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Structural and Functional Importance of Two Glutamate Residues, Glu47 and Glu146, Conserved in *N*-Carbamyl D-Amino Acid Amidohydrolases

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Abstract The mutant enzymes of *N*-carbamyl-D-amino acid amidohydrolase (*N*-carbamylase) from *Agrobacterium radiobacter* NRRL B11291, showing a negligible activity, were selected from the library generated by random mutagenesis. From the sequence analysis, these mutants were found to contain the amino acids substitutions at Cys172, Glu47, and Glu146. Previously, Cys172 was reported to be necessary for the enzyme catalysis. The chemical modification of the *N*-carbamylase by carboxyl group specific chemical reagent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), resulted in a loss of activity. The replacement of glutamic acids with glutamines by site-directed mutagenesis led to aggregation of the enzymes. Mutant enzymes fused with maltose binding protein (MBP) were expressed in soluble form, but were inactive. These results indicate that two glutamic acid residues play an important role in structure and function of the *N*-carbamylase. Multiple sequence alignment of the related enzymes revealed that Glu47 and Glu146 are rigidly conserved, which suggests that these residues are crucial for the structure and function of the functionally related C-N hydrolases.

Key words: *N*-Carbamyl-D-amino acid amidohydrolase, glutamic acid, C-N hydrolase

Optically active D-amino acids have been widely used in the pharmaceutical field as intermediates for the synthesis of semisynthetic antibiotics, peptide hormones, pyrethroids, and pesticides [1, 30]. Yamada *et al.* developed a method to synthesize D-amino acids in which a racemic mixiture of chemically synthesized D- and L-5-monosubstituted hydantoins were used as a starting substrates [1]. Of the two isomers, the D-form substrate is only hydrolyzed to the corresponding *N*-carbamyl-D-amino acid by the action of a D-hydantoinase. The resulting *N*-carbamyl-D-amino acid is further converted by either enzymatic or the chemical method. Unreacted L-form substrate is spontaneously racemized to D-form substrate under alkaline condition, which leads to 100% conversion. Enzymatic method using a *N*-carbamylase offers several advantages over chemical one, and much attention has been paid to the *N*-carbamylase to develope a fully enzymatic process.

The *N*-carbamylases have been isolated from *Agrobacterium* [20, 23, 27], *Pseudomonas* [8, 32], *Arthrobacter* [18], *Comamonas* [24], and *Blastobacter* [25], and their properties have been investigated. Recently, the gene encoding the *N*-carbamylase of *Agrobacterium* was cloned and over-expressed in *E. coli* [3, 20, 15], and co-expression or immobilization of the enzyme was attempted for the process development [6, 21, 26]. However, the structure-function relationship and physiological role of the enzyme have received a wide attention.

It has been reported that the *N*-carbamylase is generally labile under the condition of oxidation. It has much lower activity than the first-step enzyme, D-hydantoinase, which has limited the practical application of the enzyme [9]. We have been working on the development of a complete enzymatic process using the D-hydantoinase of *Bacillus stearothermophilus* SD1 and the *N*-carbamylase of *Agrobacterium radiobacter* NRRL B11291 [26]. It was observed that the *N*-carbamylase possessed a much lower stablility and activity compared to the D-hydantoinase.

In this paper, we performed the random mutagenesis of the *N*-carbamylase from *A. radiobacter* NRRL B11291 by using error-prone PCR. Mutants exhibiting a negligible activity compared to the wild type enzyme were selected for further analysis. As a result, most of the selected mutants were found to contain the substitutions at the site of Cys172, Glu47, or Glu146. The Cys172 was previously reported to be involved in enzyme catalysis [7]. To understand some insights into the functional importance

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of Glu47 and Glu146, chemical modification and site-directed mutagenesis were carried out. Details are reported herein.

MATERIALS AND METHODS

Enzymes and Reagents

N-Carbamyl-D-*p*-hydroxyphenylglycine (NC-HPG) was synthesized as described in our previous work [9]. Restriction enzymes and T4 ligase were from Boehringer Mannheim GmbH (Manheim, Germany). DNA polymerases (Vent polymerase and Taq polymerase) were purchased from New England Biolabs Inc. (Beverly, U.S.A.) and Kosco (SungNam, Korea), respectively. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was from Sigma (St. Louis, U.S.A.). Other reagents were of analytical grade.

