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A Single Amino Acid Substitution Converts γ-Glutamyltranspeptidase to a Class IV Cephalosporin Acylase (Glutaryl-7-Aminocephalosporanic Acid Acylase)

Hideyuki Suzuki,* Chinatsu Miwa, Sayaka Ishihara, and Hidehiko Kumagai†

Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto, Japan

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The aspartyl residue at position 433 of γ-glutamyltranspeptidase of *Escherichia coli* K-12 was replaced by an asparaginyl residue. This substitution enabled γ-glutamyltranspeptidase to deacylate glutaryl-7-aminocephalosporanic acid, producing 7-aminocephalosporanic acid, which is a starting material for the synthesis of semisynthetic cephalosporins.

The effective production of 7-aminocephalosporanic acid (7-ACA) is a matter of concern in the pharmaceutical industry because it is a starting material for the synthesis of semisynthetic cephalosporins, which are the best-sold antibiotics worldwide, with global sales of $8.3 billion of $466.3 billion of the total pharmaceutical market in 2003. Semisynthetic cephalosporins are made by the modification of the side chains of positions 3 and 7 of 7-ACA, which are commercially supplied mainly by the chemical deacylation of cephalosporin C (CPC), produced by the fungus *Acremonium chrysogenum*. However, the chemical process requires several complicated steps using toxic compounds and produces a lot of chemical wastes. The innovation of an enzymatic process involving two enzymes has recently become the new deacylation process of CPC on an industrial scale (12). This enzymatic process involves no toxic raw materials, proceeds under mild reaction conditions, and reduces waste significantly. In this process, D-amino acid oxidase converts CPC to 7β-(5-carboxy-5-oxopentanamido)-cephalosporanic acid, followed by autoconversion to glutaryl-7-ACA (GL-7-ACA). Cephalosporin acylase then deacylates GL-7-ACA to 7-ACA. The critical enzyme of this bioprocess is cephalosporin acylase, and extensive screening for this enzymatic activity is extremely important. However, this enzyme has been found only in a limited number of bacterial strains.

There is a surprisingly high similarity between a class IV cephalosporin acylase (GL-7-ACA acylase) (7) and γ-glutamyltranspeptidase (GGT; EC 2.3.2.2). Seventy-six of about 580 amino acid residues were completely conserved in the GGTs for which the amino acid sequences are known. Of these 76 amino acid residues, 58 were also conserved in class IV cephalosporin acylases. GGT catalyzes the hydrolysis of γ-glutamyl compounds and the transfer of their γ-glutamyl residues to other amino acids and peptides (18). Although the γ-glutamyl group [$NH_2CH(COOH)CH_2CH_2CO$] and the glutaryl group [$CH_2(COOH)CH_2CH_2CO$] have similar chemical structures and the linkage between the 7-amino group of 7-ACA and the glutaryl group is an amide linkage (Fig. 1), GL-7-ACA deacylating activity was not detected in *Escherichia coli* GGT. Asp-433 of *E. coli* GGT is one of the residues that are completely conserved in GGTs but not in class IV cephalosporin acylases. This residue corresponds to that of human GGT, postulated to interact with the α amino group of the γ-glutamyl residue of its substrate (2), and the residue is Asn in class IV cephalosporin acylases. Therefore, in this study, the GGT enzyme with the D433N mutation (GGT D433N) was obtained and studied.

The bacterial strains, plasmids, and oligonucleotide used in this study are listed in Table 1. Single-stranded DNA of pSH1248 was obtained by superinfecting strain SH1277 with M13KO7. The D433N substitution was generated to obtain pCM2 using single-stranded pSH1248 as a template and phosphorylated oligonucleotide GGT D433N as a mutagenic primer by the method of Kunkel et al. (8). The correctness of the DNA sequence of pCM2 was confirmed by the method of Sanger et al. (11) as modified by Katayama et al. (6). The 0.9-kb HindIII fragment of pCM2 was ligated with the 5.4-kb HindIII fragment of pSH253 to obtain pCM3. The 3.2-kb SmaI-SphI fragment was then ligated with the 4.0-kb EcoRV-SphI fragment of pBR322 to obtain pCM7. Strain SH641 was transformed with pCM7, and strain CM9 was obtained.

