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Active site pocket of *Streptomyces* D-stereospecific amidohydrolase has functional roles in aminolysis activity

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D-Stereospecific amidohydrolase from *Streptomyces* sp. 82F2 (DAH) recognizes D-amino acyl ester derivatives as substrates and catalyzes hydrolysis and aminolysis to yield D-amino acids and D-amino acyl peptides or amide derivatives, respectively. Crystallographic analysis has revealed that DAH possesses a large cavity with a small pocket at the bottom. Because the pocket is close to the catalytic center and is thought to interact with substrates, we examined the function of the eight residues that form the pocket in terms of substrate recognition and aminolysis via mutational analysis. Formation of the acyl-enzyme intermediate and catalysis of aminolysis by DAH were changed by substitutions of selected residues with Ala. In particular, I338A DAH exhibited a significant increase in the condensation product of Ac-D-Phe methyl ester and 1,8-diaminooctane (Ac-D-Phe-1,8-diaminooctane) compared with the wild-type DAH. A similar effect was observed by the mutation of Ile338 to Gly and Ser. The pocket shapes and local flexibility of the mutants I338G, I338A, and I338S are thought to resemble each other. Thus, changes in the shape and local flexibility of the pocket of DAH by mutation presumably alter substrate recognition for aminolysis.

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[**Key words:** D-Stereospecific amidohydrolase; Active site pocket; Aminolysis; Peptide bond formation activity; Substrate specificity]

Serine peptidases are widespread and abundant and play key roles in all living organisms. The enzymes are classified into 53 families (S1–S81 in the MEROPS peptidase database [http://merops.sanger.ac.uk/]) (1,2), and account for over 30% of all known proteolytic enzymes. The catalytic mechanism of all known serine peptidases involves a nucleophilic Ser residue that attacks the carbonyl moiety of the substrate peptide bond to form an acyl-enzyme intermediate. The substrate is an acyl donor. The intermediate is then hydrolyzed through nucleophilic attack by an activated water molecule. In the presence of high concentrations of primary amines, an amide bond is formed through nucleophilic attack by an amino group instead of a water molecule (3,4). The primary amine acts as an acyl acceptor. This enzymatic aminolysis reaction has been used for the syntheses of various biologically active peptides, such as kyotorphin (5) infusion material (6) and carnosine (7,8).

The nucleophilicity of the catalytic Ser of most serine peptidases is dependent on a catalytic triad of Asp, His, and Ser residues, commonly referred to as the charge relay system (9–11). In contrast, variations in the architecture of the active site, such as the Ser/Ser/Lys triad and Ser/Lys dyad, have also been reported (12–15). Recently, we found a new serine peptidase, D-stereospecific amidohydrolase (DAH) from *Streptomyces* sp. 82F2 (16). The enzyme belongs to the S12 peptidase family, which has a catalytic Ser/Lys dyad (Ser86 and Lys89). The most characteristic feature of DAH is the recognition of D-amino acyl derivatives as substrates and the formation of amide bonds by aminolysis. In DAH aminolysis reactions, the enzyme preferentially uses D-aminoacyl derivatives as acyl donors and L-amino acids and their derivatives as acyl acceptors, and produces dipeptides with a DL-configuration (17). In fact, DAH has been used to synthesize a cyclic dipeptide as the lead compound of the pesticide cyclo-(D-Pro-L-Arg), which inhibits family 18 chitinases (18), in a one-step/one-pot reaction (17).

Several of the enzymes of the S12 family exhibit high aminolysis activity toward various types of substrates including amides, esters, and peptides (19–21). The functions are thought to be mainly associated with the biosynthesis or remodeling of peptidoglycan. Five crystal structures of S12 family peptidases, including DAH (PDB: 3WWX), have been reported to date: D-aminopeptidase (PDB: 1EI5), D,D-peptidase (PDB: 3PTE), class C β-lactamase (PDB: 1BLS), D-amino acid amidase (PDB: 2EFX) (22–26). Among them, D,D-peptidase and D-stereospecific aminopeptidase, as well as DAH, catalyze the formation of peptide bonds by aminolysis (19,21). Their substrate specificities vary, and the structural factors responsible for the substrate specificity of hydrolysis have been identified (9,27). However, the mechanisms of substrate (both acyl donors and acceptors) recognition for aminolysis remain unclear

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but represent an important issue for understanding the characteristics of this class of enzymes and their detailed function in biosynthesis or remodeling of peptidoglycan.

