

## Role of the Amino Acid Residues in the Catalysis of Catechol 2,3-dioxygenase from *Pseudomonas putida* SU10 as Probed by Chemical Modification and Random Mutagenesis

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The catechol 2,3-dioxygenase (C23O) encoded by the *Pseudomonas putida* *xylE* gene was over-produced in *Escherichia coli* and purified to homogeneity. The activity of the C23O required the reduced form of the Fe(II) ion since the enzyme was highly susceptible to inactivation with hydrogen peroxide but reactivated with the addition of ferrous sulfate in conjunction with ascorbic acid. The C23O activity was abolished by treatment with the chemical reagents, diethylpyrocarbonate (DEPC), tetranitromethane (TNM), and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMC), which are modifying reagents of histidine, tyrosine and glutamic acid, respectively. These results suggest that histidine, tyrosine and glutamic acid residues may be good active sites for the enzyme activity. These amino acid residues are conserved residues among several extradiol dioxygenases and have the chemical potential to serve as ligands for Fe(II) coordination. Analysis of random point mutants in the C23O gene derived by PCR technique revealed that the mutated positions of two mutants, T179S and S211R, were located near the conserved His165 and His217 residues, respectively. This finding indicates that these two positions, along with the conserved histidine residues, are specially effective regions for the enzyme function.

**Key words:** C23O, Fe(II), ascorbic acid, random mutagenesis, chemical modification, active site

Catechol 2,3-dioxygenase (C23O) allows a critical step to occur in biodegradation pathways of a large share of aromatic pollutants by catalyzing the extradiol opening of catechol rings and the insertion of molecular oxygen (19, 26). This enzyme is encoded by the TOL plasmid (pWWO) present in the bacterium *Pseudomonas putida* mt-2 (22). Each of the four identical subunits composing the enzyme contains one high spin iron(II) ion essential for activity (22). The Gram-positive bacterium *Rhodococcus rhodochrous* is able to degrade 3-methylalanine via this extradiol enzyme via the meta-cleavage pathway (7, 9). This enzyme also consists of four identical subunits, each of which is of MW 39,000 and contains Fe(II) (28). These extradiol-cleavage enzymes belong to one gene family, and all members of this gene family described up to now have been reported from different *Pseudomonas* strains (7, 16). The composition of amino acid sequences of isofunctional enzymes for naphthalene (2, 10, 12), PCB (15, 28, 31), and biphenyl (8) is of particular interest, because conserved amino acid residues may be important for

enzyme function (7).

The derived amino acid sequence of the C23O has been compared to those of nine other enzymes which catalyze the extradiol cleavage of aromatic rings (1, 7). Furthermore, the roles of four strongly conserved histidines in C23O have been examined by chemical modification of the histidyl residues of the native enzyme, and it has been suggested that the histidine residue lies at an active site (7).

An examination of the crystal structure of the BphC enzyme has shown that there is only one feasible entrance route leading to the active site of the enzyme. This path in the enzyme is surrounded by Ile<sup>174</sup>, Phe<sup>201</sup>, His<sup>208</sup>, Tyr<sup>249</sup> and Thr<sup>280</sup> (29). Considering the general requirement of a ferrous iron for the enzymatic activity of extradiol type dioxygenases (4, 14), the active site seems to be located inside the barrel structure of domain 2 (3, 29). A single Fe(II) ion in the active site seems to coordinate the side chains of three amino acid residues, His<sup>145</sup>, His<sup>209</sup> and Glu<sup>260</sup> (3, 29).

The structure of 2,3-dehydroxybiphenyl 1,2-dioxygenase from a PCB-degrading strain of *Pseudo-*

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*monas cepacia* has been determined at 1.9 angstrom resolution. The iron atom has five ligands in square pyramidal geometry: one glutamate and two histidine side chains, and two water molecules (11, 32). We have already cloned the *P. putida xylE* gene encoding the C23O which consists of 307 amino acids (16). In order to characterize the enzyme biochemically and to find the active site of the enzyme by chemical modification or by random mutagenesis using PCR, we first overexpressed the *xylE* gene in the *Escherichia coli* strain BL21 and purified the enzyme to homogeneity. Chemical reagents that modify conserved amino acid residues among extradiol-cleavage dioxygenases abolished the C23O activity. In this respect, the relationship between Fe(II) requirement and these residues is discussed with respect to the active site of the enzyme.

## Materials and Methods

### Recombinant plasmid construction

For the overexpression of the *xylE* gene in *Escherichia coli*, a 2 kb *XhoI-ScaI* fragment containing the *xylE* sequence of pTY1 (16) was subcloned into pET3a (16) at the *XbaI* site to construct pET-*xylE* as shown in Fig. 1. After transformation into *Escherichia coli* BL21 (DE3) pLysS with the resulting construction, ampicillin-resistant colonies were screened for the presence of C23O activity by spraying the plate with a solution of 5 mM catechol.

