) of the catechol 2,3-dioxygenases since the \textit{K}_m values are generally below 10 \textmu M (6, 19, 23, 49). This means that the decrease in the product formation rate cannot be caused by a reduction of the substrate concentration during the assays but is due to some inactivation. Harayama and coworkers already observed that \textit{XylE} was susceptible to inactivation during conversion of 3MC and 4MC (6, 37), which most likely results from the oxidation of the Fe$$^{2+}$$ cofactor (16, 37). Since this kind of inactivation was not observed for \textit{CbzE}, this indicates that this enzyme not only is most resistant to inactivation by 3CC but also is able to resist inactivation by other catechols.

\textbf{Turnover capacity with 3CC of several catechol 2,3-dioxygenases}. The amounts of 3CC that can be converted by the enzymes were measured (Table 1). The turnover capacity with 3CC of \textit{CbzE} was as much as two orders of magnitude higher than the turnover capacities of TdnC, \textit{TomB}, C23OII, and \textit{XylE}, which shows that \textit{CbzE} is indeed very resistant to inactivation during conversion of 3CC. During these measurements the conversion of 3CC by \textit{TomB}, TdnC, and C23OII stopped within 1 min after the enzyme and substrate were mixed. For \textit{XylE}, it took approximately 20 min before no further conversion took place (Fig. 5). The turnover capacity of \textit{XylE} with 3CC was comparable to those with the other three enzymes. This means that \textit{XylE}, which belongs to the 2.A subfamily of class 1 extradiol dioxygenases (9), converted 3CC at a much lower rate than the enzymes of the 2.C subfamily. Active \textit{XylE} consists of four identical subunits, and the tetramer has a \textit{K}_cat of about 45,000 min$$^{-1}$$ with catechol (30). With the turnover capacity of \textit{XylE} of 64 nmol of 3CC per unit of activity with catechol (Table 1), this means that about 2,900 molecules of 3CC were converted per molecule of \textit{XylE} (tetramer) in the crude cell extract. This value is in the same order of magnitude as the turnover of 1,000 that was previously determined for \textit{XylE} by using oxygen depletion measurements with 3CC (50). For \textit{CbzE} it took about 30 min before the enzyme stopped converting 3CC (Fig. 5).

Both \textit{CbzE} and \textit{XylE} were inactivated when they were incubated in phosphate buffer without 3CC. This was probably due to oxidation of the ferrous iron cofactor during the incubation. Because of this, the activities of extracts with \textit{CbzE} and \textit{XylE} were monitored over time when they were incubated in phosphate buffer in the absence of 3CC. A first-order decrease in the activities of the enzymes was observed in both cases with inactivation constants (\textit{k}) of 0.028 min$$^{-1}$$ ($r^2 = 0.93$) and 0.0098
Because of this, part of the inactivation that was observed during 3CC conversion by both enzymes cannot be attributed to 3CC. The reciprocal of the coefficient \( p \), which describes the inactivation of the catechol 2,3-dioxygenase due to conversion of 3CC, would give the turnover capacity of the enzymes when aspecific inactivation is absent. The value of \( p \) is 0.033 U/mmol for CbzE and 15.1 U/mmol for XylE, which means that the turnover capacity of CbzE and XylE is 2100 and 220 nmol 3CC/unit of catechol 2,3-dioxygenase activity with catechol, respectively. Because of this, the part of the inactivation that was observed during 3CC conversion by both enzymes cannot be attributed to 3CC. The reciprocal of the coefficient \( p \), which describes the inactivation of the catechol 2,3-dioxygenase due to conversion of 3CC, would give the turnover capacity of the enzymes when aspecific inactivation is absent. The value of \( p \) is 0.033 U/mmol for CbzE and 15.1 U/mmol for XylE, which means that the turnover capacity of CbzE and XylE is 2100 and 220 nmol 3CC/unit of catechol 2,3-dioxygenase activity with catechol.

\[ \text{Activity with catechol} = \frac{1}{p} \]

\[ \text{Relative activity} = \frac{1}{p} \]

\[ \text{Turnover capacity} = \frac{1}{p} \]

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capacities would be 44 and 3% higher without aspecific enzyme inactivation for CbzE and XylE, respectively.

