Hydrolysis of Third-generation Cephalosporins by Class C β-Lactamases

STRUCTURES OF A TRANSITION STATE ANALOG OF CEFOTAXIME IN WILD-TYPE AND EXTENDED SPECTRUM ENZYMES*

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Michiyoshi Nukaga‡, Sanjai Kumar§, Kayoko Nukaga‡, R. F. Pratt§, and James R. Knox‡‡

From the Department of Molecular and Cell Biology, The University of Connecticut, Storrs, Connecticut 06269-3125 and the Department of Chemistry, Wesleyan University, Middletown, Connecticut 06459-0180

Bacterial resistance to the third-generation cephalosporins is an issue of great concern in current antibiotic therapeutics. An important source of this resistance is from production of extended-spectrum (ES) β-lactamases by bacteria. The Enterobacter cloacae GC1 enzyme is an example of a class C ES β-lactamase. Unlike wild-type (WT) forms, such as the E. cloacae P99 and Citrobacter freundii enzymes, the ES GC1 β-lactamase is able to rapidly hydrolyze third-generation cephalosporins such as cefotaxime and ceftazidime. To understand the basis for this ES activity, m-nitrophenyl 2-(2-aminothiazol-4-yl)-2-(Z)-methoxyimino]acetylaminomethyl phosphate has been synthesized and characterized. This phosphonate was designed to generate a transition state analog for turnover of cefotaxime. The crystal structures of complexes of the phosphonate with both ES GC1 and WT C. freundii GN346 β-lactamases have been determined to high resolution (1.4–1.5 Å). The serine-bound analog of the tetrahedral transition state for deacylation exhibits a very different binding geometry in each enzyme. In the WT β-lactamase the cefotaxime-like side chain is crowded against the Ω loop and must protrude from the binding site with its methyloxime branch extended. In the ES enzyme, a mutated Ω loop adopts an alternate conformation allowing the side chain to be much more buried. During the binding and turnover of the cefotaxime substrate by this ES enzyme, it is proposed that ligand-protein contacts and intra-ligand contacts are considerably relieved relative to WT, facilitating positioning and activation of the hydrolytic water molecule. The ES β-lactamase is thus able to efficiently inactivate third-generation cephalosporins.

β-Lactam antibiotics are widely used because of their effectiveness and safety. However, β-lactam-resistant bacteria have become widespread. The main cause of this resistance is the production of β-lactamases, which hydrolyze the amide bond in the β-lactam ring. β-Lactamases are grouped into four classes, A–D, on the basis of amino acid sequence. Although class B β-lactamases are metallo-β-lactamases, class C, and D β-lactamases are serine-reactive hydrolyses that function by acylation and deacylation steps employing tetrahedral intermediates (1, 2) (see Reaction 1).

To overcome β-lactam resistance, third-generation cephalosporins such as cefotaxime (1), ceftazidime (2), and ceftriaxone have been employed since the 1980s because of their relative stability to serine β-lactamases and their broad antibacterial spectrum. Third-generation cephalosporins typically contain a bulky group such as a 2-(2-aminothiazole-4-yl)-2-oxymino substitution (R1) at position C7 of the cephalosporin nucleus.

From the kinetic perspective, third-generation cephalosporins show high Km and low kcat values for class A and D β-lactamases, and low Km (Kf) and low kcat values for class C enzymes. This behavior suggests that these cephalosporins cannot effectively bind to the active site of wild-type (WT) class A β-lactamases and acylate them but can bind to and rapidly acylate class C enzymes to form stable acyl intermediates. Extensive use of third-generation cephalosporins has provided selective pressure leading to the appearance of extended-spectrum (ES) β-lactamases (3). These ES β-lactamases are able to hydrolyze third-generation cephalosporins as a result of mutations in a parental β-lactamase. Most ES β-lactamases belong to class A and are plasmid-mediated, such as those in the TEM and SHV families (4). In 1995, the first class C chromosomal ES β-lactamase was discovered in Enterobacter cloacae GC1 (6). Genetic and mutagenesis experiments with the ES GC1 enzyme revealed an improved deacylation activity that is somehow due to a unique three-residue repeat insertion (7).

