Structural Insights into Catalytic Relevance of Substrate Poses in ACC-1

Da-Woon Bae,^a Ye-Eun Jung,^a Young Jun An,^b Jung-Hyun Na,^{a*} Sun-Shin Cha^a

^aDepartment of Chemistry & Nanoscience, Ewha Womans University, Seoul, Republic of Korea

^bMarine Biotechnology Research Center, Korea Institute of Ocean Science and Technology (KIOST), Busan, Republic of Korea

ABSTRACT ACC-1 is a plasmid-encoded class C β -lactamase identified in clinical isolates of *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella enterica*, and *Escherichia coli*. ACC-1-producing bacteria are susceptible to cefoxitin, whereas they are resistant to oxyimino cephalosporins. Here, we depict crystal structures of apo ACC-1, adenylated ACC-1, and acylated ACC-1 complexed with cefotaxime and cefoxitin. ACC-1 has noteworthy structural alterations in the R2 loop, the Ω loop, and the Phe119 loop located along the active-site rim. The adenylate covalently bonded to the nucleophilic serine reveals a tetrahedral phosphorus mimicking the deacylation transition state. Cefotaxime in ACC-1 has a proper conformation for the substrate-assisted catalysis in that its C-4 carboxylate and N-5 nitrogen are adequately located to facilitate the deacylation reaction. In contrast, cefoxitin in ACC-1 has a distinct conformation, in which those functional groups cannot contribute to catalysis. Furthermore, the orientation of the deacylating water relative to the acyl carbonyl group in ACC-1 is unfavorable for nucleophilic attack.

KEYWORDS crystal structures, ACC-1 class C β -lactamase, adenylation, acyl-enzyme complex, cefotaxime, cefoxitin

The most frequently prescribed antibacterial agents in the world are β -lactams. Bacterial resistance to this class of antibiotics is directly linked to the antibiotic resistance crisis that is a global public health threat. Although several molecular mechanisms for β -lactam resistance are established, the emergence and dissemination of β -lactamases among bacteria is the most common and important resistance mechanism. β -Lactamases hydrolytically break the amide bond in the β -lactam ring to inactivate β -lactam antibiotics. There are classes A, B, C, and D β -lactamases, classified by their primary structures (1). Among them, class C enzymes, which are widely distributed in Gram-negative pathogens, prefer a particular type of β -lactams called cephalosporins as the substrates, due to which they are referred to as cephalosporinases. Production of extended-spectrum class C β -lactamases such as CMY-10 (2) and AmpC BER (3–5) can confer resistance to cephamycins (cefotaxime and cefotetan), oxyimino cephalosporins (ceftazidime, cefotaxime, and ceftriaxone), monobactams (aztreonam), and even the carbapenem imipenem (6–8). Although genes encoding class C β -lactamases are mainly located in chromosomes, plasmid-borne class C enzymes have been reported in various microorganisms (9). It is notable that plasmid-encoded class C β -lactamases can contribute to the spread of multidrug resistance among *Klebsiella pneumoniae*, *Escherichia coli*, and other *Enterobacteriaceae* species, due to the transmissible nature of plasmids (10).

ACC-1 is a plasmid-borne class C β -lactamase identified in a *K. pneumoniae* strain causing nosocomial pneumonia (11). Plasmidic β -lactamases are originated from chromosomal enzymes; the high sequence identity (94%) between ACC-1 and the chromosomally encoded AmpC-type β -lactamase of *Hafnia alvei* (12) defines the likely origin of

Citation Bae D-W, Jung Y-E, An YJ, Na J-H, Cha S-S. 2019. Structural insights into catalytic relevance of substrate poses in ACC-1. *Antimicrob Agents Chemother* 63:e01411-19. <https://doi.org/10.1128/AAC.01411-19>.

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Address correspondence to Sun-Shin Cha, chajung@ewha.ac.kr.

* Present address: Jung-Hyun Na, Division of Discovery and Optimization, New Drug Development Center, KBIO HEALTH, Cheongju, Chungbuk, Republic of Korea.

Received 12 July 2019

Returned for modification 13 August 2019

Accepted 19 August 2019

Accepted manuscript posted online 26 August 2019

Published 22 October 2019

FIG 1 The schematic drawing of cefotaxime (top) and cefoxitin (bottom) with the β -lactam nucleus numbered. The R1 and R2 side chains, attached at C-7 and C-3 positions of the β -lactam nucleus, respectively, are labeled. The carboxamide in the R2 side chain that is removed during the acylation step is colored in blue.

ACC-1. After the first identification, several clinical isolates, including isolates of *K. pneumoniae*, *Proteus mirabilis*, *Salmonella enterica*, and *E. coli*, that produce ACC-1 have been reported in Germany, France, Tunisia, Spain, and The Netherlands (13, 14). For ACC-1-producing bacteria, MIC values of oxyimino cephalosporins (ceftazidime and cefotaxime), 7-methoxy-cephalosporins (cefotetan, moxalactam, and flomoxef), dipolar cephalosporins (cefepime and ceftipime), the monobactam aztreonam, and piperacillin increased over 4-fold (11, 15).

