Structural Aspects for Evolution of β-Lactamases from Penicillin-Binding Proteins

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Abstract: Penicillin-binding proteins (PBPs), biosynthetic enzymes of bacterial cell wall assembly, and β-lactamases, resistance enzymes to β-lactam antibiotics, are related to each other from an evolutionary point of view. Massova and Mobashery (Antimicrob. Agents Chemother. 1998, 42, 1–17) have proposed that for β-lactamases to have become effective at their function as antibiotic resistance enzymes, they would have had to undergo structure alterations such that they would not interact with the peptidoglycan, which is the substrate for PBPs. A cephalosporin analogue, 3β-[N-(Acetyl-L-alanyl-D-glutamyl-L-lysine)-3-acetoxyethyl-3-cephem-carboxylic acid (compound 6), was conceived and synthesized to test this notion. The X-ray structure of the complex of this cephalosporin bound to the active site of the deacylation-deficient Q120L/Y150E variant of the class C AmpC β-lactamase from Escherichia coli was solved at 1.7 Å resolution. This complex revealed that the surface for interaction with the strand of peptidoglycan that acylates the active site, which is present in PBPs, is absent in the β-lactamase active site. Furthermore, insertion of a peptide in the β-lactamase active site at a location where the second strand of peptidoglycan in some PBPs binds has effectively abolished the possibility for such interaction with the β-lactamase. A 2.6 ns dynamics simulation was carried out for the complex, which revealed that the peptidoglycan surrogate (i.e., the active-site-bound ligand) undergoes substantial motion and is not stabilized for binding within the active site. These factors taken together disclose the set of structure modifications in the antibiotic resistance enzyme that prevent it from interacting with the peptidoglycan, en route to achieving catalytic proficiency for their intended function.

Sequence similarity, a shared protein fold, conservation of structural motifs, and mechanistic features such as active site acylation by their respective substrates at specific serine residues have been proposed as consistent with kinship of β-lactamases and penicillin-binding proteins (PBPs).1–6 β-Lactamases are bacterial resistance enzymes to β-lactam antibiotics, and PBPs are biosynthetic enzymes involved in assembly and processing of the cell wall. It is clear that, despite the divergence of the sequences, the general protein fold for these families of enzymes is preserved. The divergence of the sequences has allowed for impressive diversification of function among these proteins.

It is widely thought that the genes for the more ancient PBPs gave rise to those of β-lactamases.2,4–6 PBPs process the building blocks for the cell wall, namely the peptidoglycan (1). Two of the important PBP activities are the DD-transpeptidase and DD-peptidase reactions. The former activity is responsible for the final step of cell wall assembly by cross-linking two strands of peptidoglycan (species 4). The latter activity removes the C-terminal d-alanine from the peptidoglycan (species 5), a process that moderates the degree of cross-linking. The enzymes that carry out these activities catalyze their reactions in two steps that involve an intermediary acyl–enzyme species (2).

β-Lactamases of classes A, C, and D also go through an acyl–enzyme species in their turnover of β-lactam antibiotics. These antibiotics mimic the structure of the acyl-d-Ala-d-Ala portion of the peptidoglycan.2 However, the catalytic machineries...
ies of these three classes of \(\beta\)-lactamases are distinct and have evolved independently from different ancestral PBP progenitors.\(^2\)\(^,\)\(^4\) Designed cephalosporin molecule that provides structural evidence for the proposal of Massova and Mobashery. Molecular dynamics simulations of this complex provide additional insights into these issues, suggesting that a portion of the ligand undergoes substantial motion in the active site of this enzyme in agreement with X-ray diffraction data, which revealed the existence of two possible conformers of the bound ligand. The 2.6 ns molecular dynamics simulation provided a detailed account of the motion of the ligand within the active site, which incidentally samples both conformers of the ligand seen within the X-ray structure of the complex.