The crystal structure of the ES GC1 enzyme shows the insertion makes the Ω loop at the bottom of the binding site more flexible compared with the parental WT E. cloacae P99 enzyme (8). It was suggested that a conformational change in ES GC1 permits the enzyme to accommodate cephalosporins with large R1 side chains and allows more conformational freedom for the acyl intermediate. However, although structural information is available for cephalosporins and monobactams bound to WT class C β-lactamases (9–11), no information is available for a β-lactam bound to an ES β-lactamase.

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‡‡‡To whom correspondence may be addressed: Dept. of Chemistry, Wesleyan University, Middletown, CT 06459-0180. Tel.: 860-685-2629; E-mail: rpratt@wesleyan.edu.

§§§To whom correspondence may be addressed: Dept. of Molecular and Cell Biology, The University of Connecticut, Storrs, CT 06269-3125. Tel.: 860-486-3133; Fax: 860-486-4745; E-mail: james.knox@uconn.edu.

1 The abbreviations used are: WT, wild-type, non-ES; EDC, 1-β-(dimethylamino)propyl-3-ethylcarbodiimide hydrochloride; ES, extended spectrum; MPD, 2-methyl-2,4-pentanediol; PEG, polyethylene glycol; r.m.s.d., root-mean-squared deviation; MS, mass spectrometry; ESI, electrospray ionization.

2 G. Jacoby and K. Bush, see www.lahey.org/studies/webt.stm for a listing of ES β-lactamases.
Because the ES GC1 β-lactamase can effectively hydrolyze cefotaxime (1) and ceftazidime (2), its intermediates with these important β-lactams are difficult to visualize by x-ray crystallography. For this study, phosphonate 4 was designed to produce a tetrahedral transition state analog for cefotaxime hydrolysis. We report two crystal structures of the phosphonate complexed with both ES and WT β-lactamases from E. cloacae GC1 and Citrobacter freundii GN346, respectively. Large differences in the binding of the analog and in the conformation of the Ω loop of the enzymes are revealed, which provide better understanding of the hydrolysis of third-generation cephalosporins by ES class C β-lactamases (see Structures 1–7).

MATERIALS AND METHODS

The substrate cephalothin 3 was a gift from Eli Lilly and Co. The phosphonate 5 and the depsipeptides 6 and 7 were available from previous studies (12, 13) 3

Synthetic Methods—m-Nitrophenyl 2-(2-aminothiazol-4-yl)-2-[(Z)-methoxyimino]acetylaminomethyl phosphonate (4) was synthesized. This compound was prepared by the Scheme 1 reaction sequence (outlined in Structures 8–10). First, the hydroxysuccinimide ester of 2-(2-aminothiazol-4-yl)-2-[(Z)-methoxyiminocetic acid (8) was prepared as described previously. It was then condensed with aminomethylphosphonic acid to afford 2-(2-aminothiazol-4-yl)-2-[(Z)-methoxyiminomethylphosphonic acid (9). Thus, to a solution of aminomethylphosphonic acid (2.5 g, 22.5 mmol) in water (37 ml) was added sodium hydrogen carbonate (6.25 g, 74.4 mmol) and potassium carbonate (13.7 g, 99.4 mmol), and the mixture was stirred at room temperature until all components were dissolved. To this was added 7 (8.06 g, 27

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3 Kumar, S., Adeban, S. A., Nukaga, M., and Pratt, R. F. (2004) Biochemistry, in press.
Ethyl acetate (3 mmol) was added, and the reaction mixture was stirred at room temperature for 26 h. After quenching the reaction by the addition of water (37 ml), the resulting mixture was filtered and the filtrate was acidified to pH 3.2 with concentrated hydrochloric acid. This solution was extracted with ethyl acetate (3 mmol), and the reaction mixture was stirred at room temperature for 24 h. After quenching the reaction by the addition of acetone (total 8.5 ml) over 2 days, yielded pure product 4 in 32% (0.035 g) yield. 1H NMR spectrum (2H2O): 8.10 (m, 4H, ArH), 3.79 (d, J = 12 Hz, 2H, PCH2), 4.02 (s, 3H, OCH3), and 7.42 (s, 1H, thiazole-4H) (Structures 8–10).

The m-nitrophenyl ester 10 was then prepared as follows. To a solution of well dried 9 (0.316 g, 1.14 mmol) in pyridine (6 ml), freshly distilled from barium oxide, was added dry m-nitrophenol (0.190 g, 1.56 mmol). After the mixture had been stirred for 15 min at room temperature under nitrogen, trichloroacetonitrile (1.9 ml, 18.9 mmol) was added, and the reaction mixture was stirred at 80 °C for 6.5 h. Pyridine was removed by evaporation at room temperature with an oil pump overnight, and the dark brown crude material was taken into saturated sodium hydroxide solution, washed several times with water, through a Sephadex G-10 column. 1H NMR spectrum (2H2O): 8.1 (m, 4H, ArH); MS (ESI): 558.5.

