Short communication

Site-Directed Mutagenesis of Two Cysteines (155, 202) in Catechol 1,2-dioxygenase I₁ of *Acinetobacter lwoffii* K24

**Seung Il Kim*, Soo-Jung Kim, Sun-Hee Leem[†], Kye-Heon Oh[‡],
Soohyun Kim and Young-Mok Park**

Biomolecular Research Team, Korea Basic Science Institute, Taejon 305-333
[†]Department of Biology, Dong-A University, Pusan 604-714
[‡]Department of Life Science, Soonchunghyang University, Asan 336-745, Korea

Catechol 1,2-dioxygenase I₁ (CDI₁) is the first enzyme of the β-ketoadipate pathway in *Acinetobacter lowffii* K24. CDI₁ has two cysteines (155, 202) and its enzyme activity is inhibited by the cysteine inhibitor, AgNO₃. Two mutants, CDI₁ C155V and CDI₁ C202V, were obtained by site-directed mutagenesis. The two mutants were overexpressed and the mutated amino acid residues (Cys→Val) were characterized by peptide mapping and amino acid sequencing. Interestingly, CDI₁ C155V was inhibited by AgNO₃, whereas CDI₁ C202V was not inhibited. This suggests that Cys²⁰² is the sole inhibition site by AgNO₃ and is close to the active site of the enzyme. However, the results of the biochemical assay of mutated CDI₁s suggest that the two cysteines are not directly involved in the activity of the catechol 1,2-dioxygenase of CDI₁.

Introduction

Catechol 1,2-dioxygenase is the key enzyme of the β-ketoadipate pathway with protocatechuate 3,4-dioxygenase in soil-borne bacteria. It catalyzes the conversion of catechol to *cis,cis*-muconic acid. More than ten catechol 1,2-dioxygenases have been reported from various bacteria (Kim *et al.*, 1999; Murakami *et al.*, 1999). The enzymes are grouped into two types by different substrate specificities and amino acid sequences. While type IIs have broad substrate activity to chlorocatechols, type Is have low activity to chlorocatechols. However, all of the catechol 1,2-

dioxygenases have Fe²⁺ as a non-iron-sulfur cofactor and two conserved histidines & tyrosines, which are believed to form the active site with Fe²⁺ (Neidle *et al.*, 1988; Broderick & O'Halloran, 1991).

Acinetobacter lowffii K24, which has been reported as an aniline-assimilating bacterium, has two *cat* gene clusters. These include two type I catechol 1,2-dioxygenases (CDI₁ & CDI₂) (Kim *et al.*, 1997). CDI₁ and CDI₂ have different substrate specificities and physiochemical properties. In the previous work, we found that AgNO₃, which functions as a cysteine inhibitor, was able to inhibit the enzyme activity of CDI₁ and the DNA sequence analysis showed that CDI₁ has two cysteines (155, 202), but CDI₂ has none. These results suggest that the two sulfhydryl groups (-SH) of CDI₁ are not linked, but free to interact with other molecules. Several of the catechol 1,2-dioxygenases were also inhibited by AgNO₃, which acts as a cysteine inhibitor. It revealed that the enzymes have conserved cysteines by using a sequence homology search.

In this work, we performed a site-directed mutagenesis of CDI₁, overexpression and characterization of mutants to study the function of the two cysteines (155 and 202) in the activity of catechol 1,2-dioxygenase.

Materials and Methods

Bacterial strains and plasmids *Escherichia coli* DH5α was used for routine transformation and *Escherichia coli* BL21 for overexpression (Studier *et al.*, 1990). Plasmid pCD12 (*catA₁B₁C₁*; pUC118) was used as the DNA template for the mutagenesis of Cys²⁰² of *catA₁*, and pCD11 (*catA₁C₁*; pUC19) for Cys¹⁵⁵ of *catA₁* (Kim *et al.*, 1997), respectively.

Molecular techniques Plasmid DNA and digested DNA fragments were isolated by using a QIAGEN plasmid kit and QIA quick gel extraction kit (Santa Clarita, USA) according to the manufacturer's protocols. Restriction enzyme digestions and

---

*To whom correspondence should be addressed.
Tel: 82-42-865-3451; Fax: 82-42-865-3419
E-mail: ksi@comp.kbsi.re.kr

ligations were performed as described by the enzyme supplier (NEB, Beverly, USA). Transformations were performed by the methods of Sambrook (Sambrook et al., 1989).

