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D-Amino acid aminotransferase: fragmentation at a flexible loop is an efficient method to generate mutant enzymes with new substrate specificities and elevated activities

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

D-Amino acid aminotransferase (D-AAT) (EC 2.6.1.21) catalyzes the interconversion between various D-amino acids and α-keto acids. A subunit of the homodimeric enzyme from a thermophile, *Bacillus* sp. YM-1, consists of two distinct structural domains connected by one loop. We previously constructed an active fragmentary enzyme whose backbone was cut at the interdomain loop [J. Biochem. 124 (1998) 905]. In this work, we constructed 13 fragmentary D-amino acid aminotransferase genes by inserting a termination codon, an SD sequence, and an initiation codon into the specific positions of the gene corresponding to various loop regions and expressed in *Escherichia coli* cells. We have obtained six genes producing active fragmentary enzymes, one producing an inactive fragmentary enzyme, four producing only large peptide fragment, and another two that gave no products. The six active fragmentary enzymes purified to near-homogeneity showed various substrate specificities and thermostabilities distinct from each other and also from the wild-type enzyme: two exhibited higher catalytic activity towards D-alanine, the most efficient substrate, than the wild-type enzyme. These results suggest that cleavage at a loop region is an efficient method for the alteration of enzyme properties.

Keywords: D-Amino acid aminotransferase; Folding; Fragmentary enzyme; Pyridoxal 5′-phosphate

1. Introduction

D-Amino acid aminotransferase (D-AAT) [EC 2.6.1.21] requires pyridoxal 5′-phosphate (PLP) as a coenzyme and catalyzes the interconversion of various D-amino acids and α-keto acids (Scheme 1). The enzyme has been regarded as a target for designing specific inhibitors that serve as an antibacterial agent because the enzyme participates in the metabolism of D-glutamate, an indispensable component of the peptidoglycan layer of bacterial cell walls [1–3]. D-AAT is also important as a biocatalyst: the enzyme of a thermophile, *Bacillus* sp. YM-1 was used for the enzymatic synthesis of various D-amino acids from the corresponding α-keto acids in combination with alanine racemase, alanine dehydrogenase, and formate dehydrogenase [4,5]. In the system, D-glutamate and D-valine were produced with yields of 85%. In contrast, yields of D-phenylalanine and D-tyrosine were 15 and 5%, respectively [5].

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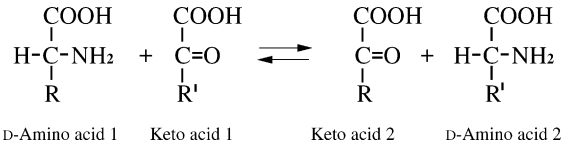

Scheme 1.

Difference in the yields reflects the substrate specificity of D-AAT. Probably, we can improve the productivities of D-phenylalanine and D-tyrosine by altering the substrate specificity of D-AAT with protein engineering.

D-AAT of *Bacillus* sp. YM-1 consists of two identical subunits with 282 amino acid residues. Each subunit of D-AAT is composed of two domains connected with a single loop [6–8]. We previously constructed a fragmentary D-AAT whose backbone was cut between Pro119 and Arg120 of the interdomain loop. The cleavage reduced the susceptibility of the enzyme to the inhibition by α-ketoglutarate and increased the catalytic efficiency towards several substrates, such as D-arginine and D-lysine, when α-ketoglutarate was used as an amino acceptor [9]. Such an increase in the catalytic efficiency is probably advantageous for the enzymatic synthesis of D-amino acids with D-AAT [4,5]. The X-ray crystallographic study of D-AAT revealed that the enzyme contains more than 10 loop structures in the enzyme monomer in addition to the interdomain one [8]. In this study, we attempted to obtain various fragmentary D-AATs with variety of catalytic properties by cleavage of the backbone at the various loop regions of the enzyme. We here showed the construction, expression, and properties of the fragmentary D-AATs cleaved at various loop regions.