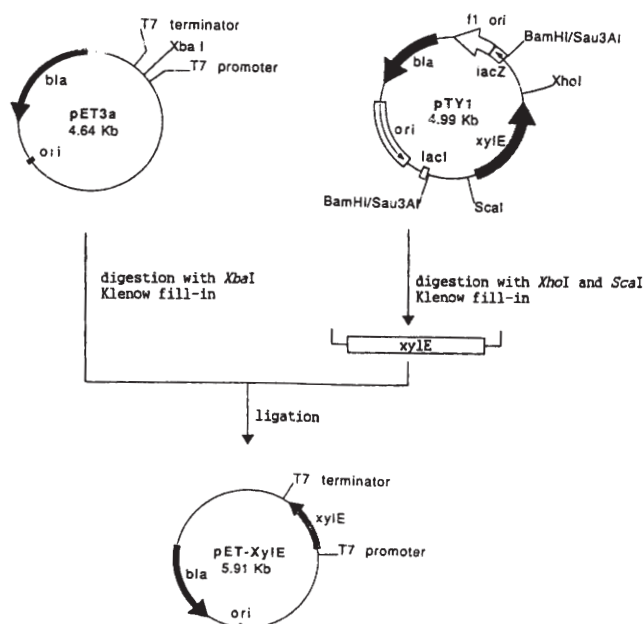


Fig. 1. Construction of recombinant plasmid pET-*xylE* for overexpression of catechol 2,3-dioxygenase.

### Inactivation and reactivation of C23O

Inactivation of C23O was carried out according to the method of Polissi and Harayama (25). The purified C23O was combined with 4 mM  $H_2O_2$  and incubated at 25°C for 20 minutes. Aliquots were then taken, and their C23O activities were determined in a 50 mM potassium phosphate buffer, pH 7.5. To reactivate the enzyme, 10 mM ascorbic acid and 2 mM  $FeSO_4$  were added to the purified enzyme or inactivated enzyme solution and incubated at 25°C for 20 minutes.

### Expression of the C23O in *E. coli*

The recombinant plasmid pET-*xylE* was transformed into *E. coli* BL21 (DE3) pLysS. The transformed cells were grown to a late exponential growth phase in TBGM9 medium (1% Tryptone, 0.5% NaCl, 0.4% glucose, 1 mM  $MgSO_4$  supplemented with  $1 \times M^9$  salts) containing 50 mM ampicillin at 30°C. At this time, 1 mM IPTG was added and the culture further incubated for 3 hours. The cells were harvested by centrifugation, washed with STE (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0), and resuspended in TA buffer (50 mM Tris-acetate containing 10% acetone, pH 7.5). The cell suspension was disrupted by ultra-sonication (Fisher sonic dismembrator, Model 300) and the soluble fraction was clarified by centrifugation.

### Purification of C23O

The enzyme overexpressed in *E. coli* was partially purified by acetone precipitation according to the procedure reported by Nakai *et al.* (3). The proteins present in the soluble fraction obtained during acetone precipitation were dialyzed overnight against TA buffer and then loaded onto a Q-Sepharose chromatography equilibrated with the TA buffer. The bound protein was eluted sequentially with TA buffer containing 0.2 M and 0.3 M NaCl, respectively. The activity of the C23O was determined in the latter eluents. The active fractions were pooled and protein concentration was determined by the method of Bradford (5) with bovine serum albumin as a standard. SDS-PAGE was performed on a 12% (w/v) polyacrylamide gel according to the method of Laemmli (18). Proteins on the gels were stained with 0.25% Coomassie Brilliant Blue R-250 for visualization of protein bands. The marker proteins used were bovine serum albumin (78 KDa), ovalbumin (47.1 KDa), carbonic anhydrase (31.4 KDa), soybean trypsin inhibitor (25.5 KDa), and lysozyme (18.8 KDa).

### Assay of the C23O activity

The activity of C23O was routinely measured by determining the absorbance increase at 375 nm

which monitors the formation of 2-hydroxymuconic semialdehyde at 25°C for 20 minutes. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.5, and 100 µl of 10 mM catechol in a 1 ml assay volume. The reaction was initiated by the addition of an appropriate amount of enzyme (23).