One remarkable feature of ACC-1-producing bacteria is their susceptibility to cefoxitin (11, 15), which contrasts with the notion that class C enzymes can confer resistance to this antibacterial agent (16–18). This unique feature is consistent with the location of ACC-1 in a distinct branch for subclass 1h in a phylogenetic tree, due to limited sequence homology to other known class C enzymes subclassed 1a to 1i (11). Even the closest sequence homolog, AmpC β -lactamase from *Serratia marcescens* belonging to subclass 1i (11, 19), shows only 52% sequence identity to ACC-1. Since the disparity in the primary structure is reflected in the tertiary structure that determines the catalytic activity, it is reasonable to assume that the specific amino acid composition affects the substrate spectrum of ACC-1. In this study, we present the high-resolution crystal structures of apo ACC-1, the adenylylated ACC-1, the ACC-1–cefotaxime complex, and the ACC-1–cefoxitin complex. The resistance and the susceptibility of ACC-1-producing bacteria to cefotaxime and cefoxitin (Fig. 1), respectively, suggest that ACC-1 may prefer cefotaxime to cefoxitin as a substrate. Structural comparison among the three complex structures provides mechanistic insights into the differing susceptibilities of ACC-1-producing bacteria toward the two β -lactam antibiotics. Structural information on noncanonical β -lactamases such as ACC-1 will help extend our knowledge about the molecular basis for the diversification of class C β -lactamases.

RESULTS AND DISCUSSION

The overall structure of apo ACC-1. ACC-1 has the typical structure of class C β -lactamases, consisting of two domains, a large α/β domain constructed by two separate segments (residues 1 to 80 and 167 to 363; numbering starts from the first residue after signal peptide) and a small α -helical domain (residues 81 to 166) (Fig. 2A).

FIG 2 Overall structure of apo ACC-1. (A) A cartoon diagram of ACC-1 shown with the nucleophilic Ser64 highlighted by a red sphere. The α/β and the α -helical domains are colored in gray and pink, respectively. Three loops (the Ω loop in purple, the F119 loop in green, and the Y149 loop in yellow) and three secondary structural elements ($\beta 8$, $\alpha 14$, and $\alpha 16$) in the active site are labeled. Phe119, Tyr149, and Ile345 are presented as green, yellow, and black sticks, respectively. (B) The superimposed C- α tracing of ACC-1 (gray) and FOX-4 (pale green; PDB code [5CGS](#)). The orientation of the molecule is similar to that in panel A. The Ω loop, the F119 loop, and the R2 loop in ACC-1 are colored in red for clarity.

The boundaries of the active-site canyon at the domain interface are residues 212 to 222 in the Ω loop, the $\alpha 2$ helix harboring the nucleophilic Ser64 residue, the $\beta 8$ strand, Ile345 at the N terminus of $\alpha 16$, the so-called R2 loop between $\alpha 14$ and $\beta 8$ (residues 289 to 309), and the Phe119 loop (residues 112 to 126)/the Tyr149 loop (residues 137 to 151) in the α -helical domain (Fig. 2A).

One of the closest structural homologs of ACC-1 identified by the Dali server is FOX-4 (Protein Data Bank [PDB] code [5CGS](#)), with 47.5% sequence identity and a root mean square deviation (RMSD) of 0.893 Å for 347 matching C- α atoms. Structural superposition between the two structures reveals that ACC-1 has local structural alterations in the active site in spite of the overall structural resemblance. The R2 loop region in ACC-1 has a different conformation due to two amino acid deletions and the low sequence identity (33.3%) (Fig. 2B and 3). There are two more regions displaying relatively large RMSD values, residues 212 to 216 in the Ω loop and the Phe119 loop. The conformational difference of the Ω loop appear to be the consequence of the replacement of the conserved proline and glycine residues in ACC-1 by Met213 and Glu214, respectively (Fig. 3). In the case of the Phe119 loop, one amino acid deletion and the low sequence identity (26.7%) (Fig. 3) are highly likely to be responsible for the positional displacement. The R2, Ω , and Phe-119 loops are all well defined in the apo ACC-1 structure; average B -factors of the three loops are 17.08, 21.12, and 22.68 Å², respectively, which are comparable to the average B -factor for the rest part of ACC-1.

Adenylylated ACC-1 and adenylylation of class C β -lactamases. The nucleophilic serine residues exist as an adenylylated form in some portion of the AmpC BER, CMY-10, and FOX-4 class C β -lactamases that are cytoplasmically expressed in *E. coli* (4, 20). In

FIG 3 Multiple-sequence alignment of class C β -lactamases. Residue numbers of ACC-1 are presented. Active site residues interacting with cefotaxime and cefoxitin are boxed, and adenylate-interacting residues are shaded in gray. The GenBank accession numbers are as follows: [WP_032491956.1](#) (ACC-1, *K. pneumoniae*), [WP_032489727.1](#) (FOX-4, *E. coli*), [WP_032489888.1](#) (MOX-1, *K. pneumoniae*), [WP_049134845.1](#) (AmpC_P99, *Enterobacter cloacae*), [WP_003454242.1](#) (AmpC_PA01, *Pseudomonas aeruginosa*), [WP_001336292.1](#) (AmpC_K12, *E. coli*), [WP_004236386.1](#) (DHA-1, *K. pneumoniae*), [WP_011872902.1](#) (CMY-2, *Salmonella enterica* serovar Typhimurium).

contrast, the same residues remain intact when they are periplasmically expressed (4, 20), leading us to hypothesize that adenylate-related metabolites in the cytoplasm of *E. coli* are responsible for the covalent modification of the nucleophiles. Actually, we demonstrated that adenosine 5'-(*P*-acetyl)monophosphate, a cytosolic metabolite composed of adenosine-phosphate-acetyl, was able to adenylate the nucleophiles of class C β -lactamases in an irreversible manner (4).