**Experimental Procedures**

Compound 14 was prepared by a literature method.\(^{10}\) The Q120L/Y150E mutant of E. coli AmpC \(\beta\)-lactamase was made by oligonucleotide-directed mutagenesis, expressed, and purified as previously described.\(^{11}\) \(N\)-Fluorenlymethoxycarbonyl-l-\(\alpha\)-alanyl-l-glutamic Acid, \(\alpha\)-tert-Butyl, \(\gamma\)-Benzy1 Diester (10). To the mixture of \(N\)-Fmoc-l-\(\alpha\)-alanine (8) (748 mg, 2.40 mmol), l-glutamic acid \(\alpha\)-tert-butyl-\(\gamma\)-benzy1 diester (9) (587 mg, 2.00 mmol), and HOBt (324 mg, 2.40 mmol) in CH\(_2\)Cl\(_2\) (20 mL) was added DCC (480 mg, 2.33 mmol). The resultant mixture was stirred at room temperature overnight. The precipitated DCU was filtered, and the filtrate was concentrated on a rotary evaporator. Ethyl acetate (30 mL) was added to the residue, and the suspension of additional DCU was filtered. The solvent was removed in vacuo, and the residue was redissolved in EtOAc. This solution was washed with 1 N HCl, water, NaHCO\(_3\), water, and brine and then was dried over MgSO\(_4\). The solvent was removed in vacuo, and the product was purified by column chromatography (silica gel, 5:3 then 1:1 hexane/EtOAc) to give a white solid (761 mg, 65\%). \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 1.39–1.43 (overlapping d and s, 12H), 1.91–2.05 (m, 1H), 2.18–2.27 (m, 1H), 2.35–2.51 (m, 2H), 4.20–4.33 (m, 1H), 4.32–4.39 (m, 3H), 4.50–4.55 (s, 1H), 5.10 (s, 2H), 5.61 (bd, 1H, J = 7.6 Hz), 6.94 (bd, 1H, J = 7.2 Hz), 7.28–7.40 (m, 9H), 7.58 (bd, 2H, J = 6.8 Hz), 7.75 (d, 2H, J = 8 Hz). \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 19.20, 27.67, 28.19, 30.45, 47.31, 50.72, 52.37, 66.77, 67.34, 71.73, 123.03, 123.7, 127.33, 129.4, 129.7, 136.81, 137.1, 171.6, 173.3, 173.8, 173.9. EI HRMS 529.1973 (M\(^+\) – C\(_6\)H\(_5\)O\(_2\)).

**N-Acetyl-l-\(\alpha\)-alanyl-l-glutamic Acid, \(\alpha\)-tert-Butyl, \(\gamma\)-Benzy1 Diester (11).** A round-bottomed flask was charged with compound 10 (453 mg, 0.772 mmol) and 10% piperidine/CH\(_2\)Cl\(_2\) (22 mL), and the resultant solution was stirred at room temperature for 1 h. The solution was washed with phosphate buffer (pH 5.5, 1 M), water, and brine and then was dried over MgSO\(_4\). The solvent was removed in vacuo, and the product was purified by column chromatography (silica gel, 1:4 hexane/EtOAc) to give a colorless oil (314 mg, 100\%). \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 2.51 (m, 2H), 4.20–4.23 (m, 1H), 4.32–4.39 (m, 3H), 4.50–4.55 (s, 1H), 5.10 (s, 2H), 5.61 (bd, 1H, J = 7.6 Hz), 6.94 (bd, 1H, J = 7.2 Hz), 7.28–7.40 (m, 9H), 7.58 (bd, 2H, J = 6.8 Hz), 7.75 (d, 2H, J = 8 Hz). \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 19.18, 27.67, 28.19, 30.45, 47.31, 50.72, 52.37, 66.77, 67.34, 71.73, 123.03, 123.7, 127.33, 129.4, 129.7, 136.81, 137.1, 171.6, 173.3, 173.8. El HRMS 529.1973 (M\(^+\) – C\(_6\)H\(_5\)O\(_2\)).