### Chemical modification of the C230 protein

Most of the procedures were carried out with slightly modified versions of the methods of Candidus *et al.* (7). For the modification of histidyl residues, diethylpyrocarbonate (DEPC) was freshly diluted in ice-cold ethanol prior to each experiment. The purified C230 was incubated with 0.2 mM or 0.4 mM DEPC in 100 mM potassium phosphate buffer, pH 6.0 at 25°C for 30 minutes. The modification was stopped by adding 10 mM imidazole (7). The progression of modification was monitored by measuring absorbance at 240 nm. For tyrosine modification, the C230 protein was incubated with 20 mM tetranitromethane (TNM) in 50 mM Tris-HCl, pH 7.5 at 25°C for 20 minutes. For glutamic acid modification, the protein was incubated with 40 mM 1-carboxyl-3-(2-morpholinoethyl)carbodiimide metho- $\rho$ -toluenesulfonate (CMC) in 50 mM potassium phosphate (pH 7.0) at 25°C for 30 minutes. For cysteine modification, the C230 protein was incubated with 0.7 mM N-ethylmaleimide (NEM) in 50 mM potassium phosphate, pH 8.0 and 50 mM KCl at 25°C for 30 minutes. Aliquots were removed from the reaction mixture at various times and assayed for enzyme activity.

### Polymerase chain reaction

The standard reaction mixtures contained 1 ng of template DNA, 20 pmoles of PCR primers, 1.5 mM MgCl<sub>2</sub>, 0.25 mM dNTPs, and 2.5 units of Taq DNA polymerase in 10 mM Tris-HCl (pH 8.3) in a 100 µl volume. Reactions were carried out in a Takara PCR thermal cycler (TP 3,000) for 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 30 seconds. The mutagenic reaction mixture contained 2.5 mM MgCl<sub>2</sub>, 0.5 mM dNTPs, 0.25 mM MnCl<sub>2</sub>, and 2.5 units of DNA polymerase (6). PCR primers, 5'-CCCCAAGCTTCATATGAACAAAGGTAATGCGAC-3' and 5'-CCCCCTCTAGATCAGGTCAGCACGGTATAGGG-3', were purchased from Bioneer, Korea, purified by polyacrylamide gel electrophoresis, and subject to Sephadex chromatography. (<sup>35</sup>S)-(α-thio)-dNTPs were purchased from Amersham. The PCR product was ligated into the pBS-TA vector at the *Xcm*I site. The resulting plasmid DNA was used to transform competent *Escherichia coli* DH5α cells, which were grown on 50 mM ampicillin-containing

plates to produce a random mutant library. The transformants were sprayed with 0.1 mM catechol solution and white colonies were selected for nucleotide sequence determination.

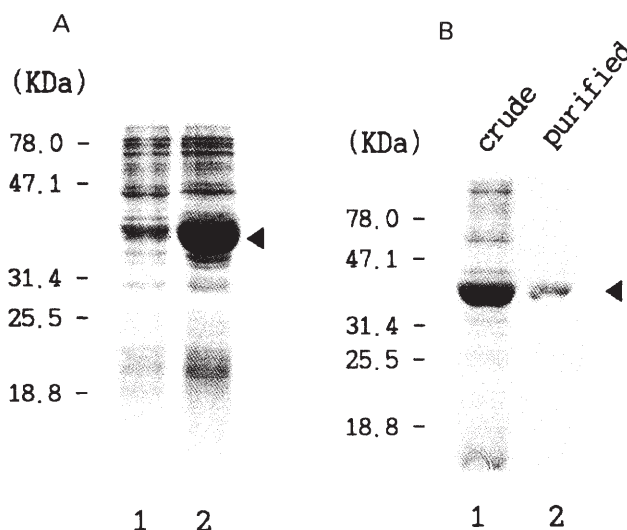
### DNA sequencing

Determination of nucleotide sequences of DNA was performed by the standard method of dideoxynucleotide chain termination (27).

## Results

### Expression and purification of *P. putida* C230

As it was previously observed that the ribosome binding sequence of *P. putida xylE* gene shows efficient expression of the gene in *Escherichia coli* (16), the *Sca*I-*Xba*I fragment containing the entire *xylE* open reading frame with its ribosome binding site was subcloned in the *Xba*I site of pET3a which is located downstream from the T7 promoter (Fig. 1). The resulting plasmid, designated pET-*xylE*, was introduced into *Escherichia coli* BL21 (DE3)pLysS in order to overproduce C230 protein. The soluble protein fraction of the disrupted cells from the IPTG-induced culture contained most portions of the overproduced C230 (data not shown). The overexpressed enzyme was purified to near homogeneity by selective acetone precipitation and Q-Sepharose chromatography as described in Materials and Methods.



**Fig. 2.** Expression and purification of *P. putida* catechol 2,3-dioxygenase. A: Induction of catechol 2,3-dioxygenase in *E. coli* BL21. Cells harboring pET-*xylE* were grown on TBGM9 medium at stationary phase, and catechol 2,3-dioxygenase was induced in absence (lane 1) or presence (lane 2) of 1 mM IPTG for 3 hours. B: SDS-PAGE analysis of the crude extract and the purified *P. putida* catechol 2,3-dioxygenase.



