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Structure of AmpC β-lactamase (AmpC^{D}) from an *Escherichia coli* clinical isolate with a tripeptide deletion (Gly286-Ser287-Asp288) in the H10 helix

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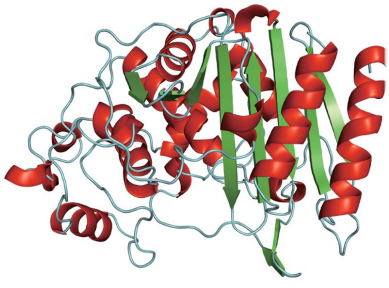

The X-ray crystal structure of AmpC β-lactamase (AmpC^{D}) with a tripeptide deletion (Gly286-Ser287-Asp288) produced by *Escherichia coli* HKY28, a ceftazidime-resistant strain, was determined at a resolution of 1.7 Å. The structure of AmpC^{D} suggests that the tripeptide deletion at positions 286–288 located in the H10 helix causes a structural change of the Asn289–Asn294 region from the α-helix present in the native AmpC β-lactamase of *E. coli* to a loop structure, which results in a widening of the substrate-binding site.

1. Introduction

AmpC β-lactamase belongs to the molecular class C β-lactamases (Ambler, 1980; Babic *et al.*, 2006) and is clinically as important as class A β-lactamases (Rice & Bonomo, 2000) as it hydrolyzes a broad range of β-lactam antibiotics, including the extended-spectrum cephalosporins such as ceftazidime, cefotaxime, cefepime and cefpirome. In addition, AmpC β-lactamase is generally not susceptible to inhibition by clavulanic acid, although tazobactam sometimes inhibits this enzyme. Therefore, the spread of AmpC β-lactamase is a serious threat to antibiotic chemotherapy for infectious diseases. Recently, many AmpC variants with extended-spectrum activity have been clinically isolated from various bacterial pathogens such as *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes* and *Serratia marcescens*. One of the main reasons for the alteration of substrate specificity in these variants is thought to be structural modification of the protein (Nordmann & Mammeri, 2007; Nukaga *et al.*, 2004; Vakulenko *et al.*, 2002; Trépanier *et al.*, 1999) such as amino-acid replacement (Raimondi *et al.*, 2001; Trépanier *et al.*, 1999; Vakulenko *et al.*, 2002), insertion (Mammeri *et al.*, 2007; Nukaga *et al.*, 1998; Crichlow *et al.*, 1999) and deletion (Mammeri *et al.*, 2004; Doi *et al.*, 2004; Barnaud *et al.*, 2001).

In 1994, Arakawa and coworkers reported a chromosomal AmpC β-lactamase produced by an *E. coli* clinical isolate from a urine specimen in Japan, HKY28 (Doi *et al.*, 2004). From a comparison of the amino-acid sequence of AmpC of *E. coli* HKY28 (denoted AmpC^{D}) with that of *E. coli* K-12 (Jaurin & Grundstrom, 1981), AmpC^{D} contained three amino-acid deletions at positions 286, 287 and 288, corresponding to Gly, Ser and Asp residues, respectively, located on the H10 helix. With respect to substrate specificity, AmpC^{D} conferred resistance to ceftazidime with a minimal inhibitory concentration (MIC) of 32 μg ml^{-1}, although *E. coli* rarely acquires resistance to this drug. Moreover, the hydrolytic activity of β-lactam antibiotics by AmpC^{D} was suppressed by the clinically available β-lactamase inhibitors sulbactam and tazobactam and to some extent by clavulanic acid.

To elucidate the structural changes in the vicinity of the substrate-binding site resulting from the tripeptide deletion, we carried out a crystallographic analysis of AmpC^{D} β-lactamase. In this paper, we report the crystal structure of AmpC^{D} β-lactamase at a resolution of 1.7 Å and a comparison with the structure of AmpC β-lactamase of *E. coli* (denoted native AmpC; PDB code 1ke4) at a resolution of 1.72 Å.

2. Materials and methods

2.1. Expression and purification

E. coli HKY28 was isolated from a culture of urine from an inpatient in Japan in 1994. The *amp*C gene of *E. coli* HKY28 was cloned between the *Eco*RI and *Bam*HI sites of the expression vector pBCKS+ (Stratagene) to yield pBE28W, which was transformed into *E. coli* CS14-2 (Doi *et al.*, 2004). For protein purification, the plasmid was re-extracted using a Wizard Plus SV Minipreps DNA-purification system (Promega) from the strain *E. coli* CS14-2 pBCKS+/AmpC^{D}, which was retransformed into competent *E. coli* JM109 cells.