The mutant GGT protein (GGT D433N enzyme) was isolated from the periplasmic fraction (17) of strain CM9 as described previously (15). The GGT activities of the wild-type GGT and mutant GGT D433N enzyme were measured as described previously using γ-glutamyl-*p*-nitroanilide and glycylglycine as substrates (16). Their protein concentrations were measured by the method of Lowry et al. (10). No transpeptidation activity was detected for GGT D433N enzyme, but its hydrolysis activity was 0.32 U/mg, about 82% of the wild-type enzyme. This might be because the γ-glutamyl residue of the

* Corresponding author. Mailing address: Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan. Phone: 81-75-753-6278. Fax: 81-75-753-6275. E-mail: hideyuki@lif.kyoto-u.ac.jp.

† Present address: Research Institute of Agricultural Resources, Ishikawa Agricultural College, Ishikawa-gun, Ishikawa 921-8836, Japan.

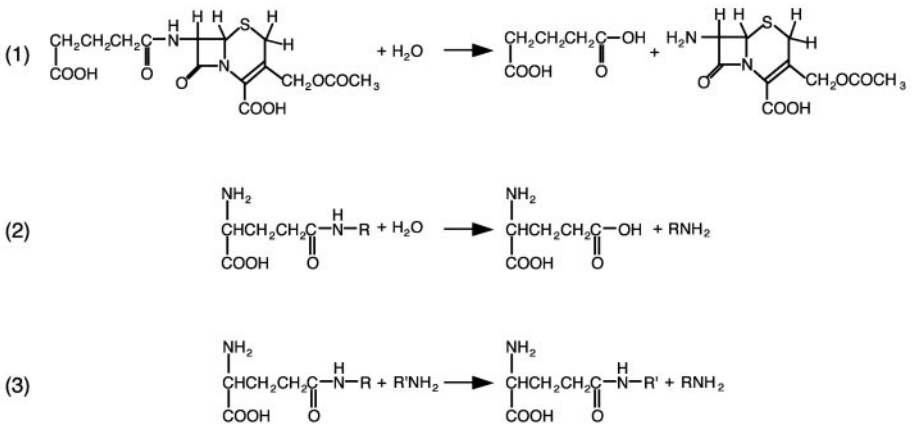

FIG. 1. Reactions catalyzed by cephalosporin (GL-7-ACA) acylase and GGT. Reaction 1 is the reaction catalyzed by cephalosporin (GL-7-ACA) acylase, reaction 2 is the hydrolysis reaction catalyzed by GGT, and reaction 3 is the transpeptidation reaction catalyzed by GGT.

γ-glutamyl enzyme intermediate formed at Thr-391 (3) wobbles without the hydrogen bond with Asp-433, and it is difficult for a γ-glutamyl acceptor to make the nucleophilic substitution from a distinct direction. On the other hand, water could attack its carbonyl carbon because of its small size.

GL-7-ACA, used to measure the activity for deacylating GL-7-ACA, was synthesized essentially by the method of Shibuya et al. (13) from glutaric anhydride and 7-ACA, purchased from Wako Pure Chemical Industry (Osaka, Japan) and Aldrich Chemical, respectively. The synthesized sample was identified as GL-7-ACA by nuclear magnetic resonance (NMR) and fast atom bombardment mass spectrometric analyses. Succinyl-7-ACA, adipyl-7-ACA, and adipyl-7-aminodeacetoxycephalosporanic acid (7-ADCA) were synthesized similarly from the corresponding acid anhydrides and 7-ACA or 7-ADCA, except for adipic anhydride, which was incubated at 60°C to dissolve in acetone.

The deacylating activity of GL-7-ACA was measured as follows. The standard reaction mixture was 50 mM Tris-HCl (pH 8.73), 2 mM GL-7-ACA, and 0.1 mg of enzyme per ml. The reaction was initiated by the addition of the enzyme solution, and the reaction mixture was incubated at 37°C. Part of the reaction mixture (200 μl) was subtracted and mixed with the same volume of 3.5 N CH_3COOH to terminate the reaction. The concentrations of both GL-7-ACA and 7-ACA after filtration through a membrane filter (pore size, 0.2 μm) were measured by a high-performance liquid chromatograph (model LC-10; Shimadzu, Kyoto, Japan) equipped with an Inertsil ODS-3 column (5 mm by 250 mm; GL Sciences, Tokyo, Japan) with gradient elution at a flow rate of 1 ml/min and at 40°C. The gradient of the mobile phase was formed with buffer A (0.05% trifluoroacetic acid) and buffer B (acetonitrile containing 0.05% trifluoroacetic acid). The concentration of buffer B was linearly increased to 50% from 0 to 25 min, kept at 50% until 34 min, and then decreased to 0% from 34 to 35 min. Both GL-7-ACA and 7-ACA were detected with a UV detec-