About 8.5 mg of purified C23O protein were recovered from *E. coli* cells, showing extraordinary efficiency at the expression level. A protein of 35 kDa, the size expected from the calculated molecular weight of *P. putida* C23O, was detected on SDS-PAGE (Fig. 2).

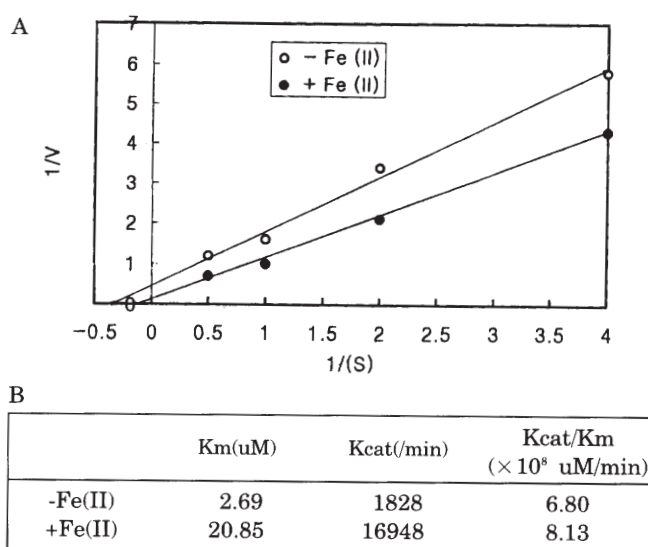
### Inactivation and reactivation of the C23O activity

It was consistently observed that C23O activity associated with the protein gradually decreased during the purification process (data not shown). Loss of C23O activity was thought to result from oxidative inactivation of the enzyme due to exposure to air oxygen as the native enzyme has been shown to manifest similar oxygen sensitivity (18). Because it has been known that C23O, like other extradiol-cleaving dioxygenases, requires Fe(II) ion for its catalytic reaction (4, 5), we examined if the spontaneous inactivation of the C23O results from oxidation of the Fe(II) ion. Preincubation of the C23O with 2 mM FeSO<sub>4</sub> alone or in conjunction with 10 mM ascorbic acid increased the enzyme activity up to 5 fold and 8 fold, respectively (Table 1). When the C23O was treated with 4 mM hydrogen peroxide, its activity was lost presumably due to complete oxidation of the Fe(II) ion. The inactivated C23O was, however, reactivated by the addition of FeSO<sub>4</sub> (Table 1). Reactivation of the C23O by the addition of ferrous ions indicates that oxidation of the Fe(II) ion in the holoenzyme is the major cause of its spontaneous inactivation. Parameters for steady-state kinetics of catechol dioxygenation catalyzed by the C23O were measured either in the presence or in the absence of FeSO<sub>4</sub>

**Table 1.** Inactivation and reactivation of *P. putida* catechol 2,3-dioxygenase<sup>a</sup>

Treatment with the enzyme	Relative activity (%)	Activation (fold)
Untreated		
None	100.0	1.00
Fe <sup>2+</sup>	498.0	4.98
Ascorbate	102.0	1.02
Ascorbate+ Fe <sup>2+</sup>	811.9	8.12
H <sub>2</sub> O <sub>2</sub>	4.5	0.04
Inactivated		
None	3.0	0.03
Fe <sup>2+</sup>	88.6	0.88
Ascorbate	19.3	0.19
Ascorbate+Fe <sup>2+</sup>	92.0	0.92

<sup>a</sup> Catechol 2,3-dioxygenase was incubated with 4 mM H<sub>2</sub>O<sub>2</sub>, 2 mM FeSO<sub>4</sub>, and 10 mM ascorbic acid, respectively, or with combination of two chemicals before or after H<sub>2</sub>O<sub>2</sub> treatment. The treated enzymes were routinely assayed for their activities.