E. coli JM109 harbouring pBE28W was cultured at 310 K for 24 h in 10 l LB broth supplemented with 30 µg ml^{-1} chloramphenicol. Cells were harvested by centrifugation at 5000*g* for 15 min at 277 K. The pellets (about 50 g wet weight) were washed by resuspension in 50 ml 20 m*M* bis-tris–HCl buffer pH 6.5 with repeat centrifugation. The supernatant was discarded. The pellets were resuspended in 50 ml of the same buffer, disrupted by sonication for 5 min and centrifuged at 100 000*g* for 75 min at 275 K. The supernatant was loaded onto an SP Sepharose Fast Flow column (GE Healthcare) and the proteins were eluted with a linear gradient of 0–0.5 *M* NaCl. The fractions were analyzed by SDS–PAGE and by their ability to turn over nitrocefin. The fractions containing the desired activity were pooled and concentrated to a volume of 10 ml using Ultracel YM-10 (Millipore). The buffer was exchanged from 20 m*M* bis-tris–HCl pH 6.5 to 20 m*M* bis-tris–HCl pH 6.5, 1 *M* ammonium sulfate. The buffer-exchanged protein was loaded onto a Sephacryl SR-100 HR column (GE Healthcare) and was eluted with 20 m*M* bis-tris–HCl pH 6.5, 1 *M* ammonium sulfate, 0.3 *M* NaCl. Fractions containing AmpC^{D} β-lactamase were pooled and concentrated to a volume of 10 ml. The protein was then again reloaded onto a Phenyl Sepharose 6 Fast Flow column (low sub; GE Healthcare) and eluted with a linear gradient of 1.0–0.5 *M* ammonium sulfate. The enzyme was further concentrated to a volume of 2 ml using both Ultracel YM-10 (Millipore) and Amicon Ultra-15 (Millipore). The enzyme was more than 95% pure as judged by SDS–PAGE. For crystallization of purified AmpC^{D}, the buffer was exchanged to 20 m*M* HEPES–NaOH pH 7.5 using Amicon Ultra (Millipore).

2.2. Crystallization

Initial screening for AmpC^{D} crystallization conditions was performed at 293 K by the hanging-drop method (Luft & DeTitta, 1992) using the screening kits Crystal Screen and Crystal Screen 2 (Hampton Research). In the initial crystallization procedure, drops were prepared by mixing 1 µl protein solution (15 mg ml^{-1}) with 1 µl reservoir solution and were equilibrated against 350 µl reservoir solution. Crystals of AmpC^{D} were first obtained in one month from condition No. 40 [20%(*w*/*v*) PEG 4000, 20%(*v*/*v*) 2-propanol and 0.1 *M* sodium citrate tribasic dihydrate pH 5.6] of Crystal Screen. Improved crystals were subsequently obtained by refining the successful conditions using the hanging-drop method in 24-well VDX plates (Hampton Research): 1 µl concentrated protein solution (10 mg ml^{-1}) in 20 m*M* HEPES–NaOH pH 7.5 was combined with 1 µl reservoir solution containing 20%(*w*/*v*) PEG 4000, 10%(*v*/*v*) 2-propanol and 0.1 *M* sodium citrate pH 5.6. This protein drop was suspended over 350 µl reservoir solution containing 20%(*w*/*v*) PEG 4000, 10%(*v*/*v*) 2-propanol and 0.1 *M* sodium citrate pH 5.6 at 293 K. Crystals formed after 10 d. The crystals were flash-frozen in nitrogen gas at 100 K after cryoprotection by brief exposure to reservoir solution containing 40%(*w*/*v*) PEG 4000, 10%(*v*/*v*) 2-propanol and 0.1 *M* sodium citrate pH 5.6.

Table 1
Crystallographic data-collection and refinement statistics for AmpC^{D}.

Values in parentheses are for the highest resolution shell.