2. Experimental

2.1. Materials

Plasmid pICT113p containing the D-AAT gene of *Bacillus* sp. YM-1 was prepared as described previously [7]. The expression vector, pKK223-3, was from Pharmacia LKB Biotechnology (Upsala, Sweden). The oligonucleotides used for mutagenesis were synthesized by means of phosphoamidite chemistry. *Escherichia coli* JM109 and enzymes used for DNA manipulation were from Takara Shuzo, Kyoto, Japan. All D-amino acids used in this study were from Nacalai Tesque, Kyoto, Japan. All other reagents and chemicals were of analytical grade.

2.2. Construction of plasmids encoding fragmentary enzymes

The expression vectors encoding 13 fragmentary D-AATs were constructed by the polymerase chain reaction (PCR) with the overlap-extension procedure [10]. These fragmentary enzymes were designed to be clipped between two residues, G39 and E40 (the mutant enzyme was labeled as F39); I60 and T61 (F60); N80 and E81 (F80); E104 and N105 (F104); N118 and P119 (F118); N172 and N173 (F172); M199 and I200 (F199); V220 and K221 (F220); Y242 and S243 (F242); and K260 and V261 (F260). The primers used for PCR are shown in Fig. 1. The N-forward primer was designed based on the sequence upstream of the promoter region of D-AAT gene and contained a *Eco*RI site. The C-reverse primer was designed based on the sequence of the C-terminal region of the D-AAT gene and contained a *Hind*III site and a stop codon. For the first PCR, 13 different sets of

Fig. 1. Primers used for the construction of fragmentary enzymes.

the C-forward and N-reverse primers were prepared. Parts of the C-forward and N-reverse primers in each set are complementary with each other and contained a stop codon, a *Pst*I site, an SD sequence, a *Sma*I site, and an initiation codon. Each C-forward primer encoded the sequence which is complementary to that corresponding to the 5′-terminal region of each C-terminal fragment. Each N-reverse primer encoded the sequence corresponding to the 3′-terminal region of each N-terminal fragment. Plasmid pICT113p was used as a template for the first PCR with each set of N-forward and -reverse or C-forward and -reverse primers. The second PCR was carried out with N-forward and C-reverse primers and the product of the first PCR as a template. Each PCR cycle consisting of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 74 °C for 1 min was repeated 30 times with a KOD DNA polymerase (Toyobo, Osaka, Japan). The obtained PCR fragment was subcloned into plasmid pKK223-3. Construction of each plasmid was verified by DNA sequencing with an Applied Biosystems 370A automated DNA sequencer. The plasmids obtained were used for the transformation of *E. coli* JM109 cells.

2.3. Protein purification

Six fragmentary enzymes, F60, F81, F104, F118, F173, and F199, which showed D-AAT activity, were purified with DEAE-Toyopearl and butyl-Toyopearl column chromatographies as described previously [9]. The homogeneity of the final preparation was judged by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined either by absorbance at 280 nm ($A^{1\%}_{1\,cm}$, 1.23) or with a BioRad protein assay kit (Biorad, CA, USA).

2.4. Enzyme assay

The overall reaction with D-alanine and α-ketoglutarate as substrates was assayed at 37 °C as follows. The assay mixture (1 ml) contained a 100 mM tris(hydroxymethyl)aminomethane–HCl (Tris–HCl) buffer (pH 8.0), 0.2 mM NADH, 50 μM PLP, 5 units of rabbit muscle lactate dehydrogenase (Sigma, USA), D-AAT (0.5–0.1 μg/ml), and various concentrations of D-alanine and α-ketoglutarate. The reaction was started by the addition of D-AAT, and the decrease in absorbance at 340 nm was followed. Reactions with other amino donors were assayed by the determination of D-glutamate produced from α-ketoglutarate. The assay mixture consisting of a 100 mM Tris–HCl buffer (pH 8.0), 50 mM D-amino acid as an amino donor, 20 mM α-ketoglutarate, 50 μM PLP, and D-AAT was incubated at 37 °C for an appropriate time. The D-glutamate formed was determined with a BEACKMAN SYSTEM7300 amino acid analyzer.

