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Crystal structure of EstSRT1, a family VIII carboxylesterase displaying hydrolytic activity toward oxyimino cephalosporins

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ABSTRACT

EstSRT1 is a family VIII carboxylesterase that hydrolyzes oxyimino third- and fourth-generation cephalosporins, first-generation cephalosporins and ester substrates. According to the crystal structure of EstSRT1 (2.0-Å resolution), this protein contains a large α/β domain and a small α-helical domain and harbors three catalytic residues (Ser71, Lys74, and Tyr160) in the cavity at the domain interface, similarly to other family VIII carboxylesterases. Comparison of the structures of EstSRT1 and EstU1, a family VIII carboxylesterase with no hydrolytic activity toward bulky oxyimino cephalosporins, revealed that EstSRT1 has a smaller active site, despite its extended substrate range. The *B*-factors of the active site segments that could potentially contact with the oxyimino groups and the R2 side chains of oxyimino cephalosporins are higher in EstSRT1 than in EstU1, thus suggesting the role of the active site's structural flexibility in the extension of EstSRT1's substrate spectrum.

1. Introduction

Carboxylesterases (EC 3.1.1.1), which exist in a wide range of organisms, including bacteria, fungi, plants, and animals, hydrolyze carboxylic esters and produce alcohols and carboxylates. On the basis of sequence homology, they are classified into eight families: I to VIII [1]. Carboxylesterases of families I–VII adopt a canonical α/β hydrolase fold with a Ser-His-Asp (or Glu) catalytic triad, and their nucleophilic serine residue resides in the consensus sequence G-X-S-X-G [2,3]. In contrast, family VIII carboxylesterases adopt a two-domain modular structure containing a large α/β domain and a small α-helical domain [4]. These proteins have a Ser-Lys-Tyr catalytic triad, and their nucleophilic serine residue is located in the S-X-X-K motif. Family VIII carboxylesterases are structurally more similar to class C β-lactamases than to carboxylesterases [1].

Class C β-lactamases break the β-lactam ring of β-lactam antibiotics through a two-step mechanism [5]. In the first acylation step, the nucleophilic serine residue attacks the carbonyl carbon of the β-lactam ring, forming the acyl-enzyme intermediate. In the second deacylation step, a water molecule attacks the same carbonyl carbon in the acyl-enzyme intermediate to release the product. The same nucleophile-mediated acylation and water-mediated deacylation reactions occur during the catalytic action of carboxylesterases [6]. Thus, it is reasonable to assume that family VIII carboxylesterases, which have β-lactamase-like features, have the ability to hydrolyze β-lactam antibiotics. In fact, several family VIII carboxylesterases have been reported to hydrolyze β-lactam antibiotics [7–11]. Among these enzymes, the hydrolytic activities of two metagenome-derived family VIII carboxylesterases (EstSRT1 and EstU1) toward diverse esters and β-lactam antibiotics, including oxyimino third- and fourth-generation cephalosporins, have been thoroughly examined by the same research group [10,11]. Although EstSRT1 and EstU1 exhibit similar activities toward the hydrolysis of ester substrates, their activities toward β-lactam antibiotics differed. EstU1 hydrolyzes first-generation cephalosporins, whereas EstSRT1 has activity toward first-, third-, and fourth-generation cephalosporins. Consequently, EstSRT1 and EstU1 are ideal proteins that can be used to reveal how promiscuous family VIII carboxylesterases extend their substrate specificities. Here, we report the crystal structure of EstSRT1 and compare it with that of EstU1 to determine the basis of the hydrolytic activity of EstSRT1 toward bulky oxyimino antibiotics.

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2. Materials and methods

2.1. Purification, crystallization, data collection, and structure determination

The expression plasmid pET-24a(+) with the EstSTR1 gene was transformed into *Escherichia coli* strain B834 (DE3), a methionine auxotroph strain, to produce selenomethionyl EstSTR1. Hereafter, EstSRT1 refers to the selenomethionyl EstSTR1. The transformed cells were cultured at 310 K in M9 minimal medium containing 0.4% glucose; 200-mM $MgSO_4$; 100-mM $CaCl_2$; 1-mg l^{-1} thiamine; 50–250-mg l^{-1} of 19 amino acids, except for methionine; 40-mg l^{-1} selenomethionine; and 50-μg ml^{-1} kanamycin. The expression of EstSRT1 was induced at an OD_{600} of 0.5 by the addition of 1-mM isopropyl-1-thio-β-D-galactopyranoside (Duchefa) and incubation for 16 h at 293 K.

The resuspended cells in 50-mM Tris (pH 8.0) buffer were disrupted by sonication and then centrifuged at 20,000*g* for 60 min at 277 K. The resulting supernatant fraction was loaded onto a column of TALON metal affinity resin (BD Biosciences, Clontech, Palo Alto, CA, USA) pre-equilibrated with 50-mM Tris (pH 8.0) buffer containing 100-mM KCl and 10% glycerol. After the column was washed with 50-mM Tris (pH 8.0) buffer containing 100-mM KCl, 10% glycerol, and 10-mM imidazole, the bound proteins were eluted with 50-mM Tris (pH 8.0) buffer containing 100-mM KCl, 10% glycerol and 300-mM imidazole. The eluted fractions containing EstSTR1 were applied to a Superdex-75 16/60 column (GE Healthcare, Picataway, NJ, USA) equilibrated with buffer containing 50-mM Tris (pH 8.0), 150-mM NaCl and 1-mM dithiothreitol (DTT). The purified recombinant EstSTR1 protein was concentrated to approximately 9 mg/ml and mixed with 1-mM phenylmethylsulfonyl fluoride (PMSF) for crystallization.