The structures comprise Scheme 1.
were grown at 37 °C in Terrific broth containing a sub-lethal concentration of kanamycin (30 µg/ml). In mid-logarithmic phase, 0.5 mM isopropyl 1-thio-β-D-galactopyranoside was added, and the culture was continued for 12 h. Crude β-lactamase was obtained by disrupting the cells with a French press in 50 mM sodium phosphate buffer, pH 7, followed by centrifugation for 1 h at 40,000 × g and 4 °C. β-Lactamase was applied to a CM-Sephadex C-50 (Amersham Biosciences) column in 10 mM sodium phosphate buffer, pH 7, and eluted with a 0–0.5 M linear NaCl gradient. Active fractions were further purified with an m-amino-phenyl boronic acid affinity column using Affi-Gel-10 (Bio-Rad) with a NaCl gradient. Active fractions were further purified with an m-amino-phenyl boronic acid affinity column using Affi-Gel-10 (Bio-Rad) with a NaCl gradient.

Active fractions were further purified with an m-amino-phenyl boronic acid affinity column using Affi-Gel-10 (Bio-Rad) with a NaCl gradient.

FIG. 1. Stereoview of the Fα – Fc electron density of the phosphonate 4 bonded to Ser-64 in the ES GC1 β-lactamase (a) and in the WT GN346 β-lactamase (b). The m-nitrophenol leaving group is absent. The contour level is 3σ. The figure was made by XtalView (23) and Raster3D (14).

### TABLE III

| Crystallographic refinements | ES GC1 | WT GN346 |
|-------------------------------|--------|----------|
| **β-Lactamase**               |        |          |
| Program used for final refinement | SHELX | CNS      |
| Resolution range (Å)          | 20–1.38| 20–1.52  |
| No. of reflections used [F > 0σ(F)] | 64,648 | 56,236   |
| Rwork/Rfree (%)               | 13.9/19.5 | 19.5/21.7 |
| Rtotal                        | 14.2  | 19.7     |
| Residues in Ramachandran zones (%) |         |          |
| Favored/allowed               | 92.2/7.8 | 93.8/6.2 |
| Disallowed                    | 0     | 0        |
| r.m.s.d. values from identity |        |          |
| Bond lengths (Å)              | 0.017 | 0.006    |
| Bond angles (deg.)            | 0.033 | 1.4      |
| Chiral volumes (Å²)/improper (deg.) | 0.062 | 0.83     |
| Mean B-factors                | 15.8 (2875) | 17.7 (2578) |
| Protein (no.)                 | 28.1 (0.75/17) | 19.9 (1.0/17) |
| Inhibitor (occupancy/no.)     | 29.1 (121) | 36.5 (8)  |
| Glycerol/MPD (no.)            | 31.2 (399) | 29.0 (414) |
| Water molecules (no.)         | 17.8 (3303) | 19.1 (3309) |

**Centre for Applied Microbiology and Research** (Porton Down, Wiltshire, UK).

**Kinetic Methods**—The inactivation rate constants (ki) for essentially irreversible inhibition of the enzymes by 4 and 5 were obtained by incubating the inhibitor (1–20 µM) with enzyme (0.25 mM) in 20 mM MOPS buffer, pH 7.5, at 25 °C. Suitable aliquots were withdrawn from the incubation mixtures with time, and the activity of the enzyme was measured by diluting the incubation mixture into the assay solution of the substrate cephalothin (200 µM). The initial rate of hydrolysis of the substrate was then monitored spectrophotometrically at 278 nm. Pseudo-first order inactivation rate constants were obtained by fitting the initial rates to an exponential function in time. Second order rate constants (ki) were determined by dividing the pseudo-first order rate constant by the inhibitor concentration.