Some recombinant ACC-1 proteins passed through the *m*-aminophenylboronic (*m*APBA) agarose resin, a clear indication of the modification of the nucleophilic Ser64. Therefore, we examined whether the adenylation occurs in the cytoplasmically expressed ACC-1 that did not bind to the resin. The 1.1-Å resolution crystal structure of the ACC-1 protein verified that the oxygen atom of the nucleophilic Ser64 is covalently linked to the phosphorous atom of the adenylate (Fig. 4). One terminal oxygen atom of the adenylate makes a hydrogen bond with Tyr149 and is also connected to Thr315 via a water molecule. The other terminal oxygen atom fits into the oxyanion hole to form hydrogen bonds with two -NH groups of Ser64 and Ser317. As a whole, four oxygen atoms bonded to the phosphorous atom in a tetrahedral arrangement. The ribose moiety sits atop of Tyr221, with its 2' OH group hydrogen bonded to the backbone carbonyl oxygen of Ser317. The 3' OH of the ribose forms a water-mediated hydrogen-bonded network with Asn342. The imidazole ring in the adenine base stacks against Tyr221, but the pyrimidine ring makes few interactions with active site residues, which is reflected in weak electron density of the adenine base (Fig. 4B).

Comparing the adenylated ACC-1 with the adenylated AmpC BER class C β -lactamase, we observed a slight difference in the pose of the adenine bases of bound adenylates (Fig. 4C). In the active site of AmpC BER, a tightly bound sulfate ion originated from the mother liquor is positioned between β 8 and the Ω loop and interacts with the N-3 atom of the pyrimidine ring to affect the conformation of the adenine base. In the case of ACC-1 whose crystals were grown in the absence of sulfate ions, there is no such sulfate ion in the active site. Therefore, it appears that adenylates in the two proteins have a similar conformation, without the crystallization condition-dependent sulfate binding to AmpC BER. This surmise is supported by the fact that active site residues (Ser64, Tyr149, Tyr221, and Ser317) interacting with the bound

FIG 4 Adenylate in ACC-1. (A) The final maximum-likelihood weighted $2F_o - F_c$ electron density maps (blue) contoured at 1σ and the simulated annealing $F_o - F_c$ electron-density maps (green) contoured at 3σ for the adenylate covalently linked to Ser64 and the adenylate is derived from the final model of the adenylylated ACC-1. (B) Interactions between active site residues and the adenylate. Water-mediated hydrogen bonds are indicated by yellow dotted lines, while black dotted lines represent direct polar interactions. The O-2 oxygen atom is pointed out by a green arrow. (C) Adenylates in ACC-1 with carbon atoms in black and AmpC BER with carbon atoms in green. To prepare this figure, adenylylated ACC-1 and AmpC BER (PDB code [5GZW](#)) are superposed and then only adenylates and nucleophilic serine residues are shown for clarity. In all figures, nitrogen, oxygen, and sulfur atoms are colored in blue, red, and yellow, respectively.

adenylates in adenylylated ACC-1 and AmpC BER are highly conserved in class C β -lactamases (Fig. 3). Consequently, adenylation of the nucleophilic serine residue is likely to occur in cytoplasmically expressed class C β -lactamases. If this is the case, class C β -lactamases with the intact nucleophilic serine should be separated from adenylylated enzymes for kinetic studies.

Common features between the ACC-1-cefotaxime and the ACC-1-cefoxitin complexes. The MIC value of cefotaxime increased 64- or 128-fold against ACC-1-producing transformant and transconjugant, whereas no increase of MICs by the acquisition of the *bla*_{ACC-1} gene was detectable against cefoxitin (11, 15). To elucidate the molecular basis for this differential susceptibility toward the second- and third-generation cephalosporins, we solved the crystal structure of ACC-1 in complex with cefotaxime and cefoxitin at 1.46-Å and 1.59-Å resolutions, respectively. The structures of the ACC-1-cefotaxime and the ACC-1-cefoxitin complexes are superposed onto that of apo ACC-1 with RMSD values of 0.166 Å and 0.147 Å for all C- α atoms, respectively,

FIG 5 Cefotaxime and cefoxitin in ACC-1. (A) The final maximum-likelihood weighted $2F_o - F_c$ electron-density maps (blue) contoured at 1σ and the simulated annealing $F_o - F_c$ electron-density maps (green) contoured at 3σ for cefotaxime (left) and cefoxitin (right) covalently linked to Ser64. Cefotaxime, cefoxitin, and Ser64 are derived from the final model of the acylated ACC-1. Stereo views showing interactions between active site residues and cefotaxime (B) and cefoxitin (C). Water-mediated hydrogen bonds are indicated by yellow dotted lines while black dotted lines represent direct polar interactions. Green spheres represent deacylating water molecules.

which indicates that substrate binding induces no significant conformational change of ACC-1.