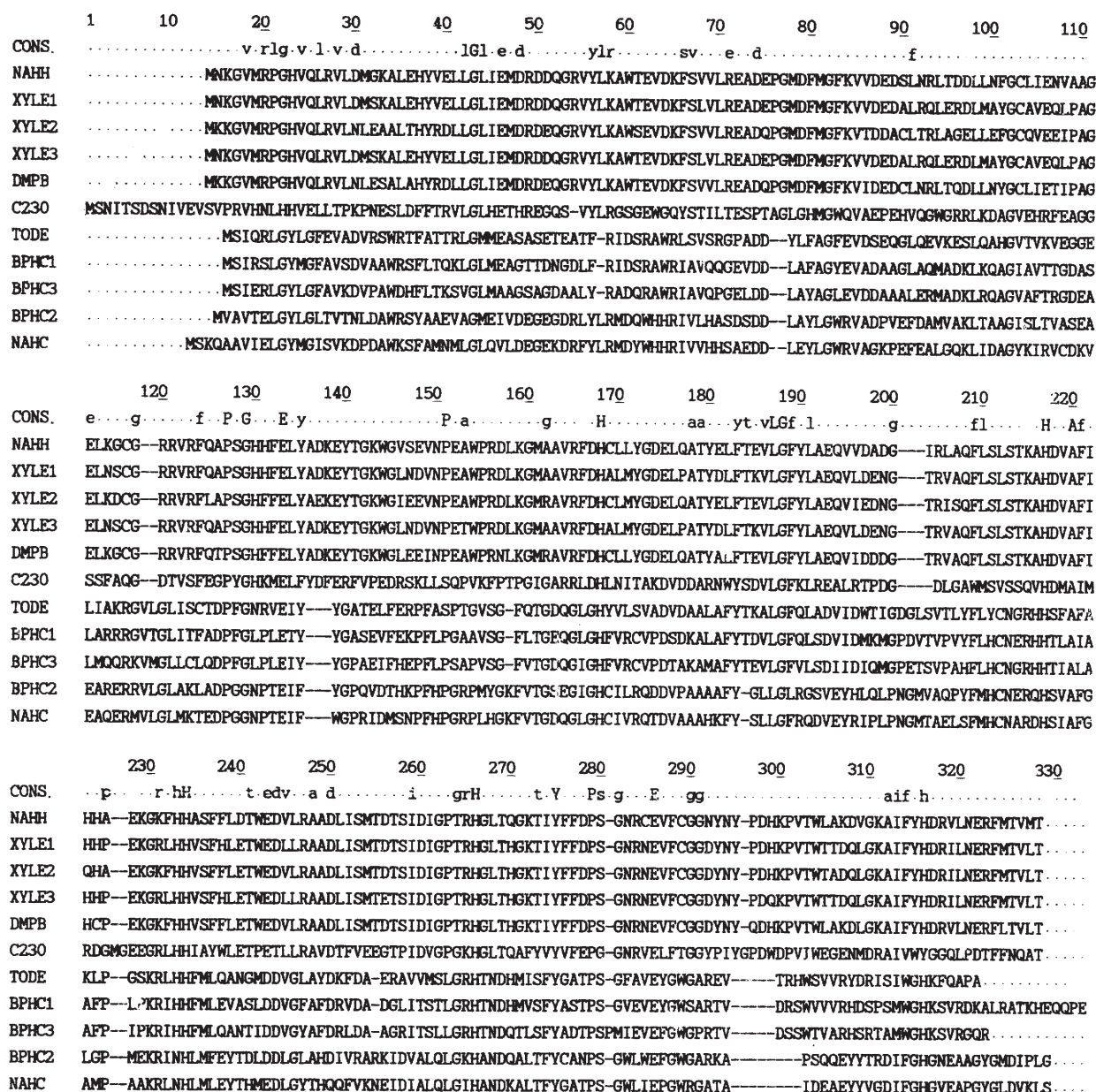
**Fig. 3.** Steady state kinetics of the reaction catalyzed by the *P. putida* catechol 2,3-dioxygenase. The kinetic parameters were measured from reaction mixture in the absence or presence of the Fe(II) ion. A: Lineweaver-Burk plot of catechol 2,3-dioxygenase in the absence of Fe<sup>2+</sup> (○) or in the presence of Fe<sup>2+</sup> (●). B: Kinetic parameters of catechol 2,3-dioxygenase

supplement. The addition of 2 mM FeSO<sub>4</sub> increased the K<sub>m</sub> value for catechol more than 7-fold and the K<sub>cat</sub> more than 9-fold (Fig. 3) with the K<sub>cat</sub>/K<sub>m</sub> consequently expressing a 1.2-fold increase. The increased turnover rate of the C23O upon addition of FeSO<sub>4</sub> with decrease in affinity of the enzyme for catechol as substrate implies a unique mode for the catalytic mechanism for C23O (see Discussion)

### Homology of C23O with other extradiol dioxygenases

The comparison of amino acid sequences of related enzymes is of particular interest because conserved amino acid residues may be important for enzyme function. The derived amino acid sequence of the C23O was compared to those of ten various extradiol dioxygenases (17) reported already, most of which are members of the same isofunctional gene family.