**N-Acetyl-l-\(\alpha\)-alanyl-l-glutamic Acid, \(\alpha\)-tert-Butyl, \(\gamma\)-Benzy1 Diester (11).** A solution of compound 14 (270 mg, 0.664 mmol) in MeOH (20 mL) was charged with Pd/C (5% Degussa type, 32 mg), and the mixture was stirred vigorously under an ambient pressure of 1 atm for 1 h. The precipitated DCU was filtered, and the filtrate was concentrated on a rotary evaporator. Ethyl acetate (30 mL) was added to the residue, and the suspension of additional DCU was filtered. The solvent was removed in vacuo, and the residue was redissolved in EtOAc. This solution was washed with 1 N HCl, water, NaHCO\(_3\), water, and brine and then was dried over MgSO\(_4\). The solvent was removed in vacuo, and the product was purified by column chromatography (silica gel, 5:3 then 1:1 hexane/EtOAc) to give a white solid (761 mg, 65\%). \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 1.39–1.43 (overlapping d and s, 12H), 1.91–2.05 (m, 1H), 2.18–2.27 (m, 1H), 2.35–2.51 (m, 2H), 4.20–4.23 (m, 1H), 4.32–4.39 (m, 3H), 4.50–4.55 (s, 1H), 5.10 (s, 2H), 5.61 (bd, 1H, J = 7.6 Hz), 6.94 (bd, 1H, J = 7.2 Hz), 7.28–7.40 (m, 9H), 7.58 (bd, 2H, J = 6.8 Hz), 7.75 (d, 2H, J = 8 Hz). \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 19.20, 27.67, 28.19, 30.45, 47.31, 50.72, 52.37, 66.77, 67.34, 71.73, 123.03, 123.7, 127.33, 129.4, 129.7, 136.81, 137.1, 171.6, 173.3, 173.8. El HRMS 529.1973 (M\(^+\) – C\(_6\)H\(_5\)O\(_2\)).


hydrogen at room temperature overnight. Some CHCl₃ (~50 mL) was added to dilute the reaction mixture, and the organic solution was washed with 5% NaHCO₃, 1 N HCl, water, and brine and then was dried over MgSO₄. The solvent was removed in vacuo, and the product was purified by column chromatography (silica gel, 50:1 CHCl₃/MeOH) to give the title compound as a white solid (200 mg). ¹H NMR (CDCl₃): δ 1.37 (d, 3H, J = 6.9 Hz), 1.45 (s, 9H), 1.93–2.48 (m, 7H), 3.49–4.66 (m, 2H), 6.81 (bd, 1H, J = 7.8 Hz), 7.30 (bd, 1H, J = 7.8 Hz). ¹³C NMR (CDCl₃): δ 18.5, 23.0, 26.8, 27.9, 30.1, 48.9, 52.5, 82.5, 170.6, 170.8, 172.7, 176.1. This crude product was redissolved in CH₂Cl₂ (12 mL), and to this solution was added 12 (256 mg, 0.762 mmol), HOBt (86 mg, 0.635 mmol), and DCC (25 mg, 0.133 mmol). The resultant mixture was stirred at room temperature overnight. Some CHCl₃ (~50 mL) was added to dilute the reaction mixture, and the organic solution was washed with 5% NaHCO₃, 1 N HCl, water, and brine and then was dried over MgSO₄. The solvent was removed in vacuo, and the product was purified by column chromatography (silica gel, 50:1 CHCl₃/MeOH) to give the title compound as a white solid (200 mg, 47%). ¹H NMR (CD₃OD): δ 0.86 (t, 3H, J = 7.5 Hz), 1.47 (s, 9H), 1.64–2.32 (overlapping s and m, 25H), 1.72–2.13 (overlapping s and m, 6H), 3.06 (m, 2H), 4.20–4.28 (m, 3H), 5.15 (s, 2H), 7.34–7.44 (m, 4H). ¹¹C NMR (CD₃OD): δ 16.90, 19.73, 21.63, 24.35, 25.77, 28.30, 28.78, 29.11, 31.43, 39.34, 49.84, 52.27, 53.40, 67.74, 81.03, 83.62, 129.31, 129.44, 129.75, 136.83, 159.01, 171.10, 171.63, 173.71, 183.22. Mp 48 °C. ESI MS: 634.36 (M⁺, calcd for C₄⁵H₃₄N₂O₉₂).
**Computational Procedures.** The X-ray structure of the acyl–enzyme complex provided the initial coordinates for the molecular dynamics simulations. Crystallographic waters were retained, and hydrogen atoms were added to the protein using the “protonate” program, which is part of the AMBER 718 suite of programs. AMBER force field parameters and atomic charges were assigned to all atoms using the “parm99” set of parameters. The Sybyl program (Tripos Inc., St. Louis, MO) was used for the manipulation and visualization of all structures and for the protonation of the bound ligand. The atomic charges of the bound ligand were determined using the RESP methodology.19 This consisted of first optimizing the molecules using the AM1 Hamiltonian, followed by an HF/6-31G* single-point energy calculation to determine the electrostatic potential around the molecule, which was subsequently used in the two-stage RESP fitting procedure. The Gaussian 98 package20 was used to carry out all ab initio calculations. The acyl–enzyme complex was immersed in a box of TIP3P21 water molecules such that no atom in the acyl–enzyme complex was within 12 Å from any side of the box; after solvation of the 5650-atom acyl–enzyme complex, the system consisted of a total of 53 830 atoms. All bonds involving hydrogen atoms were constrained using the SHAKE algorithm, and a 2-fs time step was used. The particle mesh Ewald22 method was used to treat long-range electrostatics. Water molecules were first energy minimized and equilibrated by running a short simulation with the acyl–enzyme complex, the system was subsequently slowly heated to 300 K via pressure and temperature (1 atm and 300 K, respectively) was then carried out at 300 K for further equilibration. A 2.6 ns simulation at constant temperature (1 atm and 300 K, respectively) was then carried out on an in-house beowulf cluster. Snapshots were collected every 0.2 ps.