**Crystallographic Methods**—Crystals were grown by the vapor diffusion method at room temperature. The WT GN346 β-lactamase was co-crystallized with the phosphonate 4. The protein drop containing 7.5 mg/ml protein, 8% PEG (M, 8000), 4% MPD, 0.01 m urea, 0.2 mM 4, and 0.04 mM HEPES, pH 7.5, was placed over a reservoir of 20% PEG, 10% MPD, and 0.1 mM HEPES, pH 7.5. Crystals appeared in 10–20 days. Macroseeding was necessary to get larger crystals of average size 0.3 × 0.3 × 0.5 mm. They have space group P212121 with one monomer of 39.1 kDa in the asymmetric unit. Apo-ES GC1 β-lactamase was crystallized from a protein drop containing 10 mg/ml protein, 10% PEG, and 50 mM HEPES, pH 7, placed over a reservoir of 20% PEG in 0.1 mM HEPES, pH 7 (8). Thin plate-like crystals with average size 0.05 × 0.2 × 0.8 mm appeared in 10–20 days in P212121 with one monomer of 39.4 kDa in the asymmetric unit.

Data for ES GC1 and WT GN346 crystals were collected at 100 K at MacCHESS station A1 at Cornell University or the JASRI/SPring8 beamline BL40B2 in Hyogo, Japan, respectively. A GC1 crystal was placed in a solution of 2 mM phosphonate 4, 25% PEG, and 0.1 mM HEPES, pH 7, for 90 min before mounting. After soaking, the crystal was briefly dipped into a PEG solution containing 20% glycerol to prevent ice crystal formation upon cryocooling. A GN346 crystal, co-crystallized with 4 and the cryoprotectant MPD, was directly mounted.
Inactivation Kinetics—Phosphonate monoesters such as 5, bearing a classic penicillin side chain, have been shown to inactivate WT class C β-lactamases by covalent modification of the active site serine residue (17, 18). Incorporation of a third-generation cephalosporin side chain leads to 4, which also inactivates these enzymes. Relevant inactivation rate constants are shown in Table II. The new phosphonate 4 is some 10-fold less reactive than 5 with a WT enzyme (P99). This presumably reflects the effect of the bulkier side chain of 4. This difference between the $k_i$ values of 4 and 5 is reduced to less than 3-fold in the case of the ES enzyme (GC1). This change may result from the somewhat easier fit of the rather congested penta-coordinated intermediate/transition state (17), also bearing the bulky oxyimino side chain, into the ES GC1 active site than into that of the P99 enzyme. The phosphonate 4 is somewhat more reactive with the WT C. freundii enzyme than with the WT E. cloacae P99 enzyme; this may reflect the Glu/Gln amino acid difference at position 219 (19).

Although we anticipated that the adduct formed between the phosphonates and these β-lactamases would mimic the tetrahedral intermediates/transition states of β-lactam turnover in several essential details, they would, of course, more directly resemble transient species in the hydrolysis of the depsipeptides 6 and 7, and, in particular, the deacylation tetrahedral intermediates 11 (Structure 11).

The data of Table II show that 6 and 7 are turned over by a WT and an ES class C β-lactamase with similar facility; the bulky side chain of 6 seems to have little effect. This situation contrasts sharply with the relative turnover rates of cephalosporins 1 and 3 where that of the former is greatly impeded. Apparently the resistance of third-generation cephalosporins to hydrolysis by WT class C β-lactamases requires the presence of both the oxyimino side chain and the dihydrothiazine ring, as previously suggested by Powers et al. on structural grounds (20). One solution to the problem to WT enzymes posed by third-generation cephalosporins, which is found in the ES GC1 enzyme, is revealed by the crystal structures described below for complexes formed between the phosphonate 4 and WT (C. freundii GN346) and ES (E. cloacae GC1) enzymes.

**RESULTS**

**Structure Determination and Refinement**—Molecular replacement and rigid-body refinement using the unliganded ES GC1 (Protein Data Bank entry 1GCE) and WT GN346 (1FR1) β-lactamases was done at 2 Å with CNS (21). R-factors were 35–38% for the unsolvated apo-protein models. Because the models were being optimized by simulated annealing (22), a Ser-64-bound moiety was evident in the high resolution difference density of both complexes. Based on previous work (18), phosphonate 4, devoid of the m-nitrophenol leaving group, was added to each model. XtalView (23) was employed for mapping and manipulation of the structures.

