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Site-directed mutagenesis alters DnaK-dependent folding process

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Abstract

The overproduction of D-aminoacylase (A6-D-ANase) of *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6 (*Alcaligenes* A-6) is accompanied by aggregation of the overproduced protein, and its soluble expression is facilitated by the coexpression of DnaK–DnaJ–GrpE (DnaKJE). When the A6-D-ANase gene was expressed in the *Escherichia coli dnaK* mutant *dnaK756*, little activity was observed in the soluble fraction, and it was restored by the coexpression of DnaKJE or the substitution of the R354 residue of A6-D-ANase for lysine. These results suggest that the guanidino group of the R354 residue of A6-D-ANase disturbs its proper folding in the absence of DnaK and the disturbance is eliminated by binding of DnaK to the R354 residue in the presence of DnaK. This is the first report that the DnaK-dependent folding process of the enzyme is altered by site-directed mutagenesis.

Keywords: D-Aminoacylase; *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6; DnaK; Chaperone; Folding

N-Acyl-D-amino acid amidohydrolases catalyze the hydrolysis of *N*-acyl derivatives of various D-amino acids to D-amino acids and fatty acids [1]. *N*-Acyl-D-amino acid amidohydrolases are classified into three types based on their substrate specificities. D-Aminoacylase (D-ANase), *N*-acyl-D-glutamate amidohydrolase, and *N*-acyl-D-aspartate amidohydrolase act on the *N*-acyl derivatives of various neutral D-amino acids, D-glutamate, and D-aspartate, respectively [2]. The X-ray crystallographic analysis of D-ANase from *Alcaligenes faecalis* DA1 (DA1-D-ANase) has been reported [3], and D-ANase is industrially used for the optical resolution of DL-amino acids [1].

Alcaligenes A-6 possesses the three types of *N*-acyl-D-amino acid amidohydrolases. D-ANase from the strain (A6-D-ANase) exhibits 46% and 57% sequence identity with *N*-acyl-D-glutamate amidohydrolase (D-AGase) [4] and *N*-acyl-D-aspartate amidohydrolase (D-AAase) [5] of *Alcaligenes* A-6, respectively. The high sequence identities among the three amidohydrolases could be indicative of the similarity of their structures. The overproduction of these enzymes in *Escherichia coli* results in the partial aggregation of the overproduced enzyme [6].

DnaK is a member of the highly conserved hsp70 family of heat shock proteins and functions as a molecular chaperon. Mutation in the *dnaK* gene of *E. coli* causes defects in the growth at low and high temperatures [7], and the propagation of the λ phage [8]. An *E. coli* mutant, *dnaK756*, produces a mutated DnaK protein that has defects in chaperone activity and causes similar phenotypes with the *dnaK* defective mutant [9]. DnaK and its cochaperones, DnaJ and GrpE, mediate protein folding using an ATP-controlled cycle of polypeptide binding and release [10,11]. DnaK binds to partially unfolded polypeptides and prevents their aggregation, thereby stimulating their proper folding. The results of a study using peptide display libraries [12,13] or cellulose-bound peptide libraries [14] show that peptides enriched in hydrophobic and positively charged residues are preferential DnaK substrates. Furthermore, DnaK is shown to bind to the consensus

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motif that consists of central hydrophobic residues and basic residues in both sides [14]. The DnaK-binding peptide regions have been reported in a few proteins [15], and it is considered that DnaK binds these peptide regions to facilitate the folding of the proteins. However, no peptide region has been determined as the region that is responsible for the folding of the proteins among DnaK-binding peptide regions. To study the functions of DnaK for facilitating the proper folding of a protein, it is necessary to identify the peptide region in which the DnaK binding is required for the proper folding of the protein.

Coexpression of DnaKJE increases the soluble expression of A6-D-ANase 1.2-fold [6]. As a result of the coexpression of GroEL and GroES (GroELS), and those of DnaKJE, D-AGase, and D-AAase were produced at levels that were 2.3- and 3.3-fold, respectively, higher than those of the overproduced proteins in an active form [6].

The highly conserved arginine residues among A6-D-ANase, D-AGase, and D-AAase were substituted with lysine by site-directed mutagenesis. We found that the R354K mutant of A6-D-ANase can effectually fold by itself in the absence of DnaK, though DnaK is required for the proper folding of the wild-type, and the R354 residue is one of the DnaK-binding sites that are important for its proper folding. In this report, we showed, for the first time, evidence that the DnaK-binding site facilitates the proper folding of a protein.