**Results and Discussion**

Cephalosporin 6 was conceived as a probe in exploring the potential surface interactions of peptidoglycan with the class C β-lactamase. The β-lactam nucleus of the cephalosporin mimics the two D-Ala residues of the peptidoglycan (the portions traced in red). The acyl moiety at the C7 amine (shown in blue) traces most of the remainder of the peptide in the peptidoglycan. The combined red and blue portions of the structure of 6 represent the strand of peptidoglycan that would acylate the active-site serine (i.e., E-OH). Species 7, in essence, depicts the first peptidoglycan strand that has acylated the active-site serine. On active-site acylation, the “terminal D-Ala residue” from the strand of the peptidoglycan would be eliminated (the portions of 7 shown in red).

![Scheme 1](image)

Compound 6 was synthesized according to Scheme 1. The tripeptide 13 was constructed by standard peptide coupling procedures. After the removal of the benzyl group from this peptide, it was coupled to cephalosporin 14 to give 15. A global deprotection by TFA afforded compound 6.

To explore how compound 6 would be recognized by a class C β-lactamase, the crystal structure of its complex with the Q120L/X150E mutant of AmpC was determined to 1.71 Å. This mutant is decacylation-deficient,11 and it allowed us to trap the
Despite this “patchiness,” there are clear indications that species 7 makes several hydrogen bonds with active site residues (Figure 1B). The carbonyl oxygen of the ester moiety is in the “oxyanion” hole, interacting with amide nitrogens of Ser64 and Ala318. The carbonyl oxygen of the lysine in the C7 side-chain hydrogen bonds with side-chain nitrogen of Asn152. Finally, one of the carboxylate oxygens of the C7 side-chain hydrogen bonds with the Oγ and the main-chain nitrogen of Ser211.

An intriguing feature of the complex between AmpC and species 7 was the high degree of movement observed in the ligand, especially in the distal parts of the C7 side chain. Although movement in the distal regions of ligands has been observed in other AmpC/ligand complexes,23-25 it was precisely this region of the ligand that was most informative regarding substrate recognition and differences from PBPs. We wondered if the movement observed in the crystal structure was in some sense an artifact of crystallography, for instance reflecting disorder in the crystal or problems with trapping in the decylation-deficient mutant, or whether it represented a true aspect of molecular recognition between the AmpC enzyme and this substrate. To investigate this question, we turned to the molecular dynamics simulation of the AmpC/7 complex.