2.5. Spectrophotometric measurements

Absorption spectra were obtained with a BEACKMAN DU640 spectrophotometer. Circular dichroism spectra were taken with a Jasco J-600 spectrophotometer at 25 °C with a 1 cm light path cell.

3. Results

3.1. Construction of the fragmentary D-AAT genes

Thirteen fragmentary D-AAT genes were constructed by the insertion of a stop codon, an SD sequence, and an initiation codon into the specific positions of the gene corresponding to the loop region of the enzyme. The position between the amino acid residues conserved among D-AATs was avoided as the cleavage point because conserved residues are possibly important for the catalytic function. The cleavage positions of the fragmentary enzymes on the primary and three-dimensional structures are shown in Figs. 2 and 3, respectively.

3.2. Expression and purification of fragmentary D-AATs

We previously obtained F118 by the co-expression of two plasmids encoding each N- or C-terminal fragment [9]. In this study, we prepared each fragmentary enzyme by using a single plasmid encoding the N- and C-terminal fragment genes in tandem. The amount of F118 produced with this single plasmid was 30-fold higher than that produced with the two plasmids. We measured the D-AAT activity in the soluble fraction of the host cells and found that six fragmentary enzymes are active: F60, F80, F104, F118, F172, and

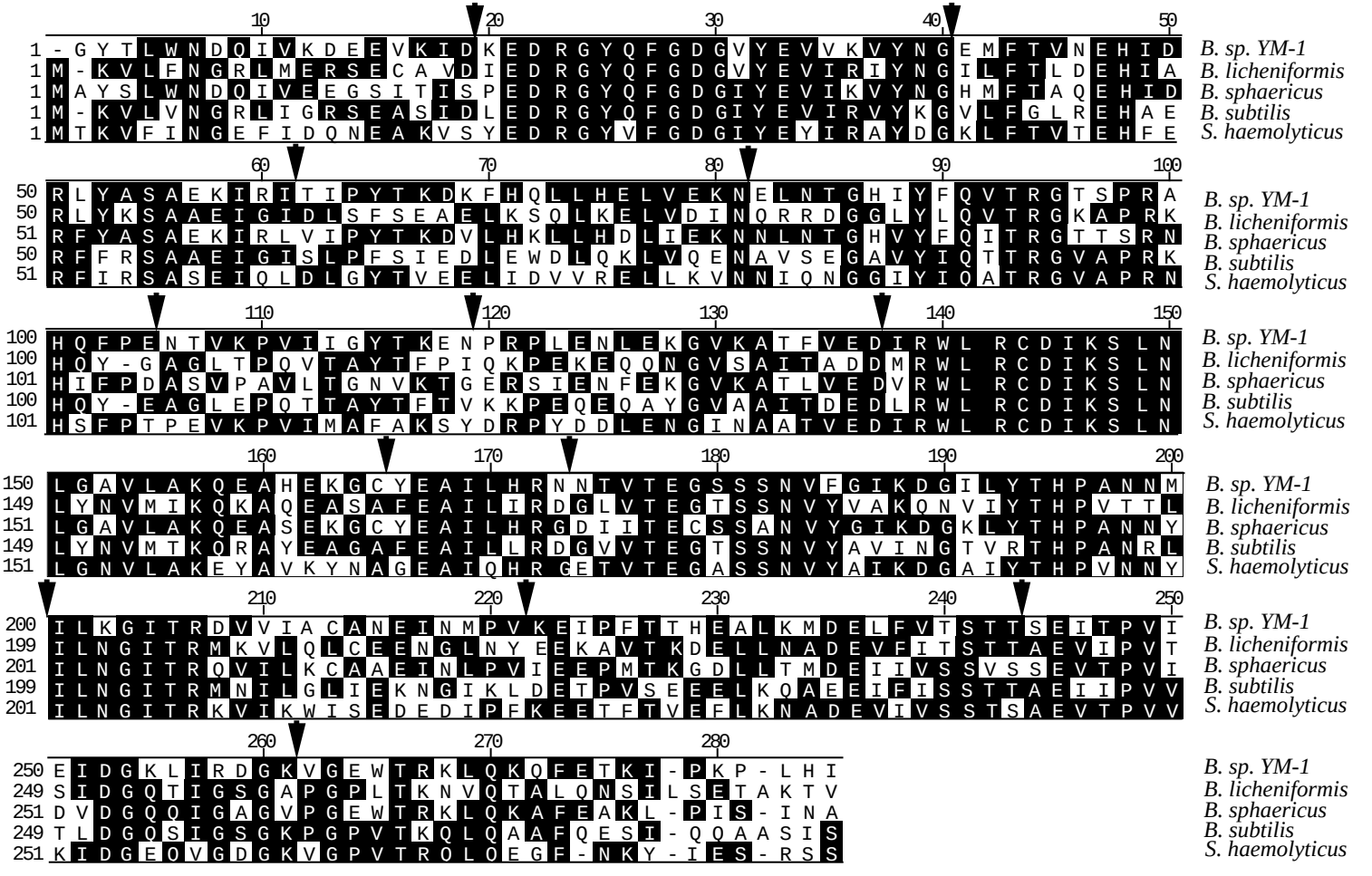