Data collection	
Resolution (Å)	50.0–1.70 (1.76–1.70)
Wavelength (Å)	1.07
Unit-cell parameters (Å, °)	a = 47.1, b = 47.4, c = 81.5, α = 82.6, β = 80.9, γ = 65.4
Space group	$P1$
Redundancy	2.2 (1.7)
Completeness (%)	94.3 (82.8)
R_{merge}†	0.062 (0.267)
No. of observed reflections	141726 (9810)
No. of unique reflections	65343 (5771)
$\langle I/\sigma(I)\rangle$	18.1 (2.6)
Refinement statistics	
σ cutoff	0
Resolution (Å)	39.2–1.70 (1.74–1.70)
No. of reflections used	62030 (3855)
B factors (Å^2)	
Average	16.9
Protein	15.6
Water	28.6
No. of non-H atoms‡	
Protein	5796
Water	611
R.m.s.d. from ideal§	
Bond lengths (Å)	0.012
Angles (°)	1.4
$R_{working}$¶	0.163 (0.228)
R_{free}††	0.206 (0.306)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity for reflection hkl and $\langle I(hkl)\rangle$ is the average intensity calculated for reflection hkl from replicate data. ‡ Per asymmetric unit. § R.m.s.d.: root-mean-square-deviation. ¶ $R_{working} = \sum_{hkl} ||F_o| - |F_c|| / \sum_{hkl} |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively. †† $R_{free} = \sum_{hkl} ||F_o| - |F_c|| / \sum_{hkl} |F_o|$ for 5% of the data not used at any stage of structural refinement.

2.3. Data collection and refinement

The data set used for structure determination was collected at SPring-8 to a resolution of 1.7 Å at a wavelength of λ = 1.07 Å. The data were integrated, merged and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The refined structure of native AmpC of *E. coli* at a resolution of 2.0 Å (PDB code 2bls; Usher *et al.*, 1998) was used as the search model for molecular replacement using *AMoRe* (Navaza, 1994), a component of the *CCP*4 program suite v.6.0.0 (Collaborative Computational Project, Number 4, 1994). Refinement was interspersed with model building using *REFMAC* v.5.2.0019 (Murshudov *et al.*, 1997), a component of the *CCP*4 program suite v.6.0.0 (Collaborative Computational Project, Number 4, 1994), and *Coot* v.0.1.2 (Emsley & Cowtan, 2004). The quality of the model was inspected using the program *PROCHECK* (Laskowski *et al.*, 1993). Figures were generated using *PyMOL* (http://pymol.sourceforge.net/) and *MolFeat* v.3.5. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB code 2zj9). The structure of AmpC^{D} was refined to a final R factor of 16.3% and a free R factor of 20.6% and the root-mean-square-deviation (r.m.s.d.) values from the ideal bond distances and angles are 0.012 Å and 1.4°, respectively. Data-collection and refinement statistics are listed in Table 1.

3. Results and discussion

The final refined structural model contains two AmpC^{D} molecules per asymmetric unit, consisting of residues Ala4–Gln361 for molecules *A* and *B*, with the exception of the deleted residues 286–288. A Ramachandran plot shows that 92.2% of the residues are in the most favoured regions, with a further 7.8% in additionally allowed regions. The r.m.s.d. value between the C$^{\alpha}$ atoms of the two monomers is

0.23 Å. As expected, each AmpC^{D} monomer adopts a mixed α/β structure of nine antiparallel β-sheets with a helical domain on one side and a mixed α/β domain on the other (Fig. 1*a*), as found in native AmpC of *E. coli* (Usher *et al.*, 1998; Powers & Shoichet, 2002; Fig. 1*b*) and other AmpC β-lactamases from *Citrobacter freundii* (Oefner *et al.*, 1990) and *Enterobacter cloacae* (Lobkovsky *et al.*, 1993).

For simplicity, only one molecule (molecule *B*) will be considered in the present discussion. The structures of AmpC^{D} and native AmpC of *E. coli*, which has 98% sequence homology (PDB code 1ke4; 1.72 Å resolution; Powers & Shoichet, 2002), were superimposed. The r.m.s.d. for the C$^{\alpha}$ atoms between Ala4 and Gln361, excluding the residues Gly286, Ser287 and Asp288 located on the H10 helix, is 0.72 Å. Upon comparison of the two overall structures, a significant difference was observed in the vicinity of the deleted resides Gly286-Ser287-Asp288. In the AmpC^{D} structure (Fig. 2*b*), the deletion causes a structural change in the 289–293 segment, corresponding to Asn289–Leu293, following the tripeptide deletion, which changes from the α-helix structure present in native AmpC (Fig. 2*a*) to a more extended loop. As a result, relative to the structure of native AmpC, the C$^{\alpha}$ atoms of residues Asn285 and Asn289, which are before and after the tripeptide deletion, move by approximately 3 Å away from the catalytically important O$^{\gamma}$ atom of Ser64, indicating that the