The hydrolysis of β -lactam antibiotics by class C β -lactamases proceeds through the acylation reaction and the following rate-determining deacylation reaction (2). In the acylation step, the nucleophilic serine residue attacks the carbonyl carbon in the β -lactam ring to break the amide bond in the ring. As a result, the active site serine is ester linked to the carbonyl carbon of the hydrolyzed β -lactam antibiotic to form the acyl-enzyme intermediate, which is then deacylated by a water molecule that attacks the same carbon to release the hydrolyzed antibiotic. In the two complex structures, the β -lactam ring carbonyl carbons of cefotaxime and cefoxitin are covalently linked to the oxygen atom of Ser64 (Fig. 5), indicating the acyl-enzyme intermediate state in which the carbonyl oxygen of bound substrates is hydrogen bonded to the main chain $-NH$ group of Ser317 in the oxyanion hole that stabilizes the negative charge devel-

oped on the carbonyl oxygen by the nucleophilic attack during the hydrolytic reaction (Fig. 5B and C). In addition, the carboxamide at C-3 of the dihydrothiazole rings of both β -lactam antibiotics is removed, and thus a methylene group is located at C-3 in the two complex structures (Fig. 5).

Active site residues of ACC-1 that directly interact with substrates in both complex structures are Leu118, Phe119, Tyr221, Ala291, Met292, Thr315, Ser317, Thr318, Asn319, and Ile345. Sequence alignment among class C β -lactamases (Fig. 3) reveals that Phe119 and Met292 are unique amino acid substitutions in ACC-1. Ala291, Asn319, and Ile345 are moderately conserved residues, while Leu118, Tyr221, Thr315, Ser317, and Thr318 are highly/absolutely conserved in class C enzymes.

Binding mode of cefotaxime. The R1 side chain of cefotaxime refers to an amide, a methoxyimino group, and an aminothiazole ring that are located at C-7 of the β -lactam ring (Fig. 1). The oxygen and nitrogen atoms in the R1 amide of cefotaxime are hydrogen bonded to the side chain –NH group of Asn151 and the main chain carbonyl group of Ser317, respectively (Fig. 5B). The bulky methoxyimino group stands against the side chain of Tyr221, while the aminothiazole ring stacks against the peptide bond between Thr318 and Asn319 in the β 8 strand with its amino group hydrogen bonded to the side chain of Asn319. Due to the vertical orientation, the peptide bond between Thr318 and Asn319 in β 8 allows for interactions with the aminothiazole ring instead of making hydrogen bonds with the lateral β 9 strand (Fig. 2A and 5B). Interestingly, the relative location of the methoxyimino group and the aminothiazole ring in the ACC-1–cefotaxime complex is totally different from that of the two groups in the active site of the *E. coli* AmpC β -lactamase (PDB code 4KG2); the aminothiazole ring faces Tyr221, and the methoxyimino group extends into the opposite space (Fig. S1). It is notable that Asn319 is replaced by Gly318 in the *E. coli* AmpC β -lactamase. In the absence of a hydrogen-bonding partner like Asn319, therefore, the aminothiazole ring seems to prefer interactions with Tyr221.

The dihydrothiazole ring of cefotaxime has an R2 side chain (a methylene group) at C-3 (Fig. 1 and 5A). The dihydrothiazole ring itself makes intramolecular T-shaped stacking interactions with the aminothiazole ring of the R1 side chain, which confines the rotation of the dihydrothiazole ring due to steric clash with the aminothiazole ring (Fig. 5B). The hydrophobic edge (S-1, C-2, and C-3 atoms) of this six-membered ring is in contact with the hydrophobic surface lined by Leu118, Phe119, Ala291, and Met292, while the carboxylate group at C-4 is hydrogen bonded to the hydroxyl side chain of Ser317 (Fig. 5B). An ordered water molecule with *B*-factor of 21.67 Å² is located near the dihydrothiazole ring; the water is 2.6 Å and 2.8 Å away from the C-4 carboxylate oxygen and the hydroxyl oxygen of Thr315, respectively (Fig. 5B). It is 3.9 Å away from the β -lactam ring carbonyl carbon and has an unobstructed path to the acyl center. The presence of a water molecule at this position is highly conserved in crystal structures of class C β -lactamases (21–23), based on which the water molecule is believed to mediate the deacylation reaction.

Binding mode of cefoxitin. The R1 side chain of cefoxitin is composed of a methoxy group, an amide, and a thiazole ring in order from C-7 (Fig. 1). The 7 α -methoxy group makes van der Waals contact with the side chain of Phe119 (Fig. 5C). Although the 7 α -methoxy groups of second-generation cephalosporins are considered to generate steric clash with the side chain of Asn150 in class C β -lactamases (24), the distance between the methoxy group and the side chain of the corresponding asparagine residue (Asn151 in ACC-1) is 3.7 Å (Fig. 5C), an indication of no steric clash. The amide group of cefoxitin is rotated $\sim 90^\circ$ compared to that in cefotaxime (Fig. 6A), resulting in no direct interactions with active site residues. Its carbonyl oxygen atom is connected to the side chain amine of Lys67 and the main chain oxygen of Ala220 through a water molecule (Fig. 5C). The thiazole ring is within the van der Waals contact distance from the aromatic side chains of Phe119 and Tyr221 (Fig. 5C).