Fig. 2. Alignment of the amino acid sequences of D-AAT from *Bacillus* sp. YM-1, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus sphaericus*, and *Staphylococcus haemolyticus*, and sites of the fragmentation. Alignment was carried out with the CLUSTAL V program of DNASTAR software (DNASTAR Inc.). Common residues are shown by shadowing. Positions of the fragmentation are shown by arrows.

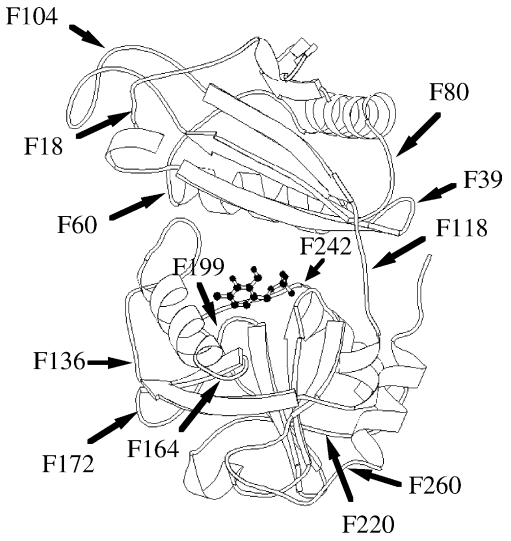

Fig. 3. Three-dimensional structure of the D-AAT monomer [8] and sites of the fragmentation. Positions of the fragmentation are shown by arrows.

F199. D-AAT proteins in the cell extracts were detected by Western blotting with an antiserum against the wild-type enzyme (data not shown). F60, F118, F172 and F199 showed two bands corresponding to the N- and C-terminal fragments by Western blotting. In contrast, F80 and F104 provided no detectable protein bands. D-AAT activities of F80 and F104 in the cell extracts were lower than those of other fragmentary enzymes. Absence in the protein bands of F80 and F104 was probably because of their inefficient expression. Both fragmentary enzymes could be partially purified from a large amount of cells as described below. F164 proteins were obtained in the soluble fraction but showed no activity. The cells with the plasmids encoding F39, F220, F242 or F260 produced only a large fragment as an insoluble form. The small fragment of these four fragmentary enzymes could not be detected even with Tricine-sodium dodecylsulfate polyacrylamide gel electrophoresis used for the separation of short peptides [11]. No fragments were observed in the soluble or insoluble fractions of the host cells containing the plasmids encoding F18 or F164. The results of expression and activity of the fragmentary enzymes are summarized in Fig. 4.