Materials and methods

Materials. Plasmid pKNSD2 encoding the A6-D-ANase was prepared as described previously [16]. An expression vector pKK223-3 was purchased from Amersham Pharmacia Biotech, Buckinghamshire, England. *N*-Acetyl-D-leucine was purchased from Sigma, St. Louis, MO, USA. The Taq DyeDeoxy Cycle Sequencing Kit was obtained from Perkin–Elmer, Wellesley, MA, USA. Primers for mutagenesis and DNA sequencing were purchased from Sawady Technology, Tokyo, Japan. A DnaK-null mutant of *dnaK756* was provided by Professor Tohru Yoshimura of the Graduate School of Bioagricultural Sciences, Nagoya University. The M13 primers M4 and RV, pGKJE7, which expresses the DnaKJE chaperone team, ExTaq DNA polymerase, restriction enzymes, and other DNA-modifying agents were purchased from Takara Shuzo, Kyoto, Japan. MonoQ HR 5/5 and the FPLC pump system were obtained from Amersham Biosciences, Piscataway, NJ, USA. All other chemicals were from Wako Pure Chemicals, Osaka, Japan.

Mutation of the arginine residue of A6-D-ANase to lysine. The expression plasmids encoding the mutant A6-D-ANase were constructed by the overlap extension method of Ito et al. [17] with pKNSD2 as a template and the mutagenic primers shown in Table 1. M13 primers M4 and RV were used as the primers hybridized with the 5′- and 3′-ends of the gene of A6-D-ANase. The obtained DNA fragment, flanked by *Eco*RI and *Hin*dIII restriction sites, was ligated into pKK223-3. Construction of the plasmid was confirmed by DNA sequencing.

Culture conditions. The *dnaK756* and JM109 strains harboring a plasmid that expresses A6-D-ANase or its arginine mutant alone or together with the DnaKJE expression plasmid (pGKJE7) were cultured for 12 h at 30 °C in 5 ml of a Luria–Bertani (LB) medium in the presence of 50 μg ampicillin per milliliter (plus 20 μg chloramphenicol per milliliter when necessary). The culture was transferred into 100 ml of a LB medium with antibiotics in 500-ml Erlenmeyer flasks and cultured for 18 h at 30 °C on a reciprocal shaker at 100 rpm.

Table 1
Oligonucleotides used in site-directed mutagenesis

Mutation	Mutagenic oligonucleotide
R26K	5′-CAGGTCGGCGCGTTTCCCCGGGGTGTT-3′
R152K	5′-AATGACCGCGGCTTTCAGCGTTGAATG-3′
R296K	5′-GGCCAGCAGCACTTTGTCCTGCTTGAG-3′
R302K	5′-GGTGATGATGGTTTTTCCGGCCAGCAG-3′
R354K	5′-GAACGCCAGGATTTTCTGCACGTCGGG-3′
R377K	5′-GGTGCCCCACAGTTTCGGATGCGGGCG-3′
R391K	5′-CAGGCCCAGGTCTTTCGCATAGTGCCC-3′

The substituted nucleotides are underlined.

Assay of enzyme activity. D-ANase activity was assayed by measuring D-leucine formed from the hydrolysis of *N*-acetyl-D-leucine as previously described [18]. One unit of the enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmol of D-leucine per minute. Specific activity is expressed as units per milligram of protein. The protein concentration was estimated using the Lowry method with crystalline egg albumin as the standard [19].

Purification of the wild-type and mutant enzymes. The recombinant wild-type A6-D-ANase was purified as previously described [16]. All the mutant enzymes were purified fundamentally by following the same procedure as that used for the recombinant wild-type enzyme, but, for the purification of mutant enzymes, additional ion-exchange column chromatography using FPLC MonoQ HR 5/5 column was performed with a 0–0.5 M NaCl linear gradient with a FPLC pump system.

Molecular modeling and structural analysis. The structure of A6-D-ANase was predicted by homology modeling. Since A6-D-ANase shares 86% sequence identity with DA1-D-ANase, the homology modeling was done on the basis of the structure of DA1-D-ANase, which was used as a reference by means of the program MODELLER, version 6 [20]. Five models were generated for each A6-D-ANase structure. The qualities of the structures were examined with PROCHECK [21], and the models with the best stereochemical parameters were selected for structural analysis.