In Fig. 4, these amino acid sequences have been optimally aligned by introducing gaps to maximize identities among the extradiol dioxygenase genes using the GENMON program. As shown in Fig. 4, the C23O of *P. putida* is highly homologous to the derived amino acid sequence of *xylE1*, *xylE2*, and *nahH*. It was assumed that residues that are important for enzyme activity may be found among the conserved amino acids. Tatsuno *et al* (32) reported that histidine and tyrosine may be the iron-



**Fig 4.** Amino acid alignment of the extradiol-cleavage dioxygenases. The top sequence shows the consensus, in which the upper case letters mean 100% conservation in all sequence and the low-case letters conservation in more the 50% of the sequences. The sequences were obtained from the several sources (7) and the XYLE3 sequence from Kim *et al.* (16).

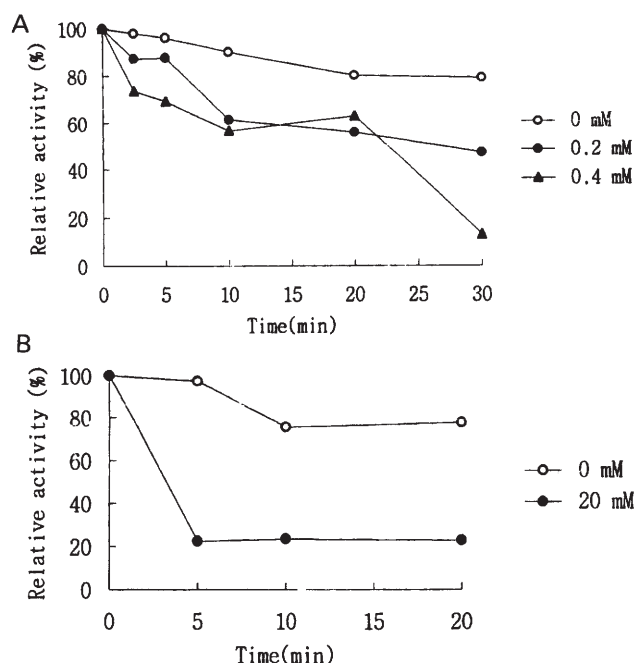
binding residues in C230. Thus, the high conservation of histidines and one tyrosine in all 11 enzymes compared is interesting.

### Chemical modification of C230

Sequence alignment of various extradiol-cleaving dioxygenase shows several invariant amino acid residues including histidine, tyrosine and glutamic acid that may play essential roles in the catalytic process (7, 23). These three amino acid residues have the chemical potential to serve as ligands for Fe(II) coordination in the assembly of a C230

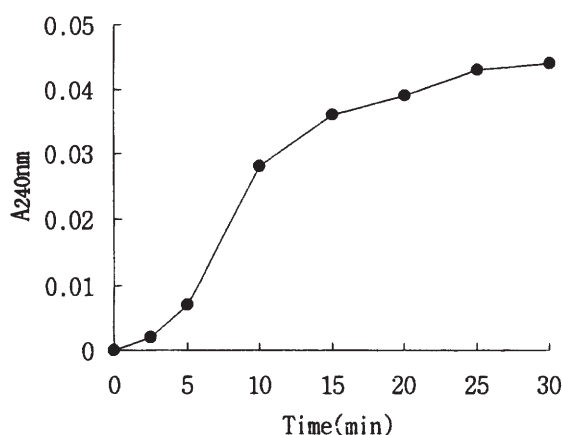
holoenzyme. We examined their possible contribution to the C230 catalysis by chemical modification of the C230 protein. Diethylpyrocarbonate (DEPC), tetranitromethane (TNM) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC), which covalently modify histidine, tyrosine and glutamic acid, respectively, were employed to specifically block each amino acid residue in the C230 protein as shown in Fig. 5 and 6.

The absorption spectra at various time intervals after the addition of 7.5  $\mu$ mol of DEPC to the purified enzyme are shown in Fig. 6. N-carbethoxyhis-