We purified F60, F118, F172, and F199 to near-homogeneity and F80 and F104 to purities of about 80 and 70%, respectively. The molecular weights of the purified fragmentary enzymes estimated by gel filtration were about 64,000, which is similar to that of the wild-type enzyme. The results suggest that these active fragmentary enzymes consist of two sets of N-terminal and C-terminal fragments.

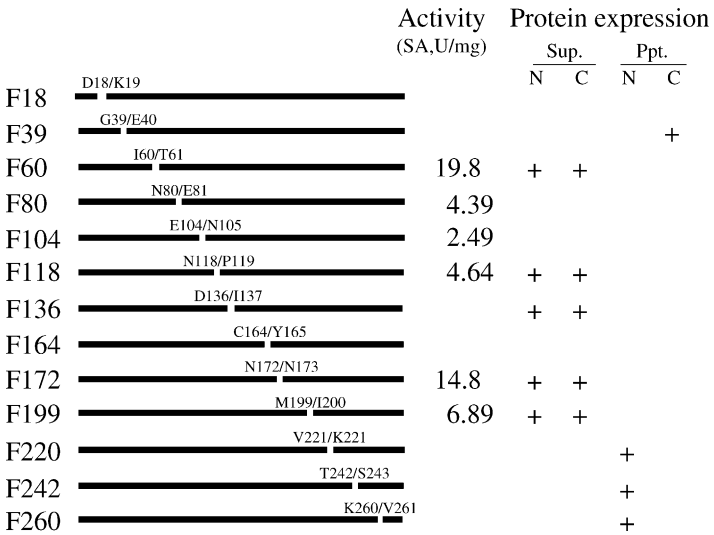

Fig. 4. Summary of the expression and activity of the fragmentary enzymes. D-AAT activity in the soluble fractions was measured with D-alanine and α-ketoglutarate as substrates.

Table 1
Overall reactions catalyzed by the wild-type and fragmentary D-AATs

Amino donor[a]	Specific activity (μmol/(mg min))						
	WT[b]	F118	F60	F80	F104	F172	F199
D-Alanine	220	160	420	89	161	270	190
D-Asparagine	84	110	110	17	30	73	14
D-Methionine	65	73	61	12	14	98	11
D-Glutamine	56	72	20	30	37	23	23
D-Serine	17	18	18	3.4	5.8	11	2.4
D-Norleucine	15	14	10	1.8	–	11	–
D-Valine	12	17	6.9	1.5	2.2	10	1.0
D-Histidine	4.9	7.1	8.1	0.60	1.2	3.8	0.60
D-Phenylalanine	3.2	4.6	1.7	0.40	0.40	1.6	0.20
D-Arginine	3.2	5.9	3.2	0.50	0.60	1.7	0.40
D-Lysine	1.6	3.2	1.3	0.20	0.30	1.6	0.30

[a] α-Ketoglutarate was used as an amino acceptor.
[b] Wild-type enzyme.

3.3. Catalytic properties of the fragmentary enzymes

We studied the substrate specificities of the wild-type and F60, F172, F118, F199, F80, and F104 enzymes in the overall reactions with α-ketoglutarate as an amino acceptor (Table 1). The substrate specificities of the fragmentary enzymes were different from each other. F60 and F172 showed higher activities towards D-alanine than the wild-type enzyme. F199 was more specific for D-alanine than other enzymes.

α-Ketoglutarate has an inhibitory effect on the wild-type enzyme [12]. The inhibition is probably caused by competition between an amino donor and an amino acceptor for binding to the same site, Arg98, the common binding site for the α-carboxyl group of substrates [12]. The inhibitory effect was reduced in the reaction catalyzed by F118 [9] but increased in that by F199. Other fragmentary enzymes were inhibited by α-ketoglutarate in the similar manner as the wild-type enzyme (Fig. 5).