Cloning of the *N*-Carbamylase

The gene encoding the *N*-carbamylase was amplified from chromosomal DNA of *A. radiobacter* NRRL B11291 by PCR using the following primers: N-terminal primer, 5'-CAAAGGTCCATG GCACGTCAGATG-3'; C-terminal primer, 5'-AAGGGATCCTTAT CGAATTCCGCGATCAG-3' (DNA International Inc., Lake Oswego, U.S.A). The amplified 0.9-kb of DNA fragment was digested with *Nco*I and *Bam*HI, and inserted into pTrc99A expression vector. The resulting plasmid pCAB1 was transformed into *E. coli* JM109 by electroporation (Gene Pulser® II Electroporation System, Bio-Rad, Hercules, U.S.A.).

Random Mutagenesis and Primary Screening

Random mutagenesis of the *N*-carbamylase was carried out by slightly modified error-prone PCR as described by Leung, D. W. *et al.* [16]. Reaction conditions were: 1 unit of Taq Polymerase, 1 mM of dATP and dCTP, 0.2 mM of dTTP and dGTP, and 1 mM of $MnCl_2$. The amplified DNA fragment was digested by *Nco*I and *Bam*HI, and inserted into pTrc99A. The resulting plasmid was transformed into *E. coli* JM109 by electroporation.

The mutant library was transferred to LB agar plate containing 0.1 mM of IPTG and incubated for 16 h at 37°C. Colonies were again transfered to Whatman filter paper and lysed with 1 mM of EDTA and 2 mg/ml of lysozyme for 30 min at 37°C. The filter paper was layed on the color developing plate as described by Park *et al.* [26] and incubated for 30 min at 37°C. Colonies showing no color change were isolated and subjected to further investigation.

Enzyme Purification

Isolated cells were grown in 500 ml of LB medium, and 1 mM of IPTG was added to the culture medium for induction when the absorbance at 600 nm reached approximately 0.6. Cells were grown further for 3 h and collected by centrifugation. Separated cells were resuspended in 20 mM of HEPES buffer (pH 7.0) containing 1 mM of dithiothreitol (DTT) and 1 mM of phenylmethanesulphonyl fluoride. Cells were disrupted by sonification (Sonifier, Branson Sonic power Co., Danbury, U.S.A.). After adding 0.1% protamine sulfate and incubating on ice for 1 h, cell debris were removed by centrifugation at 12,000 × *g* for 1 h. Supernatant was saved and loaded onto the Phenyl Superose High-Trap column (Amersham Pharmacia Biotech, Uppsala, Sweden), equlibrated with 20 mM of HEPES buffer (pH 7.0) containing 4% of sodium sulfate in FPLC system (Amersham Pharmacia Biotech). The column was eluted with reverse linear gradient (4–0%) of sodium sulfate. The active fractions were pooled and concentrated using a Centricon 100 (Amicon Inc., Bedford, U.S.A.). The concentrated protein solution was loaded on Sepharose Q Resource column (Amersham Pharmacia Biotech) equilibratd with 20 mM of HEPES buffer (pH 7.0), and the column was eluted with linear gradient (0–1 M) of NaCl. All purification steps were conducted at room temperature in the presence of 1 mM of DTT.

When the pMAL vector (New England Biolabs) was put into use, purification of the fused enzyme was carried out according to the method described by the supplier.

Chemical Modification of the *N*-Carbamylase

N-Carbamylase was chemically modified with EDC. 100 mg of purified enzyme was incubated with 25 mM of EDC in 100 mM of phosphate buffer (pH 7.0) at 40°C. Aliquots were taken from the enzyme solution at intervals and added to the reaction mixture containing 5 g/l NC-HPG in 100 mM of phosphate buffer (pH 7.0), and residual activity was determined by using HPLC system (Shimazu Co., Kyoto, Japan).