The dihydrothiazole ring of cefoxitin assumes a vertical conformation due to $\sim 90^\circ$ clockwise rotation compared to that of cefotaxime (Fig. 6A). As a result, the C-4

FIG 6 Comparison of poses among cefotaxime, ceftiofur, and adenylylate in ACC-1. (A) Superposition between cefotaxime (carbon atoms in orange) and ceftiofur (carbon atoms in teal). To prepare this figure, acylated ACC-1 in complex with cefotaxime and ceftiofur are superposed, and then only cefotaxime, ceftiofur, and Ser64 are shown for clarity. The dotted arrow indicates the $\sim 90^\circ$ clockwise rotation of the dihydrothiazolidine ring, and the dotted circle indicates the amide group in the R1 side chain. (B) Superposition between cefotaxime (carbon atoms in orange) and adenylylate (carbon atoms in black). To prepare this figure, the adenylylated ACC-1 is superposed onto the ACC-1–cefotaxime complex, and then only cefotaxime, adenylylate, and Ser64 are shown for clarity. (C) Superposition between ceftiofur (carbon atoms in teal) and adenylylate (carbon atoms in black). The adenylylated ACC-1 and the ACC-1–ceftiofur complex are superposed, and then only ceftiofur, adenylylate, and Ser64 are shown for clarity. In panels B and C, green spheres represent the deacylating water.

carboxylate is fully exposed to the solvent with no interactions with ACC-1 whereas the ring and the R2 methylene group at C-3 are in contact with hydrophobic side chains of Leu118, Phe119, Ala291, Met292, and Ile345 (Fig. 5C). The deacylating water is located with a hydrogen bonding interaction with Thr315 at a virtually identical position to where the corresponding water is in the ACC-1–cefotaxime complex. However, due to the rotation of the dihydrothiazolidine ring, the deacylating water is 7.7 Å away from the C-4 carboxylate oxygen. Instead, the deacylating water forms a hydrogen bonding network with two water molecules that occupy the space which the C-4 carboxylate of cefotaxime would be positioned (Fig. 5C).

Cefotaxime conformation for substrate-assisted catalysis. The conformation of β -lactam antibiotics at the acyl-enzyme intermediate is critical for the efficiency of the rate-determining deacylation reaction. Good substrates assume catalytically competent conformations, but poor substrates adopt strained conformations resisting deacylation. The substrate-assisted deacylation reaction has been established for class C β -lactamases in which the N-5 nitrogen and/or the C-4 carboxylate in the dihydrothiazolidine ring play as a general base to activate the deacylating water and the N-5 nitrogen contributes to electrostatic stabilization of the deacylation high-energy tetrahedral transition state (24, 25). Notably, in the ACC-1–cefotaxime complex, the deacylating water is 3.4 Å and 2.6 Å away from the ring N-5 nitrogen and the C-4 carboxylate of the dihydrothiazolidine ring (Fig. 5B), respectively, indicating that the position of the C-4 carboxylate is more adequate to serve as a general base to deprotonate the deacylating water than the N-5 nitrogen. In addition, Ile345 is 3.6 Å away from the C-4 carboxylate (Fig. 5B); the presence of a neighboring hydrophobic residue may increase the pK_a value of the carboxylate to favor its protonated state associated with the deprotonation of the deacylating water.

The crystal structure of the adenylylated ACC-1 in which the nucleophilic serine is connected to the phosphorous atom of the adenylylate shows the tetrahedral arrange-

FIG 7 Comparison of the ACC-1–cefoxitin complex with the FOX-4–cefoxitin complex. (A) Superposed structures between the two complexes. Carbon atoms in the ACC-1–cefoxitin complex are in teal, while carbon atoms in the FOX-4–cefoxitin complex are in salmon. A dotted line indicates a hydrogen bonding interaction. (B) The angles between the deacylating water and the carbonyl group of the acyl-enzyme intermediate in ACC-1 and FOX-4. Green spheres represent the deacylating water, and cefoxitin and nucleophilic serine residues are shown in sticks.

ment of four oxygen atoms around the phosphorus atom, with one oxygen atom in the oxyanion hole (Fig. 4B). One oxygen atom (O-2) is hydrogen bonded to Tyr149, which is thought to activate a water molecule for the deacylation reaction and stabilize the transition state (Fig. 4B) (26). In addition, the hydrolytic water interacting with Thr315 is hydrogen bonded to the O-2 atom (Fig. 4B), which suggests a trajectory of the water during the deacylation reaction. Taken together, the tetrahedral phosphorus in the adenylylated ACC-1 resembles the deacylation transition state. When the ACC-1–cefotaxime complex is superposed onto the adenylylated ACC-1, the ring N-5 nitrogen of cefotaxime is 2.3 Å away from the O-2 atom representing the deacylating water at the tetrahedral transition state (Fig. 6B), indicating interactions between the lone electron pair of the N-5 atom and the hydrolytic water. Consequently, in terms of the substrate-assisted catalysis, cefotaxime assumes a catalytically competent conformation adequate for both the activation of the deacylating water and the stabilization of its own high-energy tetrahedral transition state.