TABLE 1. Bacterial strains, plasmids, and oligonucleotide used in this study

Strain, plasmid, or oligonucleotide	Relevant genotype, characteristic, construction, sequence, or function	Reference or source
E. coli K-12 strains		
CJ236	pCJ105 [F′ *cat*$^+$]/*dut-1 ung-1 thi-1 relA1*	5
CM9	SH641/pCM7	
SH641	F$^-$ Δ*ggt-2 rpsL recA56 srl300*::Tn*10*	15
SH1277	CJ236/pSH1248	This study
Plasmids		
pCM2	pSH1248 but with the D433N substitution; the mutation was generated by the method of Kunkel (9)	This study
pCM3	pUC replicon f1 *ori bla*$^+$ *ggt*; the 0.9-kb HindIII fragment of pCM2 was ligated with the 5.4-kb HindIII-HindIII fragment of pSH253	This study
pCM7	ColEI replicon *rop*$^+$ *bla*$^+$ *ggt*$^+$; the 3.2-kb SmaI-SphI fragment was ligated with the 4.0-kb EcoRV-SphI fragment of pBR322	This study
pSH253	pUC replicon f1 *ori bla*$^+$ *ggt*$^+$; pUC119 with the *ggt* gene	1
pSH1248	pUC replicon f1 *ori bla*$^+$ *ggt*; the 0.9-kb HindIII fragment of pSH253 was inserted into the HindIII site of pTZ18R	14
pTZ18R	pUC replicon f1 *ori bla*$^+$	Pharmacia
Oligonucleotide GGT		
D433N	5′-AACCAGATGGATAATTTCTCCGCC-3′, to mutagenize the GAT codon for Asp-433 to AAT	

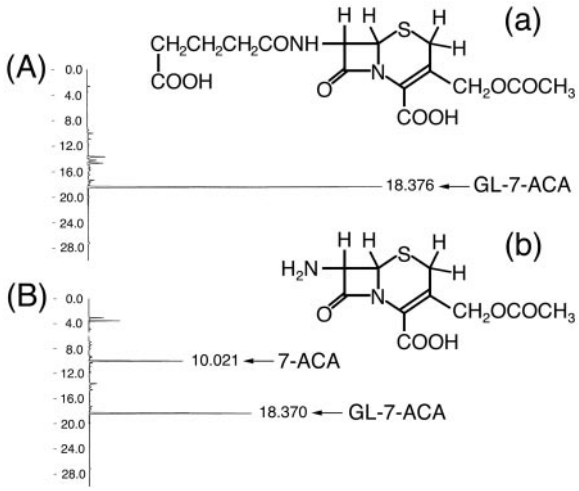

FIG. 2. Chromatograms of HPLC analysis of the reaction mixture of deacylation of GL-7-ACA by the GGT D433N enzyme. The original reaction mixture (2 mM GL-7-ACA, 50 mM Tris-HCl [pH 8.73]) was incubated with 0.1 mg of the GGT D433N enzyme per ml at 37°C for 0 h (A) and 3 h (B). The numbers shown on the left side of the chromatograms are the retention times in minutes. GL-7-ACA eluted at 18.4 min, and 7-ACA purchased from a commercial source eluted at 10.0 min. The structures of GL-7-ACA (a) and 7-ACA (b) are shown to the right of the chromatogram.

tor (model SPD-10AVP; Shimadzu) at an absorbance of 280 nm.

Enzymatic deacylation of GL-7-ACA with the GGT D433N enzyme. Whether the side chain at position 7 of GL-7-ACA could be deacylated with the GGT D433N enzyme was examined. The reaction mixture containing the GGT D433N enzyme was incubated at 37°C for 3 h and analyzed by high-performance liquid chromatography (HPLC). As shown in Fig. 2, a new peak with a retention time corresponding to the 7-ACA purchased from a commercial source was observed after a 3-h incubation. This suggested that the deacylation of GL-7-ACA was catalyzed by the GGT D433N enzyme.