The last stage of the higher resolution refinement of ES GC1 was performed with SHELX (24) using all $F > 0ou(F)$ data (Table III). At 1.38-Å resolution, anisotropic $B$-factor refinement reduced the $R$ and $R_{free}$ values by 4 and 1%, respectively, to 15.0% and 21.1%. Riding hydrogen atoms were later added in calculated positions. The Ω loop (generally residues 200–226) in the ES GC1 complex was observed to have two conformations from 219 to 225, and this part was modeled with

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**Table IV**

**Conformations and distances in the phosphorylated β-lactamases**

Distances are rounded to the nearest 0.05 Å.

| Dihedral angle (°) | ES GC1 | WT GN346 |
|-------------------|--------|----------|
| SerCO-α-D-ε-P     | 115    | 106      |
| SerOγ-γ-P-CH₃     | −164   | −162     |
| P-CH₃-NH-CO       | −128   | 155      |
| NH-CO-C-C         | 75     | −102     |
| Distance (Å, Fig. 3) |
| A                  | 2.80   | 2.80     |
| B                  | 3.10   | 2.90     |
| C                  | 3.00   | 3.10     |
| D                  | 3.20   | 2.80     |
| E                  | 2.90   | 2.75     |
| F                  | 2.70   | 2.65     |
| G                  | 3.00   | 3.10     |
| H                  | 3.40   | 3.00     |
| I                  | 5.75   | 2.80     |

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**Fig. 2.** Stereoview of the tertiary structure of the class C WT GN346 β-lactamase with the acyl-phosphonate. The ES GC1 enzyme is similar except in the Ω loop. The figures was drawn with MOLSCRIPT (5).

**Fig. 3.** Diagram of the phosphonate-enzyme distances listed in Table IV.
unequal occupancies (0.75/0.25). As described below, an “open” \Omega conformer has the higher occupancy here and is thought to occur only in a complex, whereas the lower occupancy “closed” conformer can exist in either the ligand-free or complexed enzyme (8, 25). Likewise, two sets of water molecules were modeled in the complexed and apo binding sites with 0.75 and 0.25 occupancies, respectively. Only the closed conformer was seen for the \Omega loop in the WT GN346 enzyme. Ramachandran analysis of the final ES GC1 and WT GN346 models showed 92.2% and 93.8% of backbone angles, respectively, fall in most favored regions, and none fall in disallowed regions. Final \text{R}_{\text{free}} values for the ES GC1 and WT GN346 complexes are 13.9/19.5% and 19.5/21.7%, respectively.

**Phosphonate Binding**—The electron density of the irreversibly bound (17) phosphonate in each complex is shown in Fig. 1. In the WT GN346 complex, the density of the terminal methyl group of the oxime is rather weak. The entire ligand, however, has a reasonable B-factor (20 Å²) about 35% lower than that for the ligand in the ES GC1 complex. In this ES complex, the phosphonate was modeled with a partial occupancy (0.75) that matches the occupancy of one of the two \Omega loop conformations. Fig. 2 shows the binding site in the mixed \alpha-\beta tertiary structure of the WT class C GN346 \beta-lactamase. Except in the \Omega loop, the ES GC1 molecule is folded similarly. The phosphonate lies between two domains, an all-\alpha-helical domain and an \alpha/\beta domain containing a 5-stranded antiparallel \beta-sheet surrounded by \alpha-helices.

**DISCUSSION**

**Common Features in the WT GN346 and ES GC1 Complexes**—In both complexes the catalytic Ser-64 has been phosphorylated with 4 with the departure of the \textit{m}-nitrophenol group. The terminal P-O₁ group displaces a water molecule from the oxyanion hole formed by the backbone amide group of Ser-64 and Ser-318/321 (using numbering for the WT GN346 and ES GC1 \beta-lactamases, respectively). The oxygen atom is asymmetrically hydrogen-bonded to the amides, with the stronger and more linear hydrogen bond to the NH on the B3 \beta-strand (distances E and F, Table IV and Fig. 3). The remaining P-O₂ group is exposed. It hydrogen bonds to the hydroxyl group of Tyr-150 and to a water molecule bridging to Thr-316/319 on the B3 strand (distances C and D, Table IV). Each complex has a similar conformation in the Ser-64-Oγ-P linkage, as described by the dihedral angles.