Cefoxitin conformation defective in deacylation. The conformation of cefoxitin is distinct from that of cefotaxime in ACC-1, as described above. Due to the rotation of the dihydrothiazole ring, the C-4 carboxylate of cefoxitin is 7.7 Å away from the deacylating water in ACC-1 (Fig. 5C and 7B), precluding activation of the water. In addition, in the superposed structures between the ACC-1–cefoxitin complex and the adenylylated ACC-1, the N-5 nitrogen is located at the opposite side from the O-2 oxygen atom representing the deacylating water at the tetrahedral transition state (Fig. 6C), excluding the role of the ring N-5 atom in stabilizing its own deacylation high energy intermediate. Therefore, it can be concluded that cefoxitin in ACC-1 adopts a catalytically inefficient conformation in terms of the substrate-assisted deacylation in contrast to cefotaxime in ACC-1.

FOX-4 is the structural homolog of ACC-1 (Fig. 2B), and it efficiently hydrolyzes cefoxitin, its namesake substrate. Since cefoxitin is a poor substrate to ACC-1 (11, 15) but is a good substrate to FOX-4 (20), we compared cefoxitin-bound ACC-1 and FOX-4 to reveal the structural basis for their contrasting deacylation efficiency toward the same substrate. Remarkably, the overall conformation of cefoxitin is virtually identical in the two proteins, indicating that the efficient hydrolysis of cefoxitin is independent of the substrate-assisted deacylation. This perspective prompted us to uncover the real structural determinant for the poor hydrolysis of cefoxitin in ACC-1 through a detailed

structural comparison. Cefoxitin bound to FOX-4 tilts more toward the α -helical domain due to a hydrogen bond between its C-4 carboxylate and Gln120 (Fig. 7A). In the case of cefoxitin in ACC-1, the bulky benzyl ring of Phe119 corresponding to Gln120 of FOX-4 props the dihydrothiazole ring and the 7 α -methoxy group to prevent its tilting (Fig. 7A). There is another noteworthy interaction between cefoxitin and FOX-4 that is not observed in the ACC-1–cefloxitin complex. The dihydrothiazole ring makes a close encounter with Phe293 that is the unique amino acid substitution in FOX-4 and undergoes a dramatic positional change upon cefoxitin binding (Fig. 7A). This tight interaction between the dihydrothiazole ring and Phe293 stabilizes the tilt conformation of cefoxitin in FOX-4. The R2 loop region containing Phe293 in FOX-4 has a significant difference in both primary and tertiary structures compared with the corresponding region in ACC-1 (Fig. 2B and 3 and 7A), suggesting that the tilt conformation of cefoxitin cannot be achieved in ACC-1.

The Bürgi-Dunitz angle represents the orientation of a nucleophile relative to the plane of carbonyl group in a situation of a nucleophilic attack on a trigonal unsaturated (sp^2) carbon (27). Due to the tilt conformation of cefoxitin, a more favorable geometry for the nucleophilic attack is achieved in FOX-4 with the angle of nucleophilic approach close to the general Bürgi-Dunitz angle of 105 to 107° (28). The oxygen atom of the deacylating water is at an angle of 87.0° to the carbonyl group of the acyl center in FOX-4, but the corresponding angle is just 65.6° in ACC-1 (Fig. 7B). It is notable that the acylation and deacylation reaction trajectories in serine proteases sharing enzyme mechanism with class C β -lactamases exhibit a very similar geometry with a Bürgi-Dunitz angle close to 90° (29). Consequently, the orientation of the deacylating water relative to the acyl carbonyl group is unfavorable for nucleophilic attack, which seems to be associated with the low deacylation efficiency of ACC-1 toward cefoxitin.

Conclusion. This study presents the first crystal structures of ACC-1 displaying low sequence identities of ~37.7 to 53.0% to other known class C β -lactamases. Consistently, ACC-1 has noticeable structural changes in several regions, even compared to the structural homolog FOX-4 (Fig. 2B). ACC-1-producing bacteria are susceptible to cefoxitin but resistant to cefotaxime. According to the high-resolution crystal structures of ACC-1 acylated by cefotaxime and cefoxitin, their unusual resistance phenotype is directly related to the different poses of substrates in the active site of ACC-1 (Fig. 1 and 5, and 6A). In spite of similar product-like conformations of cefoxitin in which its C-4 carboxylate group is fully exposed to the solvent, the contrasting inactivation level of cefoxitin by ACC-1 and FOX-4 can be explained on the basis of the Bürgi-Dunitz angle, which is dependent on the tilt degree of cefoxitin toward the α -helical domain (Fig. 5C and 7). In addition, structural comparison between the adenylylated ACC-1 and AmpC BER suggests that adenylylation of the nucleophilic serine residue is a general event when recombinant class C β -lactamases are produced in the cytoplasm.

MATERIALS AND METHODS

Cloning, expression, and purification of ACC-1. The coding gene of ACC-1 (residues 24 to 386) from *Klebsiella pneumoniae* with in-frame noncleavable N-terminal His₆ tag (MHHHHHH) was synthesized (Macrogen, Republic of Korea) and inserted at the NdeI and BamHI sites of the expression vector pET24a(+) (Novagen, USA). The construct was transformed into *E. coli* BL21(DE3). The transformed cells were grown to an A_{600} of ~0.6 in Luria-Bertani medium containing 50 μ g/ml at 37°C, and then the expression of ACC-1 was induced by 1 mM isopropyl β -D-thiogalactopyranoside. After an 18-h induction at 20°C, cells were harvested and resuspended in a 20 mM Tris buffer (pH 7.4) and disrupted by sonication. After discarding the cell debris by centrifugation, supernatants were successively loaded on a nickel-nitrilotriacetic acid column (GE Healthcare, USA) and a Superdex 75 HR 16/60 column (GE Healthcare, USA). After size exclusion chromatography, ACC-1 was further purified by the *m*-aminophenylboronic acid (*m*APBA) agarose resin as previously described (4, 20): β -lactamases with intact nucleophiles bind to the resin, whereas enzymes with modified nucleophiles do not. Bound and unbound ACC-1 proteins were separately concentrated to ~30 mg/ml in a 20 mM Tris buffer (pH 7.4) for crystallization.