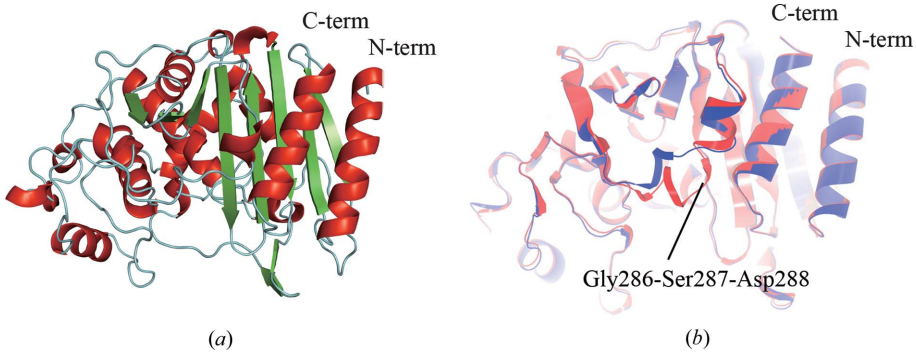

Figure 1
(*a*) Overall structure of AmpC^{D} β-lactamase. Only molecule *B* is depicted. α-Helices, β-strands and loops are shown in red, green and cyan, respectively. (*b*) Slabbed view of overall structure superposition of native AmpC β-lactamase (red; PDB code 1ke4) and AmpC^{D} β-lactamase (blue). In each structure, only molecule *B* is depicted.

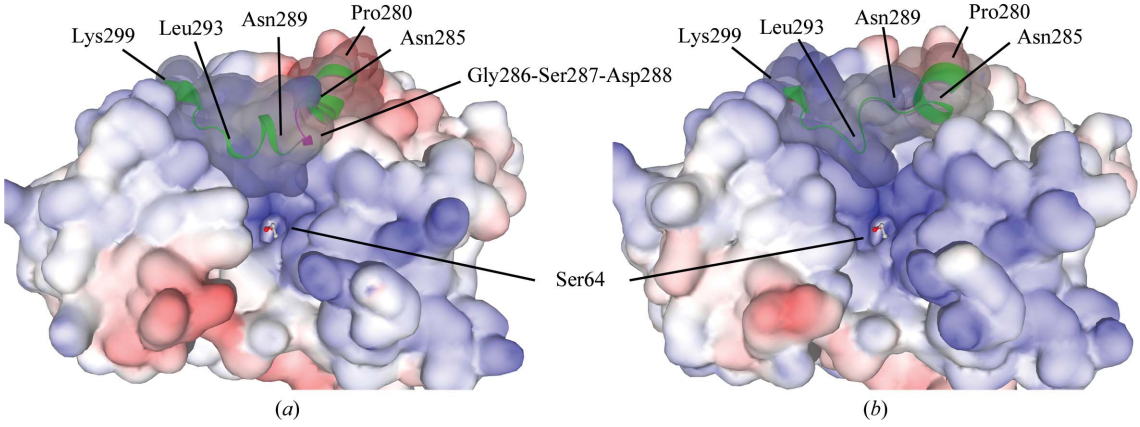

Figure 2
Electrostatic potential representations of (*a*) native AmpC β-lactamase and (*b*) AmpC^{D} β-lactamase. The catalytic residue Ser64 is represented as a ball-and-stick model (the O$^{\gamma}$ atom of Ser64 is coloured red). The Pro280–Lys299 region in native AmpC β-lactamase and AmpC^{D} β-lactamase is represented by a ribbon model coloured green on a transparent surface. In native AmpC β-lactamase, residues Gly286–Asp288 in the Pro280–Lys299 region are coloured violet.

