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The crystal structure of the amidohydrolase VinJ shows a unique hydrophobic tunnel for its interaction with polyketide substrates

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ABSTRACT

VinJ is an amidohydrolase belonging to the serine peptidase family that catalyzes the hydrolysis of the terminal aminoacyl moiety of a polyketide intermediate during the biosynthesis of vicenistatin. Herein, we report the crystal structure of VinJ. VinJ possesses a unique hydrophobic tunnel for the recognition of the polyketide chain moiety of its substrate in the cap domain. Taken together with the results of phylogenetic analysis, our results suggest that VinJ represents a new amidohydrolase family that is different from the known α/β hydrolase type serine peptidases.

1. Introduction

Peptidases cleave peptide bonds in proteins and peptide substrates, and about 2% of the genes present in the genomes of every organism encode for peptidases [1]. These enzymes are widely distributed and are critical to the effective maintenance of a variety of different biological systems such as post-translational proteolysis, as well as playing important roles in physiological processes and cellular protection mechanisms [2]. For example, the signal peptide responsible for sorting proteins during their translocation is removed from the protein by peptidase when the proteins reach their final destination [3]. As for the biosynthesis of ribosomal peptides, a peptidase cleaves off the leader peptide to allow for the generation of the bioactive peptides [4,5]. Thus, peptidases are important and intriguing enzymes that are involved in a wide variety of biological processes. Although the biochemical properties of peptidases have been well characterized, our understanding of the substrate specificities of peptidases has been limited to peptides composed of proteinogenic natural amino acids.

Enzymes belonging to the serine peptidase family recognize characteristic sequences of peptides and catalyze the hydrolysis of amide bonds within these sequences [6]. The crystal structures of serine peptidases such as chymotrypsin, trypsin and subtilisin, have provided valuable mechanistic insights into their reactions. Serine peptidases contain a catalytic triad (i.e., Ser-His-Asp) in their active site. The Ser hydroxy group attacks the carbonyl carbon of an amide moiety of the substrate to generate a tetrahedral oxyanion intermediate. Subsequent protonation of the nitrogen atom triggers the collapse of the intermediate with the release of an amino group containing product and the concomitant formation of an acyl-enzyme species, which undergoes hydrolysis to give a carboxylate-containing product and the active enzyme. In contrast to this conserved catalytic mechanism, the substrate recognition mechanism is diverse. The substrate specificity is basically controlled by the structure of the substrate binding site, including the S1 site adjacent to the catalytic triad [7].

We previously reported that the amidohydrolase VinJ, which belongs to the serine peptidase family, is involved in the biosynthesis of the macrolactam antibiotic vicenistatin, which is produced by *Streptomyces halstedii* HC34 [8]. Vicenistatin contains the unique β-amino acid unit 3-amino-2-methylpropionate at the starter position of its polyketide backbone. This starter unit is biosynthesized from L-glutamate via (2*S*,3*S*)-3-methylaspartate, which is initially transferred onto the standalone acyl carrier protein (ACP) VinL by the ATP-dependent ligase VinN. After decarboxylation with the PLP-dependent enzyme VinO, the resulting 3-aminoisobutyryl

Abbreviations: ACP, acyl carrier protein; pNA, *p*-nitroanilide; r.m.s.d., root mean square deviation; F1, tricon-interacting peptidase F1; PIP, proline iminopeptidase; PAP, prolyl aminopeptidase

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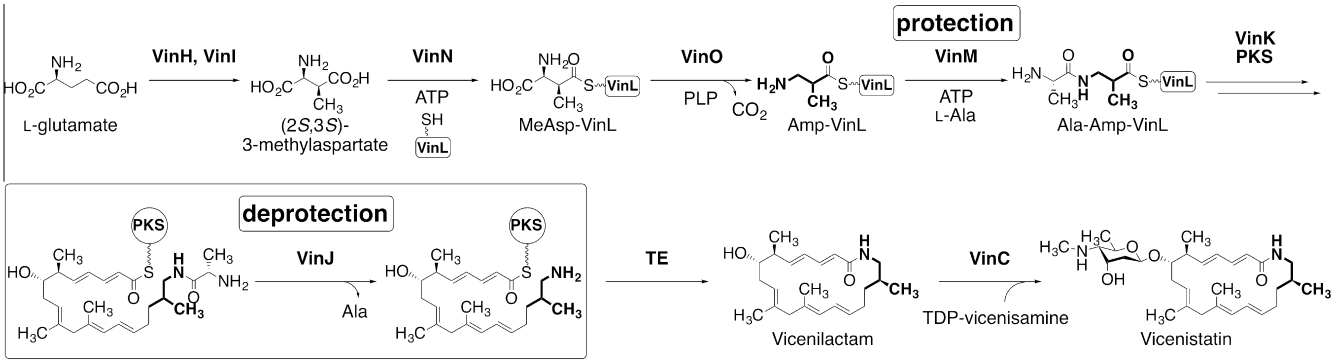

Fig. 1. Biosynthetic pathway of vicenistatin. VinJ catalyzes the hydrolysis of an amide bond in the alanyl-polyketide chain.