Optimum pH of the deacylating reaction. Since the reaction of GGT is very much influenced by the pH of the reaction mixture, its effect was determined (Fig. 3). The optimum pH of the deacylating reaction of GL-7-ACA as a substrate was 8.73, which is identical to the optimum pH of the transpeptidation reaction using γ-glutamyl-*p*-nitroanilide and glycylglycine as substrates (16), and not to that of the hydrolysis reaction using γ-glutamyl-*p*-nitroanilide as a substrate (3).

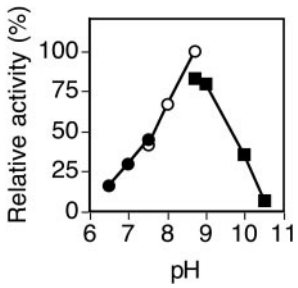

FIG. 3. Effect of reaction pH on the deacylation reaction of GL-7-ACA. The optimum pH for the GGT D433N enzyme was determined in the following buffers: 50 mM potassium phosphate buffer (filled circles), 50 mM Tris-HCl (open circles), and 50 mM NH_4Cl-NH_4OH buffer (filled squares).

Identification of the reaction product as 7-ACA. Although the retention time of the reaction product by HPLC was the same as that of 7-ACA, it was not evident that the peak indicated as the reaction product was in fact 7-ACA. The reaction was performed for 3 h with 730 ml of the reaction mixture. The reaction mixture was applied to a column (30 ml) of Dowex 1X8, which was prepared as the CH_3COO^- form. The column was washed with 150 ml of water. The possible 7-ACA was then eluted with 0.5 N CH_3COOH and lyophilized. The sample was dissolved in water and subjected to reverse-phase HPLC with a Cosmosil 5C18-AR-II column (20 mm by 250 mm) (Nacalai Tesque, Kyoto, Japan). The fraction containing possible 7-ACA was lyophilized, and 18.3 mg of possible 7-ACA was obtained. The possible 7-ACA was subjected to NMR analysis (Bruker 500-MHz spectrometer). As shown in Fig. 4, the NMR chart of the sample exactly matched that of 7-ACA purchased from a commercial source. This indicates that the GGT D433N enzyme indeed catalyzed the deacylating reaction of GL-7-ACA to produce 7-ACA.

Substrate specificity of the GGT D433N enzyme. The substrate specificity of the GGT D433N enzyme was measured using synthesized substrates and cephalosporin C purchased from a commercial source (Sigma-Aldrich). The GGT D433N enzyme cleaved GL-7-ACA at the rate of 0.12 μmol/min/mg, but did not cleave CPC, succinyl-7-ACA, adipyl-7-ACA, or adipyl-7-ADCA at a detectable rate. In this study, the detection limit was 6×10^{-5} μmol/min/mg. The cephalosporin (GL-7-ACA) acylases previously reported had some activity to CPC derivatives other than GL-7-ACA (7). Therefore, the substrate specificity of the GGT D433N enzyme is very strict in comparison with those of other enzymes. This may be because the original structure of GGT was not suited to cleave CPC derivatives.

Kinetic parameters of the GGT D433N enzyme. The kinetic parameters of the GGT D433N enzyme using GL-7-ACA as a substrate were determined. The K_m value for GL-7-ACA was 0.198 mM, and the k_{cat} value was 0.122 s^{-1}. The K_m and k_{cat} values of the class IV cephalosporin (GL-7-ACA) acylases previously reported were 6.1 mM and 17 s^{-1}, respectively (4), and those of GL-7-ACA acylase from *Pseudomonas* sp. strain GK16 were 1.05 mM and 9.48 s^{-1}, respectively (9). The k_{cat} value of the GGT D433N enzyme was low, but the K_m was very low. Therefore, the k_{cat}/K_m value was not very different from those of other enzymes.

In conclusion, the single amino acid substitution of GGT, Asp-433 to Asn, converted GGT to cephalosporin (GL-7-ACA) acylase. Although the k_{cat} value has to be increased by introducing other mutations before it can be applied in the pharmaceutical industry, this study showed that the *ggt* gene could be a new source for cephalosporin (GL-7-ACA) acylase. Since GGT is known to be distributed widely in biological organisms and eukaryotic GGTs are 500- to 1,000-fold more active than *E. coli* GGT, it is anticipated that we could find a much better *ggt* gene from other organisms.