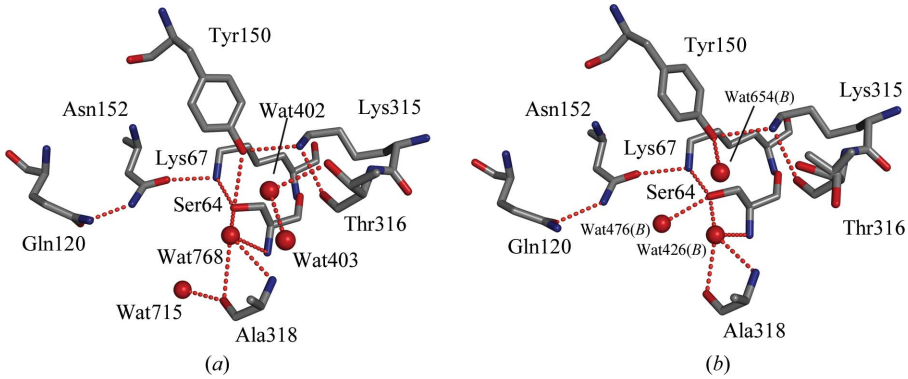

Figure 3
Substrate-binding site of (*a*) native AmpC β-lactamase determined to 1.72 Å resolution and (*b*) AmpC^{D} β-lactamase. In each structure, only molecule *B* is depicted. C, O and N atoms of the enzyme are shown in grey, red and blue, respectively. Water molecules cited in the text are labelled and are represented by red spheres. Dashed lines indicate hydrogen bonds with distances between 2.5 and 3.2 Å.

substrate-binding site of AmpC^{D} is wider than that of native AmpC. The crystal structure of AmpC^{D} also supports the previous results of molecular-modelling studies on AmpC^{D} with ceftazidime (Doi *et al.*, 2004); the tripeptide deletion in AmpC^{D} provides a more open site that can accommodate the R2 side chain of ceftazidime.

In the substrate-binding site of AmpC β-lactamases, residues Ser64, Lys67, Tyr150, Asn152, Lys315, Thr316 and the main chain of Ala318 are thought to be important in the hydrolysis reaction of β-lactam antibiotics (Lobkovsky *et al.*, 1993; Oefner *et al.*, 1990; Monnaie, Dubus & Frère, 1994; Monnaie, Dubus, Cooke *et al.*, 1994; Dubus *et al.*, 1994, 1995, 1996; Lobkovsky *et al.*, 1994). An overlay of these seven residues between AmpC^{D} and native AmpC shows a close fit, with the exceptions of the side chain of Tyr150 (the phenyl ring is rotated 19° around the $C^{\beta}-C^{\gamma}$ bond relative to that in molecule *B* of native AmpC). Thus, the structures of the key catalytic residues in the substrate-binding site are not dramatically affected, but, as mentioned above, the collapse of the α-helix on the tripeptide deletion in the H10 helix appears to give rise to an expansion of the substrate-binding site and is therefore believed to be the primary reason for the altered selectivity profile exhibited by AmpC^{D} relative to that of native AmpC (Doi *et al.*, 2004).

In the structure of native AmpC (Fig. 3*a*), four water molecules (Wat402, Wat403, Wat715 and Wat768) were observed in the substrate-binding site, where Wat402 was presumed to be the deacylating water (Powers & Shoichet, 2002) which hydrogen bonds to Thr316 $O^{\gamma 1}$ and Wat403. Wat715 is hydrogen bonded to the main-chain carbonyl O atom of Ala318 and Wat768 interacts with Ser64 and Ala318. In the substrate-binding site of AmpC^{D}, three water molecules [Wat426(*B*), Wat476(*B*) and Wat654(*B*) in molecule *B*] were located in the active site (Fig. 3*b*). As in native AmpC, a water molecule [Wat426(*B*) in the AmpC^{D} structure] is bound in the site which stabilizes the tetrahedral intermediate of the lactamase reaction (Usher *et al.*, 1998; Murphy & Pratt, 1988) and is hydrogen bonded to the main-chain N atoms of Ser64 and Ala318, Ser64 O^{γ} and the main-chain carbonyl O atom of Ala318.

In conclusion, we have determined the crystal structure of AmpC^{D} of *E. coli* with a tripeptide deletion (Gly286-Ser287-Asp288) in the H10 helix and revealed the structural changes associated with the tripeptide deletion. However, further crystallographic studies on AmpC^{D} in complexes with the hydrolyzed products of substrates and with inhibitors are required to further understand the structural correlations with enzyme activity and the altered selectivity profile.

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