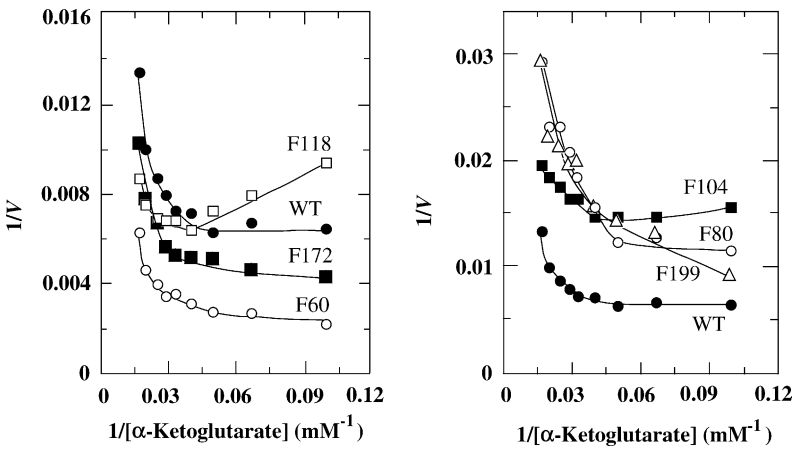

Fig. 5. Double reciprocal plots of the rates of the overall reactions catalyzed by the wild-type and fragmentary enzymes against the concentrations of α-ketoglutarate. The reaction mixture (1 ml) contained 100 mM Tris–HCl buffer (pH 8.0), 10 mM D-alanine, various concentrations of α-ketoglutarate, 0.2 mM NADH, 50 μM PLP, 10 units of lactate dehydrogenase, and the wild-type or fragmentary D-AATs. Other conditions were the same as described in the text.

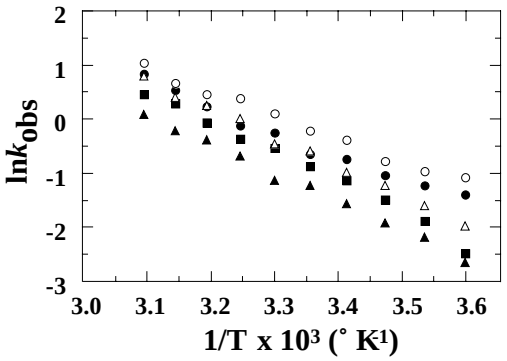

Fig. 6. Temperature dependence of the specific activity of the wild-type and fragmentary enzymes. The reaction mixture (1 ml) containing a 100 mM potassium phosphate buffer (pH 8.0), 10 mM D-alanine, 10 mM α-ketoglutarate, 0.2 mM NADH, 50 μM PLP, 10 units of lactate dehydrogenase, and an appropriate amount of the wild-type enzyme (●), F60 (○), F118 (■), F173 (△), or F199 (▲) was incubated at the indicated temperature.

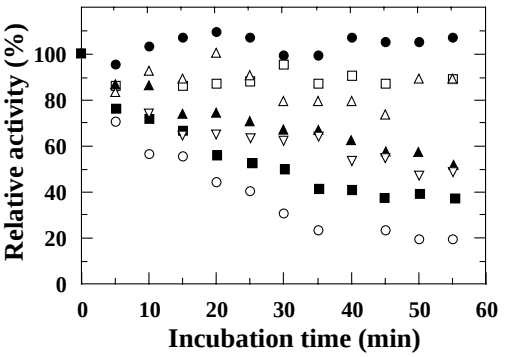

Fig. 7. Thermostability of the wild-type and fragmentary enzymes. The wild-type enzyme (●), F60 (○), F81 (□), F104 (▽), F118 (■), F173 (△) or F199 (▲) was incubated at 50 °C in a 20 mM potassium phosphate buffer (pH 7.3). The remaining activity of the enzymes at the indicated times was assayed at 37 °C as described in the text.

3.4. Effect of temperature

The specific activities of the fragmentary and wild-type enzymes in the overall transamination between D-alanine and α-ketoglutarate were plotted against the temperature. The obtained Arrhenius plots showed a similar pattern among the fragmentary enzymes (Fig. 6). The F60 fragmentary enzyme showed higher specific activities than those of the wild-type enzyme at any temperature measured. The thermostability of the enzyme was also studied. The wild-type, F80 and F172 enzymes retained the full activities after 1.5 h incubation at 50 °C. In contrast, F60, F104, and F199 gradually lost the activity under the same conditions (Fig. 7). F60 showing the highest activity was the most unstable.