**Differences in Phosphonate Binding in WT and ES Enzymes**—The orientation and conformation of the cefotaxime-like side chain of the phosphonate is markedly different in the two complexes (Fig. 4a). In the WT GN346 binding site, the oxyimino branch is solvent-exposed, but in the ES GC1 complex the oxyimino branch is quite buried. The two methyloxime positions are 10 Å apart as a result of large rotations, including an almost 180° flip about the CO–C bond (Table IV). Because the acylamide linkages have different spatial positions, the two aminothiazole rings lie in approximately the same position, however. In each complex a hydrogen bond exists between the NH group of the acylamide and the backbone CO group of Ser-318 and Asn-152 side chains (distances H and I) (10, 18), differs considerably in the two structures and is essentially absent in the ES complex, because the CO group of the phosphonate has shifted inward by 2.5 Å. It is notable that neither the oxyimino group nor the aminothiazole group engages either \beta-lactamase in direct
hydrogen bonding. The apparently looser binding of the phosphonate side chain within the less-restricted binding site of the ES enzyme, evidenced by the larger \( B \)-factor of the ligand, may provide the extra motion or conformational freedom necessary for a more facile hydrolysis of cefotaxime by this extended-spectrum \( \beta \)-lactamase.

FIG. 5. Overlay of phosphonate (blue) and boronic acid (yellow) analogs in WT GN346 and WT AmpC complexes, respectively (a), and phosphonate (blue) and acylceftazidime (yellow) complexes (b).

FIG. 6. a, schematic of potential close contacts in a class C \( \beta \)-lactamase complex with an acylated cephalosporin having a branched side chain; b, overlay of ES GC1 phosphonate complex (red) and WT AmpC acylceftazidime complex (blue).
Structural Factors Influencing Binding—The primary structural determinant of the large difference in phosphate orientation in the two complexes is the Ω loop at the bottom of the binding site (Figs. 2 and 4b). In the WT complex, the aminothiazole ring has its edge only 3.5 Å from the ring of Tyr-221, forming a perpendicular quadrupolar interaction. This contact with the Ω loop causes the phosphate to tilt out of the WT binding site, exposing the methyl end of the oxyimino branch.

In the ES complex, a quite different orientation is found because the Ω loop of the enzyme, longer by a 3-residue insertion, can adopt an alternative folding. This conformational change in the Ω loop is presumably initiated by contact between Tyr-221 (numbered 224 in GC1) and an early reaction intermediate, as seen in other GC1 complexes (25, 26). The expanded Ω loop refolds to a more open conformation so that Tyr-224 moves as much as 10 Å from its WT position, a position now occupied by the methyl group of the oxyimino branch. The cefotaxime-like side chain of the phosphonate is therefore more buried in the ES GC1 complex than in the WT complex. This new binding mode in the ES enzyme is facilitated by the absence of two hydrogen bonds to Gln-120 and Asn-152, present in WT, and by the formation of weak hydrophobic interactions, which pull the intermediate deeper into the binding pocket.

Refolding of the Ω loop in the ES β-lactamase to make more space for the side chain (Fig. 4b) does not explain the large rotation, relative to WT, about the CO-C bond of the phosphonate. It is likely that hydrophobic or polar interactions determine which rotamer is stabilized. It seems that the methyl group of the oxime, rather than the more polar aminothiazole ring, better replaces the (shifted) Tyr-224 on the flexible Ω loop of the ES enzyme. But in the WT enzyme, the other phosphate rotamer is found, because the aminothiazole ring can form a quadrupole-quadrupole interaction with the (immovable) ring of Tyr-221 on the more rigid Ω loop.

Comparison with Related Complexes—No crystal structure is available of cefotaxime (1) bound to a β-lactamase. A structure is known of the acyl complex of cefotaxime with an ancestrally related penicillin-binding protein, the R61 D-ala-n-D-ala-1-depsipeptide (27, 28). The R1 side chain of cefotaxime is found, because the aminothiazole ring can form a perpendicular quadrupolar interaction. This contact against the 290s loop at the bottom of the binding site, no mutations exist in the 290s loop. Nor is structural disorder in the 290s loop expected, and none has been observed to date (25, 26).

The open conformer of the mobile Ω loop of the extended spectrum GC1 β-lactamase permits burial of the third-generation side chain of cefotaxime into the class C β-lactamase active site. The additional binding energy thus obtained and the minimal steric disruption of the active site allow the ES enzyme to efficiently catalyze hydrolysis of branched cephalosporins; bacterial resistance to these antibiotics is thus achieved. Should the ES GC1 β-lactamase become a more widespread clinical problem, new cephalosporin derivatives bearing even larger oxyimino branches might be explored.

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