Crystallization, data collection, and structure determination. Crystal screenings were performed using the microbatch crystallization method at 22°C as described previously (30, 31). With ACC-1 proteins that did bind to the *m*APBA resin, we grew crystals of apo ACC-1 by using a precipitant solution consisting of 0.1 M sodium cacodylate (pH 5.5), 0.2 M sodium chloride, and 30% polyethylene glycol (PEG) 400. Crystals of the adenylylated ACC-1 that did not bind to the *m*APBA resin were grown in a

precipitant solution containing 0.1 M sodium cacodylate (pH 5.5), 0.2 M sodium chloride, and 35% PEG 1500. In the case of the ACC-1–cefotaxime and the ACC-1–cefoxitin complexes, crystals of apo ACC-1 were soaked for 1 min at room temperature in the same precipitant solution supplemented with 25 mM cefotaxime and cefoxitin. For data collection, crystals were frozen after a brief soak in a cryoprotectant solution containing 20% ethylene glycol in the same precipitant solution. A 1.59-Å resolution data set of apo ACC-1, a 1.46-Å resolution data set of the ACC-1–cefotaxime complex, and a 1.59-Å resolution data set of the ACC-1–cefoxitin complex were collected from a flash-cooled crystal at 100 K using an ADSC Q315r charge-coupled device (CCD) on beamline 5C of Pohang Light Source, Republic of Korea (Table S1). A 1.1-Å resolution data set of adenylylated ACC-1 was collected from a flash-cooled crystal at 100 K using a Dectris Pilatus3 S6M detector (Dectris Eiger X16M) on beamline 5A of Photon Factory, Japan.

Diffraction data of apo ACC-1, the ACC-1–cefotaxime complex, and the ACC-1–cefoxitin complex were processed and scaled using DENZO and SCALEPACK from the HKL-2000 program suite (HKL Research, Inc.). Diffraction data of adenylylated ACC-1 were processed and scaled using XDS and XSCALE (32). Molecular replacement was performed with MOLREP (33) using the structures of the β -lactamases (PDB code 5EVI) for apo ACC-1 as a search model (Table S1). In the cases of the adenylylated ACC-1, the ACC-1–cefotaxime complex, and the ACC-1–cefoxitin complex, the structure of apo ACC-1 was used as a search model. The subsequent refinements and manual refitting of the models were performed with Phenix (34) and Coot (35), respectively.

Data availability. The atomic coordinates and structure factors of the final models have been deposited in the Protein Data Bank under identification codes 6K8X (apo ACC-1), 6KBY (adenylylated ACC-1), 6K9T (the ACC-1–cefotaxime complex), and 6KA5 (the ACC-1–cefoxitin complex).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01411-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank the staffs of beamlines at Pohang Light Source (Republic of Korea) and Photon Factory (Japan) for help with data collection.

This study was supported by grant NRF-2015M1A5A1037480 (the National Research Foundation of Korea), the project entitled “Development of Biomedical materials based on marine proteins” (the Ministry of Oceans and Fisheries, Republic of Korea), and a grant from the Collaborative Genome Program 20180430 (the Ministry of Oceans and Fisheries, Republic of Korea).