Site-Directed Mutagenesis of the *N*-Carbamylase

The gene encoding the *N*-carbamylase was inserted into the *Nco*I/*Bam*HI site of mutation vetor pALTER-EX2 (Promega), and the resulting plasmid was used for site-directed mutagenesis with a kit from Promega accorgding to the method described by the supplier. The sequences of the synthetic oligonucleotide (DNA International Inc.) for site-directed mutagenesis were as following: 5'-TCGTCT-TTCCCC**A**GCTTGCGCTC-3' for change of Glu47 to Gln; 5'-TCCAGCATCTTC**A**AAAGCGTTATTT-3' for change of Glu146 to Gln. Mutation sites are denoted in bold. The mutated gene was cloned into the expression vector, pTrc99A or pMAL C2, and expressed in *E. coli* JM109 by adding 1 mM of IPTG. The mutations were confirmed by DNA sequencing.

Analysis

The activity of *N*-carbamylase was determined as described in our previous work [9]. Analytical SDS/PAGE in slab

gels was performed according to the method of Laemmli [17]. Acrylamide gels were stained with Coomassie Brilliant Blue R250. The protein concentration was determined by the method of Bradford [4].

RESULTS AND DISCUSSION

Cloning, Expression, and Random Mutagenesis of the *N*-Carbamylase Gene

The gene enconding the *N*-carbamylase was amplified from the chromosomal DNA of *A. radiobacter* NRRL B11291 and inserted into the pTrc99A expression vector. The resulting plasmid was transformed into *E. coli* JM109 and expressed by adding 1 mM of IPTG. The gene was sequenced and found to be identical to that reported [3, 7]. Expression level of the *N*-carbamylase was approximately about 8-10% of the total soluble proteins (data not shown).

Mutant library was generated by using error-prone PCR, and colonies showing a negligible enzyme activity were isolated to examine the specific amino acid residues involved in catalysis or structure of the enzyme. From the DNA sequence, it was found that most of the selected mutants contain the modifications at Cys172, Glu47, or Glu146. It was recently reported that Cys172 is critical for enzyme catalysis [7].

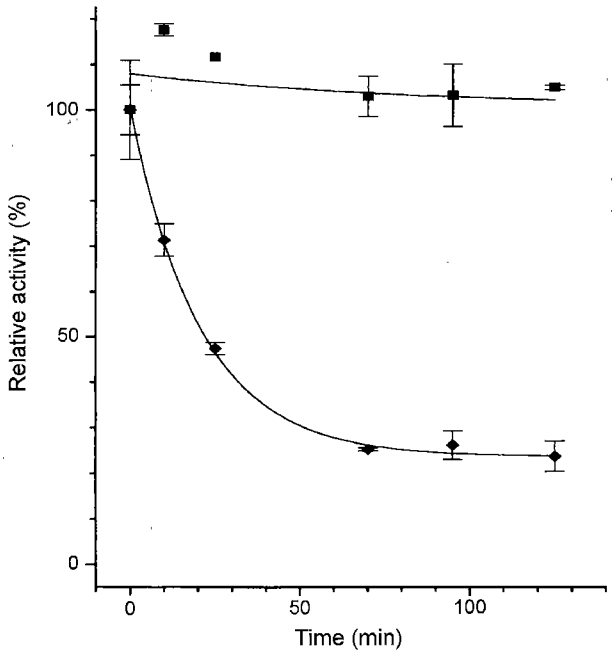

Fig. 1. Inhibition of *N*-carbamoylase activity by EDC.
Purified enzyme was incubated in the presence and absence of 25 mM EDC, and remaining activity was determined as a function of time. Symbols are: (■) in the absence of EDC; (◆) in the presence of EDC.

Chemical Modification of the *N*-Carbamylase

Chemical modification is useful method to investigate the role of certain amino acids [28]. To test whether the carboxyl groups of Glu47 and Glu146 are linked with the loss of enzyme activity, we investigated the inhibition effect of EDC on the N-carbamylase activity. EDC is known to react specifically with the carboxyl group [33]. As shown in Fig. 1, residual enzyme activity decreased to 20% within 2 h when incubated with 25 mM EDC in the absence of nucleophiles. From the above result, it is likely that carboxyl groups of the N-carbamylase definitely play a crucial role in catalysis or structure of the enzyme.