Crystallographic analysis of DAH revealed that the enzyme possesses a large cavity. The side chains of Ser86 and Lys89 that create the catalytic Ser/Lys dyad are positioned at the center bottom of the cavity (22). In addition, there is a pocket close to this catalytic center (Fig. 1). The overall structures of S12 enzyme family members are similar, although there are significant differences in terms of the shapes and sizes of the cavities and active site pockets among them. DAH recognizes L-aminoacyl derivatives specifically as acyl acceptors. However, the exact positions that recognize L-amino acids in the cavity of DAH have not been found. Okazaki et al. (28) reported that the structure of the D-amino acid amidase pocket fits L-Phe and D-Phe. In this structure, unlike the bound D-Phe, the bound L-Phe does not form an acyl-enzyme intermediate. Thus, we assumed that the pocket functions to recognize acyl acceptors for aminolysis. In addition to L-aminoacyl derivatives, DAH recognizes 1,8-diaminooctane (1,8-DAO) as an acyl acceptor in aminolysis (22), and the crystal structure of DAH has been refined in the presence of 1,8-DAO. Although the bound 1,8-DAO in the DAH crystal structure is positioned at the side of the cavity (Fig. 1), the distance between Ser86 and 1,8-DAO is 7.3 Å, which is too great for nucleophilic attack. Therefore, the 1,8-DAO binding form is thought not to reflect the location of the nucleophilic attack on the acyl acceptor (22). For that reason, an investigation into the effects of mutations of residues constituting the pocket on aminolysis could provide new insights. The aim of this study was to evaluate the role of the residues constituting the DAH pocket via mutations. We used site-directed mutagenesis to analyze the potential effects of altered residues in the DAH pocket, which is thought to bind substrates, and investigated any changes in substrate recognition, especially in aminolysis. The data indicated that Ile338 is an important residue for substrate recognition among the DAH pocket residues.

MATERIALS AND METHODS

Materials, bacterial strains, and plasmids Peptides and aminoacyl ester derivatives were purchased from Bachem AG (Bubendorf, Switzerland), Sigma-Aldrich Co. (St. Louis, MO, USA), Merck KGaA (Darmstadt, Germany), and Wako Pure Chemical Industries Ltd. (Osaka, Japan). *Escherichia coli* JM109 was used as a host strain for general cloning procedures and *E. coli* Rosetta(DE3) was used as a host strain for gene expression. Plasmid pET-82F2DAP (with the *DAH* gene inserted into the MscI-NcoI gap of pET-22b (17) was used for the expression of wild-type DAH (WT) and as a template for mutagenesis.

Mutagenesis Site-directed mutagenesis for the construction of mutant enzymes was conducted by inverse PCR using two pairs of primers containing a point mutation (Table S1). The PCR program consisted of 18 cycles for 1 min at 95°C, 1 min at 65°C, and 8 min at 68°C. The PCR product was treated with Dpn I at 37°C overnight. Thereafter, it was transfected into competent *E. coli* JM109 cells according to the manufacturer's protocol. After the plasmid was extracted, accurate cloning was confirmed by sequencing.

Expression and purification of WT and mutant enzymes *E. coli* Rosetta(DE3) harboring pET-82F2DAH or the expression vector for mutant DAH production was cultivated at 25°C for 48 h in 50 mL of Overnight Expression Instant TB medium (Novagen Inc., Madison, WI, USA). Since DAH is an extracellular enzyme (16), the culture was centrifuged to remove the cells and the recombinant enzyme was purified using the following procedures. The culture supernatant was dialyzed against 20 mM sodium citrate (pH 5.5). The dialyzate was loaded onto a Vivapure-S spin column (Sartorius AG, Göttingen, Germany) equilibrated with 20 mM sodium citrate (pH 5.5). After washing with the same buffer containing 0.1 M NaCl, the bound protein was eluted with the same buffer, this time containing 0.4 M NaCl. The homogeneity of the purified proteins was confirmed by 12% SDS-PAGE under denaturing conditions (29).