Expression of CbzE and TdnC in pGEF. Both cbzE and tdnC were cloned behind the T7 promoter of pGEF, to give pGFcbzE and pGFtdnC, respectively. The plasmid was transformed into E. coli BL21(DE3), which carries the T7 RNA polymerase gene behind the lac promoter which can be induced with IPTG. E. coli BL21(DE3) also expresses some T7 RNA polymerase without IPTG (48). When the lac promoter was induced with IPTG, a severe formation of inclusion bodies of CbzE and TdnC was observed when whole cells were analyzed on SDS-polyacrylamide gels. This did not occur in uninduced cells. Therefore, cells were grown without IPTG, and this resulted in expression levels that were comparable to that observed with pBScbzE in E. coli JM101 (Tables 1 and 2).

Upon cloning, the second amino acid of cbzE was changed from a serine to a glycine residue. The substrate range and turnover capacity with 3CC of CbzE that was expressed from pGFcbzE were somewhat different from the substrate range and turnover capacity of CbzE that was expressed from pBScbzE (Tables 1 and 2). When the turnover measurements were repeated with a lower initial concentration of 3CC (70 μM), the turnover capacity increased more than twice to 18,000 nmol/U, which indicates that the enzyme is very sensitive to high concentrations of 3CC. The increase in turnover capacity was approximately one-third for CbzE that was expressed from pBScbzE, which is much less. Also, the enzyme that was expressed from pBScbzE, which is much less. Also, the enzyme that was expressed from pBScbzE, which is much less. Also, the enzyme that was expressed from pBScbzE, which is much less. Also, the enzyme that was expressed from pBScbzE, which is much less. Also, the enzyme that was expressed from pBScbzE.
pressed from pGFcbzE was less stable in phosphate buffer. The first-order inactivation constants were 0.058 min$^{-1}$ ($r = 0.99$) and 0.022 min$^{-1}$ ($r = 0.93$) for CbzE expressed from pGFcbzE and pBScbzE, respectively (results not shown). Besides the Ser-2-to-Gly-2 mutation, the absence of CbzT in extracts from *E. coli* BL21(DE3)(pGFcbzE) could be responsible for this difference, since this protein is similar to ferredoxins that are involved in reactivation of catechol 2,3-dioxygenases (16, 37). Nevertheless, CbzE expressed from pGFcbzE had a turnover capacity with 3CC that was at least 35-fold larger than the turnover capacities of other catechol 2,3-dioxygenases, showing that the overexpressed enzyme is highly resistant to 3CC and that the resistance is due not only to CbzT or Gly-2. The substrate range of TdnC that was expressed from pGFtdnC and pBScbzE determined the observed differences between both enzymes. This was done by exchanging smaller parts of the C-terminal domain, as is depicted in Fig. 6.

The hybrids H3 and H4, in which the exchanges were made in the beginning of the C-terminal domains, were normally active, although a large part of H3 that was produced was insoluble (Table 2). Compared with TdnC, the replacement of amino acids 148 to 189 of TdnC with amino acids of CbzE (H4) resulted in the loss of the substrate preference for 3MC. This hybrid was even worsened in its capacity to convert 3CC, although it obtained a part of CbzE. The complementary hybrid protein (H3), in which the corresponding fragment of CbzE was replaced by that of TdnC, did not have an improved relative rate for 3MC, while the rate by which 3CC was converted was reduced. Thus, the exchange of these peptide segments influenced the substrate specificity of the enzymes in such a way that both hybrid enzymes lost their preference for substituted catechols. The amount of 3CC that could be converted by CbzE dropped significantly when amino acids 148 to 189 of CbzE were replaced by those of TdnC in H4, while the rate by which 3CC was converted was almost the same as that of TdnC, which indicates that the regions that were exchanged were not similar enough to yield properly folded hybrid enzymes. The active site of catechol 2,3-dioxygenases is located in that domain (9).