Molecular dynamics simulations provided insights into the conformational states as a function of time. Over the course of the 2.6 ns of simulation, snapshots were collected and superimposed on the initial X-ray structure. The resulting structures were used to determine the root-mean-square (rms) deviation and atomic fluctuations, which provide a measure of the flexibility for various regions of the protein and ligand.

Evolution of the rms deviation with respect to time of the Cα atoms of the protein and of species 7 are shown in Figure 2A. The rms deviation of the protein is found not to exceed 1.5 Å, consistent with the crystallographic result of a stable protein structure. The rms deviation value of the ligand, on the other hand, fluctuated around 2 Å for the first 1.4 ns of simulation, which subsequently gradually increased reaching a maximum of 4.2 Å at 1.8 ns. This increase indicates that the ligand is prone to substantial movement in the active site of the protein.

Figure 2B depicts more closely the dynamics of different parts of the ligand. The portion of the ligand highlighted in red was found to be the most mobile, with its rms deviation reaching 2.3 Å, which is more pronounced than the 1.3 and 0.9 Å rms deviations experienced by the lysine (in blue) and the dihydrothiazine ring, highlighted in green. To illustrate the range of movement that the ligand experiences in the active site of the β-lactamase, 26 structures were collected at 100 ps intervals and are shown superimposed in Figure 2C. The L-Ala-γ-D-Glu region of the ligand (white arrows in Figure 3C) shows significant movement and samples a wide variety of conformations. At the initial stages of the simulation, the acetyl carbonyl oxygen of the ligand was pointing toward the guanidinium moiety of Arg-201, and the carboxylate moiety of d-Glu was in correlated movement to accommodate repositioning of species 7 in the active site, and they are also modeled with two alternative conformations. The conformation of the ligand in the active site of monomer A is very similar to one of the alternative conformations of the compound in monomer B (Figure 1A). Species 7 makes several hydrogen bonds with active site residues (Figure 1B). The carbonyl oxygen of the ester moiety is in the “oxyanion” hole, interacting with amide nitrogens of Ser64 and Ala318. The carbonyl oxygen of the lysine in the C7 side-chain hydrogen bonds with side-chain nitrogen of Asn152. Finally, one of the carboxylate oxygens of the C7 side-chain hydrogen bonds with the Oγ and the main-chain nitrogen of Ser211.

AmpC/6 complex (species 7). Evaluation of the structure by Procheck23 showed that all residues are in the most favored and additionally allowed regions of the Ramachandran plot. The refined model is well fit in 2Fo−Fc electron density, except three residues at positions 284–286 of monomer A, which have disconnected density and were removed from the final model. The two monomers in the structure are very similar, except for three residues at positions 284–286 of monomer A, which have found to be the most mobile, with its rms deviation reaching 2.3 Å, which is more pronounced than the 1.3 and 0.9 Å rms deviations experienced by the lysine (in blue) and the dihydrothiazine ring, highlighted in green. To illustrate the range of movement that the ligand experiences in the active site of the β-lactamase, 26 structures were collected at 100 ps intervals and are shown superimposed in Figure 2C. The L-Ala-γ-D-Glu region of the ligand (white arrows in Figure 3C) shows significant movement and samples a wide variety of conformations. At the initial stages of the simulation, the acetyl carbonyl oxygen of the ligand was pointing toward the guanidinium moiety of Arg-201, and the carboxylate moiety of d-Glu was


pointing toward Ser-209 (3.9 Å). The orientation of these moieties corresponded to that adopted by conformer 1 (see compound colored according to the atom types in Figure 1A) in its acyl-enzyme complex with AmpC (conformer 1/AmpC complex X-ray structure was used as the initial structure for the molecular dynamics simulation). As the trajectory progressed, the acetyl moiety gradually flipped over to eventually occupy a large cavity in the active site (12 o’clock in Figure 2C), while the D-Glu carboxylate occupied the initial position of the acetyl moiety to form hydrogen bonding interactions with Arg-201. The white arrow points to the inserted loop specific to class C β-lactamases that prevents binding of the second strand of the peptidoglycan to the active site.