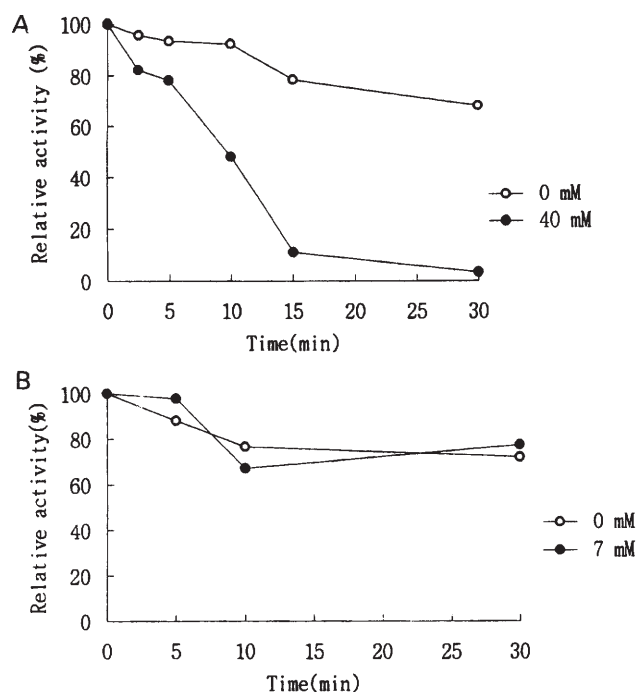


**Fig. 5.** Effect of the treatment with amino acid modifying reagents on the C23O. Chemical modification of histidine (A) and tyrosine (B) residues of the enzyme by diethylpyrocarbonate and tetranitromethane, respectively.

tidines formed after the addition of DEPC is plotted against time. The enzyme was totally inactivated within 30 min after the addition of 7.5  $\mu$ mol DEPC. As a comparison, *N*-ethylmaleimide (NEM) was additionally used in the C23O modification in order to block the cysteine residues that are not conserved in the dioxygenases. The C23O proteins treated with DEPC, TNM, and CMC lost their enzyme activity within 30 minutes of incubation time while NEM had no adverse effect on the enzyme activity.



**Fig. 6.** Effect of diethylpyrocarbonate treatment on the enzyme to produce *N*-carboethoxyhistidine during incubation period.



**Fig. 7.** Effect of the treatment with amino acid modifying reagents on the C23O. Chemical modification of glutamic acid (A) and cysteine (B) residues of the enzyme by 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate and *N*-ethylmaleimide, respectively.

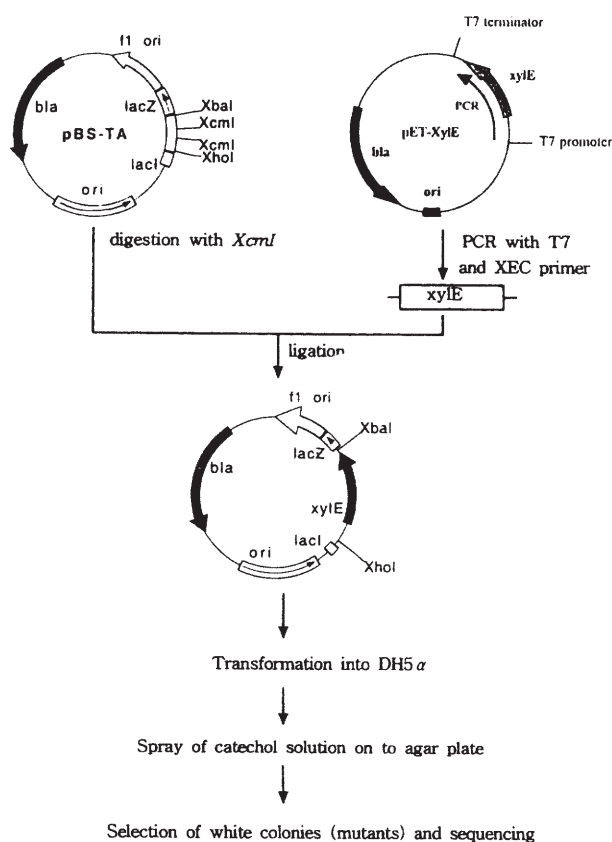
### Analysis of nucleotide sequence of the *xylE* gene

The determined nucleotide sequence and deduced amino acid sequence of C23O have been shown by Kim *et al* (16). For detection of an active site in the amino acid residues of the enzyme, each single point mutation was randomly introduced into the *xylE* gene by PCR. In order to perform this experiment, recombinant plasmid for PCR mutagenesis and nucleotide sequencing analysis was constructed as shown in Fig. 8. The total DNA nucleotide sequence of the gene randomly mutated by PCR technique was determined in both directions.

The results of nucleotide sequence determination are shown in Fig. 9. When colonies on LB plates were sprayed with 0.1 mM catechol solution, the parental strain developed a yellow color while mutant strains retained white colonies (Table 2). We isolated four mutants, H23Q, L57P, T179S, and S211R, which had single amino acid substitutions. Three mutants showed single amino acid substitutions in the *xylE* gene; His<sup>23</sup> to glutamine, Leu<sup>57</sup> to proline, Thr<sup>179</sup> to serine, and Ser<sup>211</sup> to arginine, respectively (Fig. 9). These amino acid residues appear to be important for the enzymatic activity of C23O.