4. Discussion

In this study, we constructed 13 fragmentary enzyme genes. *F60*, *F80*, *F104*, *F172*, *F118*, and *F199* genes produced active fragmentary enzymes; *F39*, *F220*, *F242*, and *F260* produced only a large peptide fragment; *F136* produced an inactive fragmentary enzyme; and *F18* and *F164* gave no products. The shortest peptide fragment observed by SDS-PAGE was the N-terminal fragment of F60 consisting of 60 amino acid residues. The small peptide fragment of F18, F260, F39, F242, and F220 consisting of 18, 22, 39, 40, and 61 amino acid residues, respectively, was obtained in neither soluble nor insoluble fractions of the host cells. The length of the peptide fragment probably affects its expression efficiency and/or stability. F136 proteins were obtained in the soluble fraction but showed no activity. Nakamura et al. demonstrated that treatment of phosphoenolpyruvate carboxylase from *Thermus* sp. (ThPEPC) with trypsin and chymotrypsin resulted in the cleavage of the enzyme into two fragments of about 70 and 20 kDa [13]. The cleavage site by trypsin and that by chymotrypsin were apart from each other by only one residue. Nevertheless, trypsin-treated ThPEPC remained active, but the chymotrypsin-treated enzyme was inactive although both cleaved enzymes showed similar tertiary structures and thermostabilities. Whether a fragmentary enzyme is active or not is probably determined by a subtle difference in its local structure.

F60 catalyzes the D-alanine transamination with about twice the efficiency of the wild-type enzyme. The residues, Ile60 and Thr61, at the clipping site of F60 are located at a distance of at least 11 Å from the cofactor and from the residues known to participate in catalysis (e.g. Lys145 and Arg98). Elevation of the activity is probably derived from an indirect linkage between the distant loop and the active-site region. Hayashi et al. [14] reported that the mutation of Val39 distant from the active site of *E. coli* aspartate aminotransferase (AspAT) to leucyl residue resulted in the

k_{cat}/K_m value increasing twice more than that of the wild-type enzyme. The elevation is explained by the shift of the conformational equilibrium of the enzyme towards the closed form, which shows the higher affinity to the substrate than the open form. Gekko et al. [15] demonstrated that the adiabatic compressibility of AspAT was influenced markedly by the mutation of Val39 and showed the proportional relationship with the logarithm of k_{cat}/K_m value of the mutant enzymes. The adiabatic compressibility is related to the flexibility of the enzyme. The results suggest that increase in the flexibility of the overall structure of AspAT makes the enzyme catalytically more efficient. It is possible that the fragmentation at a loop region also affects the catalytic properties of D-AAT through increase in the flexibility. In this context, it is noteworthy that F60 showing the highest activity was the most unstable. Increase in flexibility probably affects the stability of a protein less favorably.

In this study, we obtained various fragmentary D-AATs with altered substrate specificities by fragmentation. Among them, F118 with the elevated catalytic efficiency towards D-phenylalanine is probably useful to improve the yield of D-phenylalanine production from phenylpyruvate in a coupling system with D-AAT, alanine racemase, alanine dehydrogenase, and formate dehydrogenase [4,5]. Elevation of the catalytic efficiency of the enzyme is one of the aims of the protein engineering. However, the rationally designed mutagenesis to manipulate the enzyme, especially mutations of the active-site residues involved in the catalysis, sometimes inactivates the enzyme rather than improves its catalytic property. This is probably due to the disruption of the specialized structure–function relationship, for example, the intricate hydrogen-bonding network that each enzyme has gained during its evolution. Fragmentation at the loop region is probably a convenient tool for obtaining mutant enzymes with improved catalytic properties.

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