Site-directed mutagenesis Mutagenesis of catA₁ was carried out with the LA PCR in vitro Mutagenesis kit of Takara (Shiga, Japan) according to the manufacturer's instructions. Two oligonucleotides were designed for the 155 and 202 cysteine mutation: 5'-CGG GAC [Cys²⁰²→Val] GCC ATA GCC GAC C-3' and 5'-CCA GAC [Cys¹⁵⁵→Val] TTC CAC TAC GGC GCC-3'. The oligonucleotides were provided by the Bioneer Corporation (Taejon, Korea). PCR was performed with the following conditions: 94°C 30 sec, 55°C 2 min, 72°C 3 min. Mutated PCR products were digested with SacI & KpnI or XbaI & KpnI and ligated with pCD12 or pCD11, which had also been digested with the same enzymes. Newly constructed vectors were designated as pSK255 and pSK257, respectively.

DNA sequence analysis The nucleotide sequences of catA₁ genes were determined by a modification of the dideoxy-chain termination methods using the Prism DyeDeoxy Terminator Cycles Sequencing Kit of Perkin-Elmer (Foster City, USA). Sequencing reactions were prepared according to the supplier's instructions and analyzed by electrophoresis using the Perkin Elmer Model 377 DNA sequencer.

Overexpression and partial purification E. coli BL21 containing pSK255 (catA₁-C202V), pSK257 (catA₁-C155V) and pCD12 (catA₁) were cultured in 1 liter of LB broth with 50 μg/ml ampicillin at 37°C. When the A₆₀₀ value reached 0.4, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added in the medium. After harvesting the cells after 4-6 hours, the cells were suspended in 20 ml of 50 mM Tris-HCl (pH 8.0) and broken by two cycles through a French pressure cell (SLM-AMINCO, Urbana, USA) at 20,000 lb/in² by Leems method (Leem et al., 1997). The crude extract was centrifuged at 15,000 × g for 50 min. Supernatant fractions were loaded in a HR5/5 MonoQ column of FPLC (Pharmarcia, Uppsala, Sweden). Protein was eluted with a linear gradient of NaCl from 0.1 to 0.5 M at the flow rate of 0.5 ml/min for 30 min. The 1.0 ml fractions were collected and used for an enzyme assay. Protein concentration was determined by the method of Bradford (Bradford, 1976). A SDS-polyacrylamide gel electrophoresis was performed by the methods of Laemmli (Laemmli, 1970).

Peptide mapping For the verification of mutated amino acids, mutant CDI₁s were analyzed by peptide mapping. Partially purified enzymes by MonoQ were further separated by a HR 5/10 ProRPC of FPLC according to previous methods (Kim et al., 1992; Kim et al., 1997). Digestion of enzymes with V8 protease (Pierce, Rockford, USA) was performed according to the Matsudaira's protocol (Matsudaira, 1993). The digested peptides were applied to an OD-300, Aquapore C-18 column (2.1 × 300 mm) of PE Brownlee (Foster City, USA) in Model 172 HPLC of Perkin Elmer. The digested peptides were separated with an increasing acetonitrile gradient of 16%-72% at the flow rate of 210 μl/min for 60 min. Each peak was manually collected at 210 nm and used for N-terminal sequencing.

N-terminal amino acid sequencing N-terminal amino acid sequences of V8 protease digested peptides were analyzed by an automatic sequencer (Perkin-Elmer model 476A and 491A).

Enzyme activity assay The activity of catechol 1,2-dioxygenase was assayed according to Aoki's methods (Aoki et al., 1984) by measuring the cis,cis-muconic acid product. The activity of mutated CDI₁s on catechol analogues was measured according to the previous methods (Aoki et al., 1984, Broderick & O'Halloran, 1991). To study the effects of cysteine inhibitors on mutant and wild type CDI₁s, enzyme solutions were treated with AgNO₃, Iodoacetate, p-hydroxymercuribenzoate (PHMB), dithionitrobenzoic acid (DTNB) and HgCl₂ for 20 min and the enzyme activity was assayed by adding catechol as the substrate. Kₘ values were measured by Lineweaver-Burk methods by using different substrate concentrations (1 to 5 μM of catechol).

Results and Discussion

Mutagenesis of two mutant catA₁ (C155V and C202V) genes Two mutated catA₁ genes (catA₁-C155V and catA₁-C202V) were sequenced and confirmed to have a valine codon (GTC) at 155 and 202 with no other mutation site. The crude extracts of E. coli BL21 containing pSK255, pSK257 and pCD12 showed the specific activities of 0.87-1.35 U/mg (Table 1). A 12% SDS-PAGE of these extracts revealed that CDI₁s of about 36 kD were overexpressed (Fig. 1). Three crude extracts were partially purified up to specific activities of 3.51-4.20 U/mg by MonoQ column. To verify the mutated amino acid residues (Cys→Val), CDI₁s were digested with a V8 protease (Glu-C endonuclease). From the amino acid sequence analysis, 17 digested peptide peaks were expected when using the V8 protease (Kim et al., 1997), but more than 20 peaks were seen because of incomplete digestion (data not shown). Major peaks were collected and used for the N-terminal sequencing. Both GluC-peak #7 of CDI₁ C155V and GluC-peak #18 of CDI₁ C202V were found to have Val residues at the Cys residue site (155 and 202).