Site-Directed Mutagenesis of the *N*-Carbamylase

Site-directed mutagenesis is useful method to investigate the role of certain amino acid. [11] Glu47 and Glu146 were replaced with glutamines by site-directed mutagenesis. However, the mutant enzymes, E47Q and E146Q, were found to aggregate when analyzed on SDS/PAGE (data not shown). Thus, we cloned the genes encoding E47Q and E146Q into pMAL vector to express the mutant enzymes as fusion proteins with a maltose binding protein (MBP). The MBP is generally known to have a high folding ability, leading to the efficient production of unstable fusion partner. As shown in Fig. 2, fusion proteins were expressed with a correct molecular mass on SDS/PAGE. The MBP-fused mutant enzymes were purified according to the method described by the supplier, but purified fusion enzymes did not exhibit a detactable activity (<0.1% compared to wild

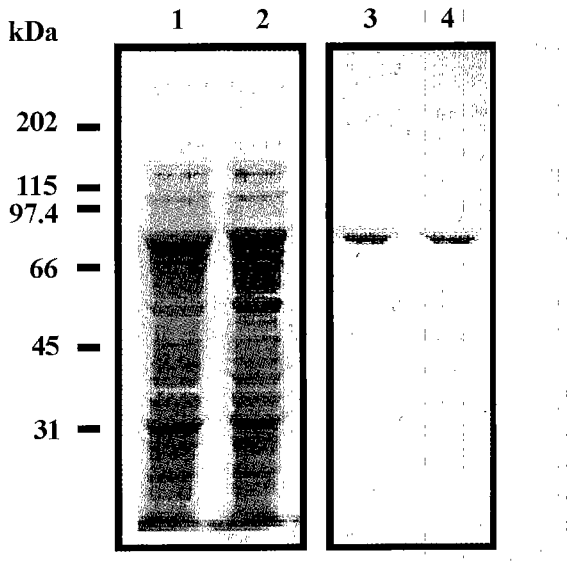

Fig. 2. SDS/PAGE analysis of the *N*-carbamoylase fused with MBP.
Lanes: 1. crude extract of *E. coli* JM109 with pE47Q; 2. crude extract of *E. coli* JM109 with pE146Q; 3. purified fusion protein of MBP-E47Q; 4. purified fusion protein of MBP-E146Q.