Results

Expression of the wild-type and arginine mutants of A6-D-ANase genes

Since *E. coli* host cells show no D-ANase activity, the activity represents the existence of the enzyme in the soluble fraction of the cells. Plasmids expressing wild-type or arginine mutants of A6-D-ANase genes were constructed by inserting these genes into pKK223-3, and the expressions were under the control of the *tac* promoter, which are inducible using isopropyl 1-thio-β-D-galactoside (IPTG). Our preliminary experiments showed that 1.0 mM IPTG in the culture reduced the production of active A6-D-ANase and that 0.1 mM IPTG was ineffective for inducing the enzyme (data not shown). In subsequent experiments, *E. coli* cells harboring the expression plasmid were cultured in the absence of IPTG. The conserved arginine residues R26, R152, R296, R302, R354, R377, and R391 were individually replaced by lysine. Table 2 shows the

Table 2
Specific activities of the wild-type and arginine mutants in the crude extract and kinetic properties of the purified enzymes

Enzyme	Specific activity (U/mg)	K_m (mM)	V_{max} (U/mg)	k_{cat} (1/min)	k_{cat}/K_m (1/mM min)
Wild-type	195	15.7	945	4.91×10^4	3.13×10^3
R26K	0.14	ND	ND	ND	ND
R152K	0.39	1.75	0.55	28.6	16.3
R296K	0.01	ND	ND	ND	ND
R302K	2.70	9.26	348	1.81×10^4	1.95×10^3
R354K	122	24.3	951	4.95×10^4	2.04×10^3
R377K	0.67	46.1	6.67	347	7.53
R391K	37.6	13.8	220	1.14×10^4	826

The wild-type and arginine mutants were expressed in *E. coli* JM109. ND, not determined.

specific activities of the JM109 cells harboring the A6-D-ANase or its arginine-mutant expression plasmids. All the arginine mutants of A6-D-ANase showed greatly decreased specific activities as compared with the wild-type A6-D-ANase except for R354K.

Kinetic parameters of the purified wild-type and arginine mutant enzymes

The kinetic parameters for the purified wild-type and the purified arginine mutants of A6-D-ANase are shown in Table 2. We could not purify R26K and R296K due to their low specific activities and their instabilities. The K_m and V_{max} for the activity of the purified R354K were similar to those of the purified wild-type. This result suggests that the R354 residue is not important for the activity of A6-D-ANase.

Expression of the wild-type and arginine mutants in dnaK756

We previously showed that the soluble expression of A6-D-ANase was increased 1.2-fold by the coexpression of DnaKJE [6]. To show the effect of the coexpression of DnaKJE on the production of the wild-type and arginine mutants of A6-D-ANase, these genes were expressed in *dnaK756*. When the wild-type enzyme was expressed in *dnaK756*, the specific activity of the cells was little. The expression level was restored when DnaKJE were coexpressed (Table 3). On the contrary, the *dnaK756* cells harboring the R354K gene showed considerable activity, and the activity was decreased by the coexpression of DnaKJE.

Discussion

The wild-type A6-D-ANase required DnaK for its proper folding. The highly positive charged guanidino group of the R354 residue might cause the disturbance in the course of the folding process in the absence of DnaK. On the other hand, in the presence of DnaK, A6-D-ANase might be able to properly fold as a result of the masking of the R354 residue by DnaK, which prefers to bind positively charged peptides [12–14]. Since DnaK is highly conserved among bacteria, it is possible that the folding of A6-D-ANase is also assisted by *Alcaligenes* DnaK in *Alcaligenes* A-6 cells.

The substitution of the R354 residue with lysine restored the ability for the folding even in the absence of DnaK (Table 3), suggesting that the lysine residue does not cause the disturbance for the folding of A6-D-ANase.

As shown in Table 2, the kinetic parameter of the purified R354K was almost the same as that of the purified wild-type A6-D-ANase. This result is consistent with the fact that the R354 residue locates in the outside of the active center of DA1-D-ANase and suggests that the R354 residue is not important for the catalysis of A6-D-ANase. However, when the wild-type enzyme or the R354K gene was expressed in the *dnaK756* cells, their expression levels differed markedly (Table 3). The substitution of the R354 residue with lysine increased the activity of A6-D-ANase in the absence of DnaK. This result suggests that the R354 residue but not the K354 residue disturbs the folding process of the enzyme in the absence of DnaK.

The specific activity of the *dnaK756* cells harboring the R354K gene was greatly decreased by the existence

Table 3
Effects of DnaKJE coexpression on the expression of A6-D-ANase or its arginine mutant gene cloned into *dnaK756*

	Specific activity of D-ANase (U/mg)							
	Wild-type	R26K	R152K	R296K	R302K	R354K	R377K	R391K
−DnaK	0.06	0.04	0.11	0.03	0.04	20.6	0.07	0.07
+DnaK	52.2	0.05	1.13	0.04	0.11	2.51	0.05	0.06

The *dnaK* mutant strain, *dnaK756* harboring the D-ANase expression plasmid (−DnaK) or the plasmid together with the DnaKJE expression plasmid (+DnaK) was grown at 30 °C in an LB medium in the presence of 50 μg ampicillin per milliliter (plus 20 μg chloramphenicol per milliliter when necessary).