Attempts were made to elucidate which amino acids mainly influenced the substrate specificity of the enzymes in such a way that both hybrid enzymes lost their preference for substituted catechols. The amount of 3CC that could be converted by CbzE dropped significantly when amino acids 148 to 189 of CbzE were replaced by those of TdnC in H4, while the rate by which 3CC was converted was almost the same as that of TdnC, which indicates that the regions that were exchanged were not similar enough to yield properly folded hybrid enzymes. The active site of catechol 2,3-dioxygenases is located in that domain (9).

The turnover capacities with 3CC were determined by the C-terminal domains, as is depicted in Fig. 6. The hybrids H3 and H4, in which the exchanges were made in the beginning of the C-terminal domains, were normally active, although a large part of H3 that was produced was insoluble (Table 2). Compared with TdnC, the replacement of amino acids 148 to 189 of TdnC with amino acids of CbzE (H4) resulted in the loss of the substrate preference for 3MC. This hybrid was even worsened in its capacity to convert 3CC, although it obtained a part of CbzE. The complementary hybrid protein (H3), in which the corresponding fragment of CbzE was replaced by that of TdnC, did not have an improved relative rate for 3MC, while the rate by which 3CC was converted was reduced. Thus, the exchange of these peptide segments influenced the substrate specificity of the enzymes in such a way that both hybrid enzymes lost their preference for substituted catechols. The amount of 3CC that could be converted by CbzE dropped significantly when amino acids 148 to 189 of CbzE were replaced by those of TdnC in H4, while the rate by which 3CC was converted was almost the same as that of TdnC, which indicates that the regions that were exchanged were not similar enough to yield properly folded hybrid enzymes.

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for 3CC, although the conversion stopped within 30 s. The turnover capacity measurement also suggested that the ability to convert 3CC was improved for H8 as compared to the ability to convert TdnC. Besides, both hybrids seemed to have an improved relative rate with 4CC.

A mutant of XylE, in which a single amino acid in the last region of the C-terminal domain was substituted (Val-291 to Ile-291), had a fourfold increase in the turnover capacity with 3CC and other substituted catechols, whereas the affinity for 3CC was lowered (50). Like this mutant of XylE, CbzE has a valine at the corresponding position (Val-297) (Fig. 2), while all the other catechol 2,3-dioxygenases have the isoleucine at that place. The increased turnover capacity with 3CC of H8 compared with TdnC might be caused by a similar effect of this substitution, and this amino acid might play a role in the resistance of CbzE to inactivation with substituted catechols.

None of the hybrids H3, H4, H7, and H8 had an improved resistance to inactivation with the various catechols, since the plots of the formation of product in time were always curved (results not shown).

The properties of other hybrid catechol 2,3-dioxygenases have been studied by Cerdan et al. (6), who constructed several hybrids of XylE and NahH. They concluded that the amino acid at position 250 (His-250 in XylE, Gln-250 in NahH) mainly determined the sensitivity of the enzyme to inhibition with 3MC and the relative $k_{cat}$ with this substrate. However, the amino acids of the catechol 2,3-dioxygenases of the 1.2.C subfamily that correspond to His-250 in XylE are always arginines (Arg-256), which means that this arginine cannot be responsible for the improved resistance of CbzE to inactivation with the various catechols.

Hybrid catechol 2,3-dioxygenases were also made of XylE of the TOL plasmid and XylE of P. aeruginosa J1104 by using a SalI site that both corresponding genes had in common. Although the enzymes are 94% identical, the substrate range of the former is much more relaxed than that of the latter, which is highly specific for catechol (21). The results indicated that the last 43 C-terminal amino acids might be involved in the substrate specificity, since eight of the nine mutations that determined the substrate range of the hybrids are located in that region. The same strategy was used by Williams et al. (51), who made hybrids of two catechol 2,3-dioxygenases that were encoded by the TOL plasmid pWW53. The amino acid sequences of these enzymes are unknown, but these researchers also concluded that the C-terminal region determined the binding and catalytic specificity of the enzymes.