We recently reported the synthesis of compound 16 and its evaluation by X-ray in a complex with a bifunctional DD-carboxypeptidase/DD-transpeptidase.26 The portion in blue of cephalosporin 16 mimics the first strand of peptidoglycan that acylates the active-site serine of the PBP. On enzyme acylation, the portions in blue and red of species 17 represent the two strands demonstrated within the active site, which includes conformers 1 and 2, both seen by the X-ray diffraction data. The fact that this portion of the ligand was found to be highly mobile in the molecular dynamics simulation is also consistent with the X-ray diffraction data, which indicated that electron density in that region was such that the structure could not be unambiguously resolved into one conformer.

Movements in the other regions of species 7 were more attenuated, as shown by the rms deviations in Figure 2B and as depicted in Figure 2C. The lysine residue (red arrow, Figure 2C) does not undergo any major conformational change over the course of the trajectory. The ligand ring (yellow arrow in Figure 2C) also does not show any large conformational change, although it is of interest to note that most of the fluctuations occurs near the carboxylate moiety of the ring.
of peptidoglycan poised for the formation of the “cross-linked” cell wall. The 1.2 Å X-ray structure for this complex from the Kelly laboratory provided the first glimpse of how DD-transpeptidases may organize two strands of the bacterial peptidoglycan in their active sites en route to cross-linking. Figure 3A shows an image of the high-resolution structure of this complex, with the active site depicted as a Connolly surface (in green). To the right of the active site, it is open to accommodate the second strand of the peptidoglycan and the “wall” at 11 o’clock is the surface for interactions of the first peptidoglycan. The same perspective is shown for the complex of species 7 within the active site of AmpC β-lactamase in Figure 3B, as reported in this manuscript. It is noteworthy that the “wall” for interactions with the first strand of the peptidoglycan is entirely absent in the β-lactamase complex. This gives a simple structural perspective for why the crystallographic data and dynamics simulations could not fix the “first strand of peptidoglycan” in species 7 in an effective binding geometry, as the enzyme surface here has evolved away from such binding ability. It is also revealing that the insertion of the peptide within the active site at 3 o’clock (shown as a translucent orange/red water-accessible surface in Figure 3B; pointed to by the blue arrow in Figure 2C) clearly has abolished the ability of a second strand of the peptidoglycan to bind to this subsite. This inserted peptide is not known to play any catalytic role in the reaction of the β-lactamase. The exocyclic methylene group of the six-membered ring in species 7 is flush against the side of the insertion peptide (white arrow in Figure 3B), and there is no room for a larger ligand such as species 17, seen in the active site of the PBP. These two crystallographic images provide evidence for the structural reasons behind the lack of recognition of the peptidoglycan by at least class C β-lactamases, which must have paved the way for the emergence of a highly competent catalyst in the destruction of β-lactam antibiotics in resistant bacteria.

Concluding Remarks. Because of the kinship of β-lactamases and PBPs, much effort has been dedicated to demonstrating that they could potentially carry out each other’s reaction(s). This has not been an easy task, although there exist evidence that some DD-transpeptidases can hydrolyze β-lactam antibiotics$^{27–29}$ and some β-lactamases have marginal peptidase activities. From the present report, it would appear that at least class C β-lactamases have fully divested from the ability to recognize the peptidoglycan. In essence, the utility of cephalosporin 6 was in the fact that it acylates the active-site serine, forcing sequestration of the “peptidoglycan strand” (i.e., the C7 substituent) into the active site. Despite this forced coexistence within the complex, binding of the surrogate for the peptidoglycan is not stabilized by the protein. The requisite surface for the interaction with the peptidoglycan that acylates the active site serine in PBPs no longer exists in the β-lactamase active site. Similarly, the peptide insertion within the active site of β-lactamase abolishes the potential binding site for the second peptidoglycan in the active site, such as it is in DD-transpeptidases. The X-ray structure for the cephalosporin ligand indicates at least two binding modes. Molecular dynamics simulations showed that the ligand samples a substantial conformational space, which incidentally included the two species seen in the X-ray structure. The evidence presented here provides structural insight into how this antibiotic resistance enzyme has divested itself from interaction with the substrate for the parental enzymes, en route to achieving full catalytic effectiveness in its intended function in living bacteria.

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