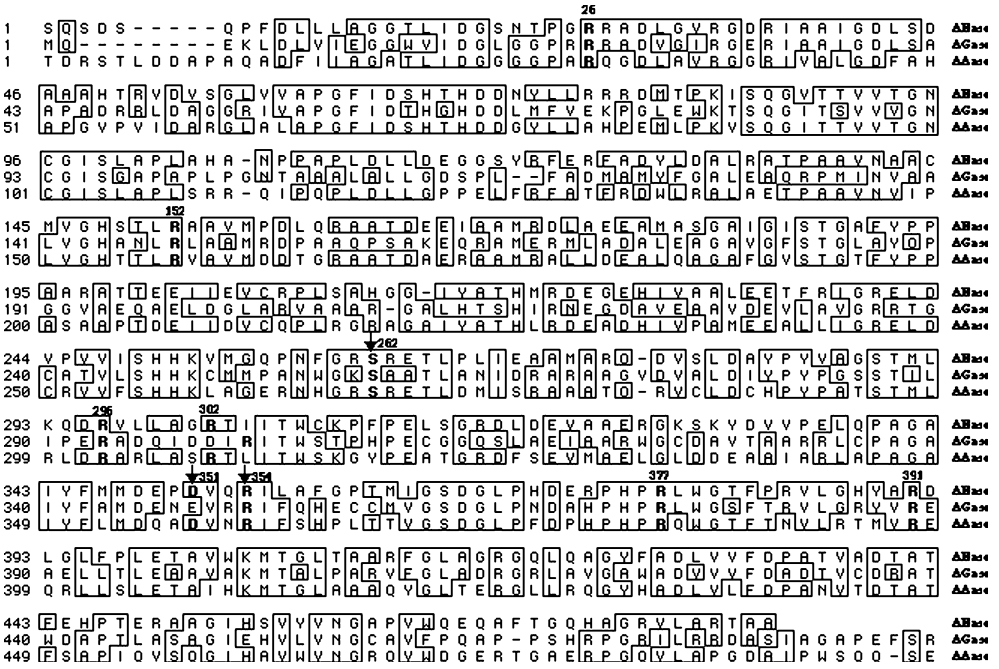

Fig. 1. Alignment of the amino acid sequences of A6-D-ANase (ANase), D-AGase (AGase), and D-AAase (AAase). The conserved amino acid residues are boxed, and the residue numbers for each enzyme are shown. The mutated arginine residues are in bold type. The arrows indicate the R354, S262, and D351 residues.

of pKJE7 even in the absence of arabinose, the inducer of DnaKJE (Table 3). We previously reported that the soluble expression of A6-D-ANase is decreased by the addition of 2 mg/ml arabinose to the culture medium [6]. However, the soluble expression was increased in the absence of the inducer. Since R354K does not require the function of DnaK for its proper folding, DnaK can have an influence only on the inhibition of folding. Thus, the soluble expression level of R354K may be more sensitive to the high amount of DnaKJE than that of the wild-type enzyme.

As shown in Fig. 1, the R354 residue is conserved among A6-D-ANase (GenBank Accession No. S80683), D-AGase (GenBank Accession No. AFAA6), and D-AAase (GenBank Accession No. AFAA61). The R354 residue is also conserved in DA1-D-ANase. In the structure of DA1-D-ANase, the S262 and D351 residues are located near the R354 residue outside of the active site. The distances between Nη1 of the R354 residue and Oγ of the S262 residue, and between Nη1 of the R354 residue and Oδ1 of the D351 residue are 2.72 and 3.29 Å, respectively. The R354 residue seems to be hydrogen-bonded to S262 and D351 residues. On the basis of the 3D structure model of A6-D-ANase, the R354 residue is also located near the S262 and D351 residues. These hydrogen bonds seem to occur in A6-D-ANase. The S262 residue is conserved among A6-D-ANase, D-AGase, and D-AAase, and the D351 residue is conserved between A6-D-ANase and D-AAase (Fig. 1). Although D-AGase does not contain the aspartate residue corresponding to the D351 residue, its E348 residue may function as a negatively charged residue in a similar manner to the D351 residue of A6-D-ANase (Fig. 1). Since the soluble expressions of D-AGase and D-AAase in *E. coli* JM109 cells are also increased by the coexpression of DnaKJE [6], the conserved R354 residue is one of the candidates that DnaK binds to in order to facilitate the folding of D-AGase and D-AAase.

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