FIG. 6. Schematic representation of the construction of hybrid catechol 2,3-dioxygenases (H1 to H10). The primers (P1 to P14) that were used in PCRs to generate DNA fragments that contained overlapping regions for the PCR fusions are indicated with arrows. △, polypeptide fragments derived from CbzE; □, polypeptide fragments derived from TdnC. The amino acids of CbzE and TdnC between which the fusions were made are indicated.
for the resistance of the CbzE enzyme. For 3CC, irreversible suicide inactivation was observed with XyIE (5), while Klečka and Gibson (22) observed reversible inactivation of TodE of *P. putida* F1, which was probably due to the chelation of the Fe$^{3+}$ cofactor of TodE by 3CC. TodE is only distantly related to the catechol 2,3-dioxygenases used in this study (9), and its mechanism of inactivation seems to be different from that of XyIE (5, 22).

When XyIE was inactivated with 3CC, activity could not be restored by addition of Fe$^{3+}$ and dithiothreitol (5), which suggests that the mechanism of inactivation does not involve the oxidation state or the loss of the iron cofactor. Another possible cause of the observed inactivation is the nuclophilic attack of one or more groups in the enzyme on an electrophilic intermediate in the reaction cycle. Preliminary experiments with XyIE showed that the homodimeric protein band of native enzyme migrated slightly faster on SDS-polyacrylamide gels than that of enzyme that was inactivated with 3CC, provided that the samples were not boiled prior to application on the gel. Modification of XyIE by 3CC also made the enzyme less susceptible to digestion with proteinase LysC. Complete digestion of dialyzed, inactivated XyIE was possible only in the presence of 1 M urea, while native XyIE could be digested in the absence of urea (unpublished results). These results might indicate that inactivation by 3CC involves a labile covalent modification which influences the multimeric form of XyIE. The inactivation that was observed for XyIE and NahH with methylated catechols (6) was also related to conversion of the catechols and not to the catechols themselves since the inactivation constants were usually similar at 50 and 300 μM.

The increased resistance of CbzE to inactivation by both methyl- and chlorocatechols might suggest that the mechanisms of inactivation by both compounds are similar. However, Hugo et al. (16) showed that the conversion of 4MC by XyIE resulted in the oxidation of the catalytic iron. The oxidized iron (Fe$^{3+}$) can be reduced by XyIT, which resulted in a reactivation of the enzyme (16, 37). The inactivation of XyIE by methylated catechols thus seems to be a reversible process, in contrast to the inactivation observed with 3CC (5). However, the EPR signal of the oxidized iron in XyIE after inactivation by 4MC was clearly distinct from the signal of the catalytic iron that was aspecifically oxidized by H$_2$O$_2$ (16). The authors suggested that a putative ligand that resulted from the conversion of 4MC remained in the active site, which might even hinder complete reactivation by XyIT (16). Perhaps such a ligand is also formed during the conversion of 3CC, which might bind in an irreversible manner and prevent the regeneration of the iron cofactor. It should be noted that the resistance of CbzE to 3CC is not exclusively caused by a modified interaction with CbzT, since increased resistance was also found with enzyme that was expressed from *E. coli* in the absence of CbzT, while purified CbzE also efficiently converts 3CC (19).

EPR studies of XyIE that is inactivated with 3CC would provide more insight in the mechanism that causes the inactivation. This would also help us to understand the biochemical basis for the extraordinary resistance of CbzE to methyl- and chlorocatechols. If a specific group in the enzyme is attacked by the electrophiles that are formed during the reaction with 3CC, the resistance of CbzE to 3CC might result from a modification of this target. Alternatively, substitutions that lead to a more rapid conversion of the toxic intermediate could reduce the lifetime of the reactive electrophiles, which reduces the chance of damage of the enzyme and increases the resistance to inactivation.

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