The EstSTR1 protein was crystallized by the vapor batch crystallization method at 295 K [12]. Crystal screening was performed with all screening kits available from Hampton Research, Emerald BioSystems and Axygen Biosciences. The initial crystals were grown in a precipitant solution containing 25% w/v polyethylene glycol 3350, 100-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), and 200-mM NaCl (condition No. 21 of the CP-CUSTOM-SGC screen from Axygen Biosciences). Without further optimization, the initial crystals were used for data collection. A crystal was frozen at 100 K after brief immersion in a cryoprotectant solution containing 5% glycerol in the same mother liquor. A 2.0-Å single-wavelength anomalous diffraction (SAD) data set (Table 1) was collected at a wavelength of 0.97903 Å (peak) at the 44 XU beamline at Spring-8 (Japan). The SAD data set was integrated and scaled with *HKL2000* [13]. The crystals belonged to the space group $P2_12_12_1$ with cell dimensions of $a = 80.13$, $b = 95.15$, and $c = 99.94$ Å. The asymmetric unit contained two molecules of EstSRT1. Phasing and refinement were performed using *PHENIX* [14], and the model building was completed using *COOT* [15]. The final model contains residues 5–219 and 234–387 of EstSRT1, 267 water molecules, and 2 PMSF molecules. Residues 1–5, 220–233, and 387–390 are completely disordered. The Ramachandran plot of the final model indicates that 98.1% of the non-glycine residues are in the most favored regions and that the remaining 1.9% residues are in allowed regions.

Table 1
Data collection and refinement.

Data collection	
Diffraction source	Spring-8 (BL44XU)
Wavelength (Å)	0.97903
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1.2
Space group	$P2_12_12_1$
a, *b*, *c* (Å)	80.13, 95.15, 99.94
α, β, γ (°)	90, 90, 90
Resolution range (Å)	50–2.0
Total No. of reflections	1,234,919
No. of unique reflections	52,188
Completeness (%)[a]	99.3 (97.4)
Redundancy[a]	4.5 (2.5)
I/σ (I)[a]	22.6 (3.5)
Overall *B* factor from Wilson plot (Å^2)	20.77
R_{sym} (%)[a,b]	10.6 (33.9)
Refinement statistics	
Resolution range (Å)	44.2–2.0
No. reflections	51,168
No. atoms	
Protein	5,584
PMSF	20
Water	267
B-factors	
Protein	23.4
PMSF	43.8
Water	25.9
R (R_{free}) (%)[a,c]	18.2 (22.0)
R.m.s. deviations[d]	
Bonds length (Å)	0.007
Bond angles (°)	1.070

[a] The number in parentheses is for the outer shell.
[b] $R_{sym} = \sum_h\sum_i |I_{h,i} - I_h| / \sum_h\sum_i I_{h,i}$, where I_h is the mean intensity of the i observations of symmetry related reflections of h.
[c] $R = \sum |F_o - F_c| / \sum F_o$, where $F_o = F_p$, and F_c is the calculated protein structure factor from the atomic model. R_{free} was calculated with 10% of the reflections.
[d] R.m.s. deviations in bond length and angles are the deviations from ideal values.

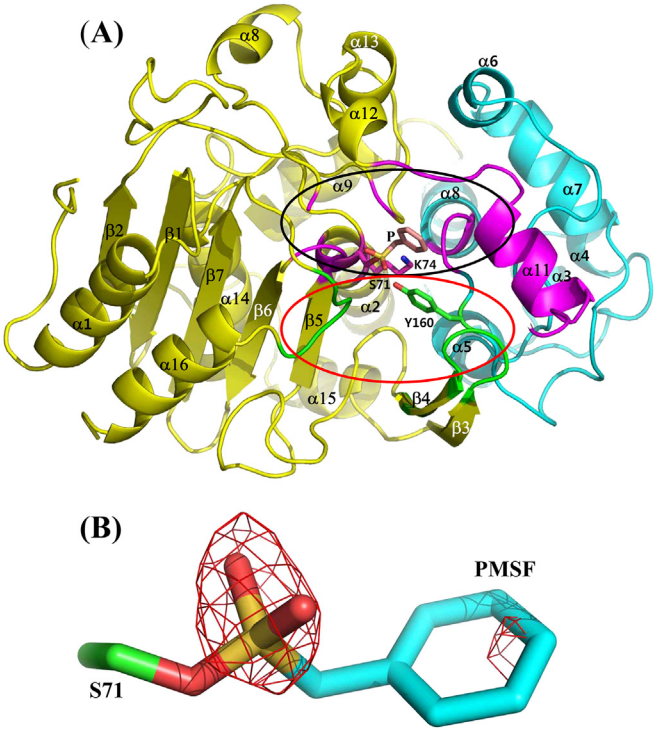

Fig. 1. The structure of EstSRT1. (**A**) A ribbon diagram of EstSRT1 shown with the secondary structures labeled. The α/β domain and helical domain are shown in yellow and cyan, respectively. The residues constituting the R1 subsite indicated by a black ellipse are shown in magenta, whereas those constituting the R2 subsite in the red ellipse are shown in green. The three catalytic residues (Ser71, Lys74, and Tyr160) and PMSF (P) are shown as sticks. (**B**) The initial $F_o - F_c$ electron density map contoured at 3σ showing PMSF in the final model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)