Table 1. Partial purification of catechol 1,2-dioxygenases

Purification step	Total protein (mg)	Total activity (Uª)	Specific activity (U/mg)
Cell extract			
CDI₁	158	137.5	0.87
CDI₁C157V	136	183.6	1.35
CDI₁C202V	178	158.4	0.89
MonoQ fraction			
CDI₁	25.4	106.7	4.20
CDI₁C157V	18.4	65.5	3.56
CDI₁C202V	27.6	96.9	3.51

ªOne unit of enzyme activity was defined as the amount, which produced 1 mmol of cis,cis-muconate per min at 24°C.
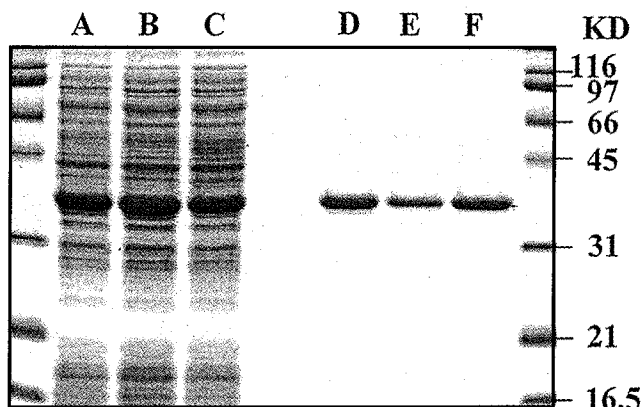
Fig. 1. Overexpression and partial purification of mutant CDI₁s (C155V and C202V). Lanes A-C are crude extracts and lanes D-E are MonoQ fractions of CDI₁. Lanes A and D; CDI₁, lanes B and E; C155V CDI₁, lanes C and F; C202V CDI₁.

Table 2. Effects of cystein inhibitors on catechol 1,2-dixoygenases

Inhibitors		Relative activity (%)		
		CDI₁	CDI₁C155V	CDI₁C202V
Catechol	100	100	100	100
AgNO₃	0.1 mM	25.4	25.1	133.4
Iodoacetate	0.1 mM	110.2	122.5	112.7
	1.0 mM	112.2	133.7	116.2
PHMB	0.1 mM	95.2	104.4	116.0
DTNB	0.1 mM	104.6	159.4	173
HgCl₂	0.1 mM	44.5	57.3	128.5
	1.0 mM	44.9	46.7	81.1

Table 3. Substrates specificities of catechol 1,2-dioxygenases

Substrates	Relative activity (%)		
	CDI₁	CDI₁C155V	CDI₁C202V
Catechol	100	100	100
3-Methylcatechol	1.8	3.0	4.2
4-Methylcatechol	50.9	56.8	59.1
4-Chlorocatechol	4.4	4.8	4.3
3-Methoxycatechol	1.7	1.2	2.1
4-Nitrocatechol	0	0	0

Effects of cysteine inhibitors on WT CDI₁ and two mutant CDI₁s (C155V and C202V) CDI₁ was about 75% inhibited by AgNO₃. This result is similar to a previous finding (Kim *et al.*, 1997). HgCl₂ also showed about 55% inhibition, but unexpectedly other cysteine inhibitors such as iodoacetate, PHMB and DTNB had no inhibitory effects on enzyme activity (Table 2). These results suggest that the sulfhydryl groups of the native CDI₁ may not interact with bulky inhibitors due to the burial of the sulfhydryl groups in protein. Even if the sulfhydryl groups of the two cysteines interacted with cysteine inhibitors, enzyme activity was not affected except when AgNO₃ and HgCl₂ were used as the inhibitors. CDI of *Frateuria* sp. was also reported not to react with DTNB due to the sulfhydryl groups being buried in the protein (Aoki *et al.*, 1984).

In an inhibition study of the two mutants, C155V CDI₁ had a similar inhibition pattern to CDI₁, but interestingly the enzyme activity of C202V CDI₁ was not inhibited by AgNO₃ and HgCl₂. This means that the interaction of Cys²⁰² with AgNO₃ and HgCl₂ can inhibit enzyme activity of CDI₁ but not Cys¹⁵⁵. Catechol 1,2-dioxygenases were reported to have Fe²⁺ as a prosthetic group in the active site. Spectroscopic studies indicated that the two conserved tyrosines and histidines are linked with Fe²⁺ (Neidle *et al.*, 1988; Broderick & O'Halloran, 1991) (Fig.1). In the case of CDI₁ of *A. lowffii* K24, the conserved tyrosines are 164 and 202 residues. From these results, the sulfhydryl (-SH) group of Cys²⁰² was assumed to interact with Ag²⁺ (AgNO₃ in solution), and the Ag bound to cysteine (-S-Ag) may prevent the catalytic activity of Fe²⁺ linked with Tyr²⁰⁰, because the two metals are very close and repulse each other. The fact that Ag²⁺ (AgNO₃) and Hg²⁺ (HgCl₂) are small molecules having the same electric charge, while other cysteine inhibitors are more bulky with no electric charge, supports this assumption. Several CDIs containing cysteine at the same site have also been reported to be inhibited by AgNO₃ (Patel *et al.*, 1976; Aoki *et al.*, 1984).