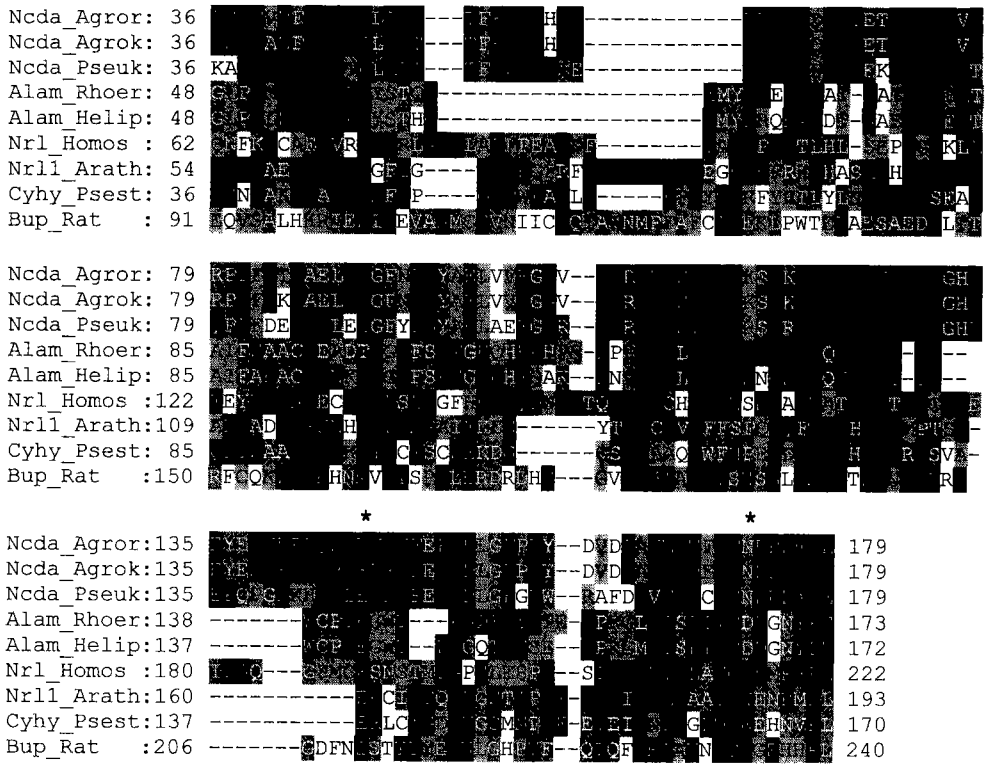

Fig. 3. Multiple sequence alignment of the *N*-carbamylase and functionally related C-N hydrolases by ClustalX program.
Ncda_Agror, *N*-carbamylase from *A. Radiobacter* NRRL B11291 [3,7]; Ncda_Agrok, *N*-carbamylase from *Agrobacterium* sp. KNK712 [20]; Ncda_Pseuk, *N*-carbamoylase from *Pseudomonas* sp. KNK003A [8]; Alam_Rhoer, aliphatic amidase from *Rhodococcus erythropolis* [28]; Alam_Helip, aliphatic amidase from *Helicobacter pylori* 26695; Nrl_Homos, Nitrilase from *Homo sapiens*; Nrl1_Arath, Nitrilase1 from *Arabidopsis thaliana* [2]; Cyhy_Psest, Cyanide hydratase from *P. stutzeri* [30]; Bup_Rat, β-alanine synthase from *Rattus norvegicus* [13]. Gene sequence without reference was obtained by BLAST program (http://www.ncbi.nlm.nih.gov). Mutation sites in this work and Cys172 are denoted by astericks. Highly conserved amino acid residues are shown in the black boxes with white letters. Relevant amino acid similarities are indicated by grey boxes.

type). The MBP-fused mutant enzymes were treated with factor Xa for further purification, but the fused enzymes were not cleaved by factor Xa. It is known that misfolded protein is resistant to proteolytic action of factor Xa. Based on the results that functional expression of the mutant enzymes in *E. coli* is difficult, and that the fused enzymes are not cleaved with factor Xa, it seems that Glu47 and Glu146 are closely aligned to maintain functional structure of the *N*-carbamylase.

Multiple Sequence Alignment of the *N*-Carbamylase and Functionally Related C-N Hydrolases

The comparative study of enzymes that catalyse the same type of reaction but have a different substrate specificity and kinetic properties is a useful approach to understand the molecular mechanism of the enzyme reaction when no structural data are available [10]. In this context, we aligned the amino acid sequences of the *N*-carbamylase with the C-N hydrolase family enzymes, including aliphatic amidase [22, 29], nitrilase [19, 12], β-alanine synthase [13], and cyanide hydratase [31] as suggested by Bork *et al.* [5]. These enzymes are known to share a similar reaction mechnism and structural conservation. Multiple sequence alignment revealed that Glu47 and Glu146 are rigidly conserved among the enzymes, and previously identified Cys172 is also conserved (Fig. 3). Hydrophobicity and secondary structure around these regions were predicted by using the GCG program, and they were observed to be similar in the related enzymes (data not shown). Mutation of the *N*-carbamylase at Glu47 or Glu146 resulted in the aggregation of the enzymes, which strongly implies that these amino acid residues play a critical role in the structure and function of enzymes which belong to the C-N hydrolase family.

The enzymes belonging to the C-N hydrolase family have high potential for biotechnological applications [14]. In this regard, the experimental data presented here are expected to make a contribution to generating an enzyme with greater potential.

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