moiety is aminoacylated with L-alanine to give L-alanyl-3-aminoisobutyryl-ACP by the unique ATP-dependent ligase VinM, presumably to avoid spontaneous formation of a six-membered lactam during the elongation of the polyketide chain at module 1 of VinP1. The dipeptide moiety is transferred to the ACP domain of VinP1, which initiates the elongation of the polyketide chain. It has been proposed that polyketide synthases VinP1, P2, P3, and P4 elongate polyketide chains harboring a terminal alanyl moiety, and that this moiety is subsequently hydrolyzed by VinJ prior to the macrolactam formation, which is catalyzed by the thioesterase domain of VinP4 (Fig. 1). We have previously shown that VinJ catalyzes the hydrolytic cleavage of the terminal L-alanyl group from *N*-alanyl-secovicenilactam ethyl ester, which was used to mimic the elongated polyketide substrate attached to ACP [9]. VinJ homologous genes are present in the other gene clusters responsible for the biosynthesis of β-amino acid containing macrolactams, suggesting that VinJ-type amidohydrolases are commonly used in the biosynthesis of macrolactams [10–14]. Herein, we report the crystal structure of VinJ to determine the structural basis of polyketide substrate recognition. Interestingly, VinJ has an unusual hydrophobic tunnel that accommodates the polyketide moiety of the substrate through hydrophobic interactions.

2. Materials and methods

2.1. Preparation of purified VinJ protein

Escherichia coli BL21(DE3) cells harboring a pColdI-*vinJ* plasmid [9] were grown at 37 °C in Luria Bertani broth containing ampicillin (50 μg/mL). After the optical density at 600 nm reached 0.6, protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (0.2 mM), and the cells were then cultured for an additional 16 h at 15 °C. The recombinant VinJ protein, which was collected from cell-free extracts prepared by sonication, was purified on a TALON affinity column (Clontech, Mountain View, CA, USA). For the construction of the S110A mutant, the pColdI-*vinJ* plasmid was used for the site-directed mutagenesis. Site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using the following oligonucleotides: 5′-ctcttcggccaggcctggggcgggat-3′ and its complementary oligonucleotide. The mutation was confirmed by determining the nucleotide sequence. This plasmid was introduced into *E. coli* BL21(DE3) cells, and the mutated enzyme was prepared as described above.

2.2. Kinetic analysis of VinJ using N-alanyl-secovicenilactam ethyl ester

N-alanyl-secovicenilactam ethyl ester and secovicenilactam ethyl ester were synthesized as described previously [9]. *N*-alanyl-secovicenilactam ethyl ester (varied between 1 and 100 μM) was reacted with purified VinJ (1 nM) in a 50 mM Tris buffer (pH 7.5) containing 10% glycerol (100 μL total volume), and the resulting solution was incubated at 28 °C for 10 min before being quenched with ethyl acetate. The reaction product was extracted with ethyl acetate (3 × 100 μL) and the combined organic extracts were evaporated to dryness to give a residue, which was dissolved in methanol (10 μL) and subjected to HPLC analysis. HPLC analysis was performed on a Hitachi HPLC system (Hitachi, Tokyo, Japan) equipped with a L-4000 UV Detector and L-6250 Intelligent Pump, using a Pegasil ODS column (4.6 × 250 mm) (Shenshu Scientific, Tokyo, Japan). Solvents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) were used as the mobile phases according to the following conditions: 0–20 min, 50% B; 20–25 min, 50–70% B linear gradient; 25–30 min, 70% B. The HPLC analysis was performed with a flow rate of 1.0 mL/min. Each reaction was carried out in triplicate. Secovicenilactam ethyl ester was used to generate standard curves for the quantification of the products. Steady-state parameters were estimated by fitting the initial velocities calculated from the HPLC-UV detection results to the Michaelis–Menten equation.

2.3. Kinetic analysis of VinJ using L-alanine p-nitroanilide

A continuous spectrophotometric assay using L-alanine *p*-nitroanilide (Ala-pNA) was carried out as follows. The reaction was initiated by the addition of VinJ (10 nM) to a mixture containing 50 mM Tris buffer (pH 7.5), 10% glycerol and Ala-pNA (varied between 100 and 4000 μM) (500 μL total volume) at 28 °C, and the increase in absorbance at 405 nm attributable to the release of *p*-nitroaniline per second was monitored using a UV-2450 spectrophotometer (Shimadzu, Tokyo, Japan). The initial velocity was determined from the linear portion of the optical density profile ($\varepsilon_{405\ nm}$ = 10 600 $M^{-1}cm^{-1}$).

2.4. Crystallization, data collection and structural determination

Crystals of VinJ were grown from a 1:1 (v/v) mixture of a VinJ protein solution [10 mg/mL in 5 mM Tris–HCl (pH 7.5)] and a reservoir solution (0.2 M lithium acetate, pH 7.0, 20% polyethylene glycol 3350) using the sitting-drop vapor diffusion method at 5 °C. Prior to the collection of the X-ray data, the crystals were soaked in a reservoir solution containing 25% glycerol as a cryoprotectant and flash-frozen in a stream of liquid nitrogen. The X-ray diffraction data were collected on a beamline BL-5A at the Photon Factory (Tsukuba, Japan), and were subsequently indexed, integrated, and scaled using the iMosflm program [15]. The initial phases were determined by the molecular replacement method using the Molrep program [16], with the crystal structure of the putative proline iminopeptidase *Mycobacterium smegmatis* (PDB code: 3NWO) being used as a search model. The ARP/wARP