Characterization of two mutant CDI₁s (C155V and C202V) The enzyme activities of two CDI₁s to catechol analogues were measured. CDI₁ C155V and CDI₁ C202V had 56.8-59.1% relative activities to 4-methyl catechol, but less than 5% relative activity to other catechol analogues. Compared with the wild type CDI₁, the two mutant CDI₁s showed similar patterns of substrate specificity. K_M values of wild type CDI₁, CDI₁ C155V and CDI₁ C202 were 1.14, 1.19 and 1.02 µM, respectively.

In this mutagenesis study, we suggest that Cys²⁰² is the sole AgNO₃ inhibition site and is close to the active site of CDI₁, but Cys¹⁵⁵ is not involved in AgNO₃ inhibition. Results of the biochemical assay of two mutant CDI₁s (C155V and C202V) showed that although the two cysteines have a free sulfhydryl group in solution, they were not involved in the enzyme active site and showed no influence on enzyme activity, substrate specificity and the K_M values of CDI₁.

References

Aoki, K., Konohana, T., Shinke, R. and Nishira, H. (1984) Purification and characterization of catechol 1,2-dioxygenase from aniline-assimilating *Rhodococcus erythropolis* AN-13. *Agric. Biol. Chem.* **48**, 2087-2095.

Aoki, K., Konohana, T., Shinke, R. and Nishira, H. (1984) Two

catechol 1,2-dioxygenases from an aniline-assimilating bacterium *Frateuria* ANA-18. *Agric. Biol. Chem.* **48**, 2097-2104.

Bradford, M. M. (1976) Rapid and sensitive methods for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Broderick, J. B. and O'Halloran, T. V. (1991) Overproduction, purification, and characterization of chlorocatechol dioxygenase, a non-heme iron dioxygenase with broad substrate tolerance. *Biochemistry* **30**, 7349-7358.

Kim, J., Kim, H. and Kim, J. (1992) A study of the two-dimensional high-performance liquid chromatography for total amino acid sequencing of rbST. *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **25**, 224-230.

Kim, S. I., Ha, K.-S. and Leem, S.-H. (1999) Differential organization and transcription of the *cat₂* gene cluster in aniline-assimilating *Acinetobacter lwoffii* K24. *J. Biosci. Bioeng.* **88**, 250-257.

Kim, S. I., Kweon, S. M., Kim, S. and Ha, K.-S. (1997) Expression and characterization of *catA₁* (catechol 1,2-dioxygenase I₁) of *Acinetobacter lwoffii* K24 in *Escherichia coli. J. Biochem. Mol. Biol.* **30**, 342-345.

Kim, S. I., Leem, S.-H., Choi, J. S., Chung, Y. H., Kim, S., Park, Y. M., Park, Y. K., Lee, Y. N. and Ha, K.-S. (1997) Cloning and characterization of two *catA* genes in *Acinetobacter lwoffii* K24. *J. Bacteriol.* **179**, 5226-5231.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Leem, S.-H., Shin, I., Kweon, S.-M., Kim, S. I., Kim, J.-H. and Ha, K.-S. (1997) Phospholipase D is not involved in Rho A-mediated activation of stress fiber formation. *J. Biochem. Mol. Biol.* **30**, 337-341.

Matsudaira, P. (1993) A practical guide to protein and peptide purification for microsequencing, pp. 54-59, Academic Press, Inc. San Diego.

Murakami, S., Takashima, A., Takemoto, J., Takenaka, S., Sinke, R. and Aoki, K. (1999) Cloning and sequence analysis of two catechol-degrading gene clusters from the aniline-assimilating bacterium *Frateuria* species ANA-18. *Gene* **226**, 189-198.

Neidle, E. L., Hartnett, C., Bonitz, S. and Ornston, L. N. (1988) DNA sequence of the *Acinetobacter calcoaceticus* catechol 1,2-dioxygenase I structural gene *catA*: evidence for evolutionary divergence of intradiol dioxygenases by acquisition of DNA sequence repetitions. *J. Bacteriol.* **170**, 4874-4880.

Patel, R., Hou, C. T., Felix, A. and Lillard, M. O. (1976) Catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus*: purification and properties. *J. Bacteriol.* **127**, 536-544.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendroff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60-89.