Assay of aminolysis activity Aminolysis and hydrolysis reactions are competitive and hydrolysis tends to occur at high temperatures; thus, the reaction was conducted at 4°C to suppress hydrolysis. In addition, pH 8.5 was adopted for the aminolysis reaction to avoid the non-enzymatic degradation of ester substrates. The aminolysis activity of DAH was assayed as follows. First, 5 μL (or 50 μL) of 0.1 mg mL^{-1} enzyme solution was added to 40 μL (or 400 μL) of the mixture containing 35 μL (or 350 μL) of 0.5 M Tris–HCl (pH 8.5) and 5 μL (or 50 μL) of 0.5 M 1,8-DAO. The reaction was initiated by adding 5 μL (or 50 μL) of an acyl donor substrate (aminoacyl derivative solutions dissolved in dimethyl sulfoxide at an appropriate concentration [approximately 0.5 M]). The reaction was then continued at 4°C for 2–30 min. The reaction was terminated by adding 50 μL (or 500 μL) 0.5 M HCl to the mixture. The reaction mixture was then analyzed by measuring the released methanol and the weight of the reaction product, and by using mass spectrometry (MS).

Measurement of the weight of the aminolysis product DAH reaction mixtures for aminolysis sometimes appear as white precipitates. Before the reaction, the weights of the reaction tubes were measured and used as the basis weight of the reaction container. After the reaction, the precipitates were collected by centrifugation (13,000 × *g*, 10 min) and dried at 60°C for around 1 day. The weights of the dried products were measured by using an analytical balance (ATX84; Shimadzu).

MS analysis The molecular masses of the products of DAH catalytic activity were determined via matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS and electrospray ionization (ESI–TOF) MS. For MALDI-TOF MS analysis, the precipitates were collected by centrifugation (13,000 × *g*, 10 min) and washed twice with distilled water. They were then suspended in 5 μL of MALDI matrix solution (150 mM 2, 5-dihydroxybenzoic acid in 50% acetonitrile). A small amount of each sample (approximately 1 μL) was dropped onto the target frame and dried, and the samples were analyzed using Autoflex TOF (Bruker Daltonics Inc.).

For ESI-TOF MS analysis, the reaction mixture was diluted with a 200-fold volume of 0.1% formic acid. After the solution was filtered, 5 μL from each sample was analyzed by using an ESI-TOF MS system (LCT Premier XE or Quattro Micro API; Waters Corp., Tokyo, Japan). The data were processed using a computer program (MassLynx; Waters Corp.).

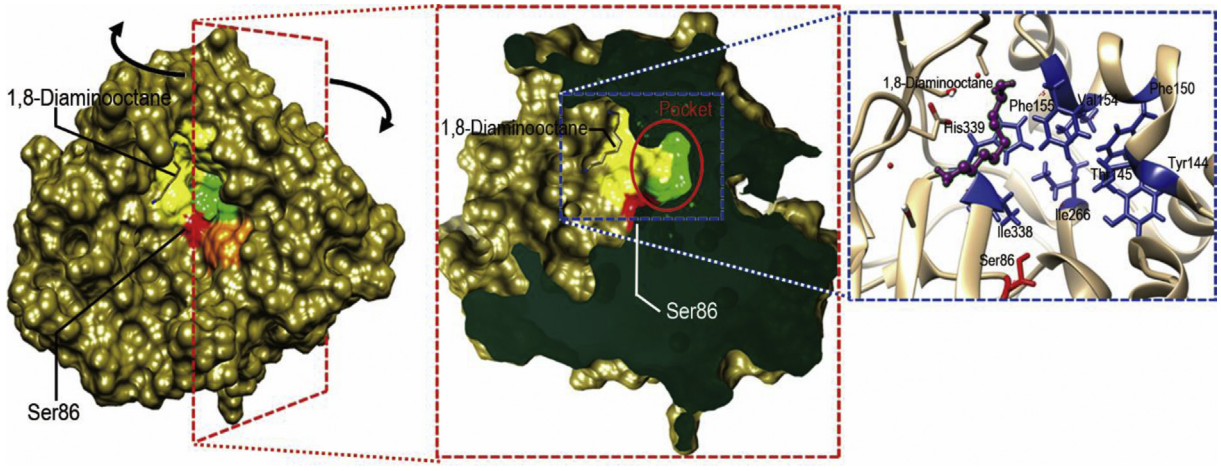

FIG. 1. Cavity shape and residues in the active site pocket. The pictures show the overall structure (left), a cross-sectional view (center), and a close-up view of D-stereospecific amidohydrolase (DAH). The bound 1,8-diaminooctane (1,8-DAO) molecule is shown as a stick or ball and stick. The active site Ser86 residue is shown in red, the residues composing the active site are shown in green (left and center) or blue, and the region associated with 1,8-DAO binding is shown in yellow. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

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