Even if all the mutated sites of the nucleotide sequences among the four mutants did not cor-





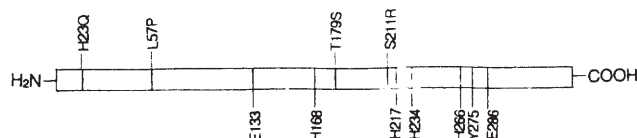
**Fig. 8.** Cloning strategy of the *xylE* mutants for nucleotide sequencing analysis.

respond to the conserved amino acids, the mutated positions of two mutants, T179S and S211R, were located near the conserved histidine residues.

This result suggests that amino acid changes at Thr<sup>179</sup> or Ser<sup>211</sup> of the enzyme effects changes in its conformation or substrate binding region.

## Discussion

In this study we characterized the *P. putida* C230 enzyme and, via chemical modification, examined several amino acid residues that might constitute the active site of the C230. Like other extradiol-cleaving dioxygenases, the *P. putida* C230



**Fig. 9.** Random point mutagenesis of C230 by PCR. Conserved amino acid residues of extradiol dioxygenases (7) are indicated in the low-line numbers and amino acid residues of *xylE* mutants are indicated in the upper-line numbers.

**Table 2.** Color development in the colonies of parental and mutant strains\*

Strain	Yellow color development
Parental strain	
<i>P. putida</i> SU10	+
Mutant strain	
<i>P. putida</i> H23Q	—
<i>P. putida</i> L57P	—
<i>P. putida</i> T179S	—
<i>P. putida</i> S211R	—

\* For the detection of mutant strains, colonies were sprayed with 0.1 mM catechol solution.

purified from *E. coli* requires Fe(II) ion for optimal enzyme activity. When Fe(II) ions were added to the inactivated C230, its catalytic activity increased about 9-fold, most presumably by replacement of the Fe(III) ions in the enzyme with the Fe(II) ions. The reactivated enzyme manifested an elevated  $K_m$  value, about 7.8-fold, indicating that the Fe(II) ion does not contribute to the enhancement of substrate binding affinity.

In the *in vivo* environment, the inactivated C230 can be reactivated by a chloroplast-type ferredoxin encoded in the *xylT* gene (25). The key step of ring cleavage is catalyzed by an Fe(II)-dependent extradiol dioxygenase.

The active site of C230 contains a mononuclear high-spin Fe<sup>2+</sup> center that has been implicated in the binding and catalytic activation of molecular oxygen (20). It has been suggested that histidine or tyrosine may be the iron-binding residues in C230 (32), as in the case of protocatechuate 3,4-dioxygenase and intradiol-cleaving enzyme (24). Therefore the high conservation of four histidines and one tyrosine in all ten enzymes was found among several sources (7). Their results have suggested that the main active-sites of these enzymes are present in the C-terminal region. Three of the four conserved histidines and the conserved tyrosine are situated in the C-terminal region, indicating their possible involvement in enzyme activity.

To elucidate the role of the conserved histidine residues, we examined the effect of chemical modification by DEPC on the activity of the C230. For this purpose, we used purified C230 through several steps. Inactivation of an enzyme by DEPC may be correlated with the modification of histidyl residues if hydroxylamine reactivates the enzyme. The time-dependent formation of N-carbethoxyhistidine has been monitored by the increase in absorption between 230 nm and 250 nm (21). In our experiment, C230 was also inactivated by DEPC in a time-dependent manner. Therefore, inactivation of C230 by incubation with DEPC is exclu-

sively due to the modification of histidyl residues.

Our results imply that at least one of the four strictly conserved histidines is important for enzyme activity. This finding is supported by a site-specific mutagenesis experiment reported by Tira *et al.* (31). In this experiment, the strictly conserved His<sup>233</sup> of *todE* (33), *bphC1* (8), *bphC2* (30) and *bphC3* (15) and the nonconserved His<sup>233</sup> and His<sup>316</sup> of *bphC1* were exchanged with alanine. The exchange of the conserved His<sup>234</sup> completely abolished enzyme activity. On the other hand, mutagenesis of the nonconserved histidines had no effect on enzyme activity. It was concluded that His<sup>234</sup> is a part of the binding site for Fe<sup>2+</sup> (31).

According to our chemical modification experiments, all three conserved histidine, tyrosine, and glutamic acid residues appear to be essential for enzyme activity. Moreover, from the results of the random mutagenesis experiments, we can conclude that Thr<sup>179</sup> and Ser<sup>211</sup> near the conserved His<sup>168</sup> and His<sup>217</sup> residues, respectively, are also important residues for enzyme activity and substrate binding conformation. Hirose *et al.* (13) have suggested that four His residues and one Glu at invariant positions could coordinate the Fe<sup>2+</sup>. We are currently searching for amino acid residues essential for enzyme function by PCR-mediated mutagenesis.

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