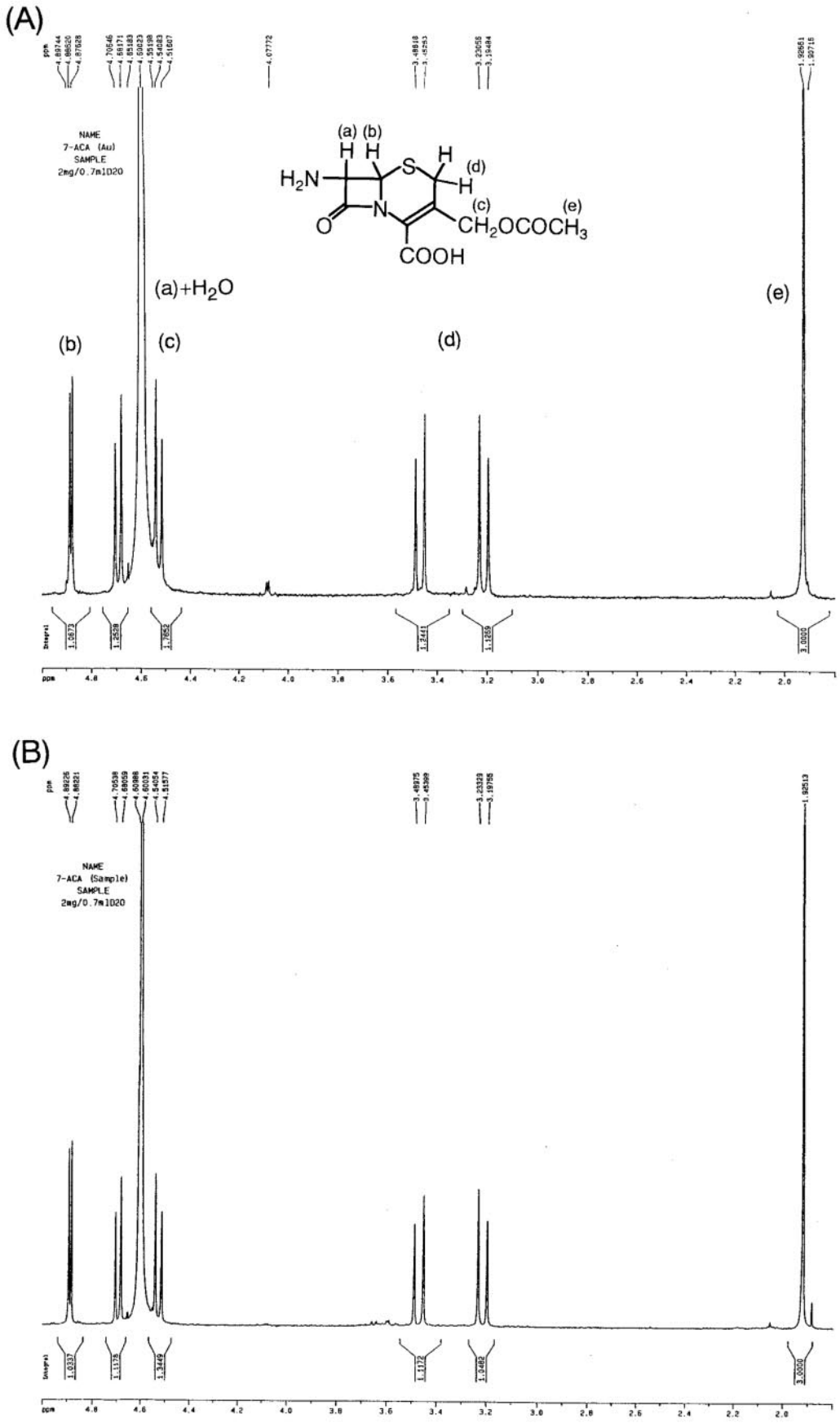

FIG. 4. ^{1}H-NMR spectrum of 7-ACA obtained from a commercial source (A) and that obtained as described in the text (B). Both were measured in D_2O (2 mg/0.7 ml) with a Bruker 500-MHz spectrometer. Each peak is assigned to a proton of 7-ACA and assigned a letter.

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