REFERENCES

- Hall BG, Barlow M. 2005. Revised Ambler classification of β -lactamases. *J Antimicrob Chemother* 55:1050–1051. <https://doi.org/10.1093/jac/dki130>.
- Kim JY, Jung HI, An YJ, Lee JH, Kim SJ, Jeong SH, Lee KJ, Suh P-G, Lee H-S, Lee SH, Cha S-S. 2006. Structural basis for the extended substrate spectrum of CMY-10, a plasmid-encoded class C β -lactamase. *Mol Microbiol* 60:907–916. <https://doi.org/10.1111/j.1365-2958.2006.05146.x>.
- Na J-H, Cha S-S. 2016. Structural basis for the extended substrate spectrum of AmpC BER and structure-guided discovery of the inhibition activity of citrate against the class C β -lactamases AmpC BER and CMY-10. *Acta Crystallogr D Struct Biol* 72:976–985. <https://doi.org/10.1107/S2059798316011311>.
- Kim M-K, An YJ, Na J-H, Seol J-H, Ryu JY, Lee J-W, Kang L-W, Chung KM, Lee J-H, Moon JH, Lee JS, Cha S-S. 2017. Structural and mechanistic insights into the inhibition of class C β -lactamases through the adenylation of the nucleophilic serine. *J Antimicrob Chemother* 72:735–743. <https://doi.org/10.1093/jac/dkw491>.
- Mammeri H, Poirel L, Nordmann P. 2007. Extension of the hydrolysis spectrum of AmpC β -lactamase of *Escherichia coli* due to amino acid insertion in the H-10 helix. *J Antimicrob Chemother* 60:490–494. <https://doi.org/10.1093/jac/dkm227>.
- Rodríguez-Martínez JM, Poirel L, Nordmann P. 2009. Extended-spectrum cephalosporinases in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 53:1766–1771. <https://doi.org/10.1128/AAC.01410-08>.
- Nordmann P, Mammeri H. 2007. Extended-spectrum cephalosporinases: structure, detection and epidemiology. *Future Microbiol* 2:297–307. <https://doi.org/10.2217/17460913.2.3.297>.
- Bradford PA. 2001. Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev* 14:933–951. <https://doi.org/10.1128/CMR.14.4.933-951.2001>.
- Philippon A, Arlet G, Jacoby GA. 2002. Plasmid-determined AmpC-type β -lactamases. *Antimicrob Agents Chemother* 46:1–11. <https://doi.org/10.1128/aac.46.1.1-11.2002>.
- Papanicolaou GA, Medeiros AA, Jacoby GA. 1990. Novel plasmid-mediated β -lactamase (MIR-1) conferring resistance to oxyimino- and α -methoxy β -lactams in clinical isolates of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 34:2200–2209. <https://doi.org/10.1128/aac.34.11.2200>.
- Bauernfeind A, Schneider I, Jungwirth R, Sahly H, Ullmann U. 1999. A novel type of AmpC β -lactamase, ACC-1, produced by a *Klebsiella pneumoniae* strain causing nosocomial pneumonia. *Antimicrob Agents Chemother* 43:1924–1931. <https://doi.org/10.1128/AAC.43.8.1924>.
- Girlich D, Naas T, Bellais S, Poirel L, Karim A, Nordmann P. 2000. Heterogeneity of AmpC cephalosporinases of *Hafnia alvei* clinical isolates expressing inducible or constitutive ceftazidime resistance phenotypes. *Antimicrob Agents Chemother* 44:3220–3223. <https://doi.org/10.1128/aac.44.11.3220-3223.2000>.
- Doloy A, Verdet C, Gautier V, Decré D, Ronco E, Hammami A, Philippon A, Arlet G. 2006. Genetic environment of acquired *bla*_{ACC-1} β -lactamase gene in *Enterobacteriaceae* isolates. *Antimicrob Agents Chemother* 50:4177. <https://doi.org/10.1128/AAC.00619-06>.
- Bidet P, Burghoffer B, Gautier V, Brahimi N, Mariani-Kurkdjian P, El-Ghoneimi A, Bingen E, Arlet G. 2005. *In vivo* transfer of plasmid-encoded ACC-1 AmpC from *Klebsiella pneumoniae* to *Escherichia coli* in an infant and selection of impermeability to imipenem in *K. pneumoniae*. *Antimicrob Agents Chemother* 49:3562. <https://doi.org/10.1128/AAC.49.8.3562-3565.2005>.
- Ohana S, Leflon V, Ronco E, Rottman M, Guillemot D, Lortat-Jacob S, Denys P, Loubert G, Nicolas-Chanoine M-H, Gaillard J-L, Lawrence C. 2005. Spread of a *Klebsiella pneumoniae* strain producing a plasmid-

- mediated ACC-1 AmpC β -lactamase in a teaching hospital admitting disabled patients. *Antimicrob Agents Chemother* 49:2095. <https://doi.org/10.1128/AAC.49.5.2095-2097.2005>.
16. Moritz VA, Carson P 1986. Cefoxitin sensitivity as a marker for inducible beta-lactamases. *J Med Microbiol* 21:203–207. <https://doi.org/10.1099/00222615-21-3-203>.
 17. Beceiro A, Bou G. 2004. Class C β -lactamases: an increasing problem worldwide. *Rev Med Microbiol* 15:141–152. <https://doi.org/10.1097/00013542-200410000-00003>.
 18. Livermore DM, Shannon KP, Winstanley TG. 2001. Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. *J Antimicrob Chemother* 48:87–102. https://doi.org/10.1093/jac/48.suppl_1.87.
 19. Nomura K, Yoshida T. 1990. Nucleotide sequence of the *Serratia marcescens* SR50 chromosomal ampC β -lactamase gene. *FEMS Microbiol Lett* 70:295–300. <https://doi.org/10.1111/j.1574-6968.1990.tb13992.x>.
 20. Lefurgy ST, Malashkevich VN, Aguilar JT, Nieves E, Mundorff EC, Biju B, Noel MA, Toro R, Baiwir D, Papp-Wallace KM, Almo SC, Frere JM, Bou G, Bonomo RA. 2016. Analysis of the structure and function of FOX-4 cephamycinase. *Antimicrob Agents Chemother* 60:717–728. <https://doi.org/10.1128/AAC.01887-15>.
 21. Lobkovsky E, Billings EM, Moews PC, Rahil J, Pratt RF, Knox JR. 1994. Crystallographic structure of a phosphonate derivative of the *Enterobacter cloacae* P99 cephalosporinase: mechanistic interpretation of a β -lactamase transition-state analog. *Biochemistry* 33:6762–6772. <https://doi.org/10.1021/bi00188a004>.
 22. Usher KC, Blaszcak LC, Weston GS, Shoichet BK, Remington SJ. 1998. Three-dimensional structure of AmpC β -lactamase from *Escherichia coli* bound to a transition-state analogue: possible implications for the oxyanion hypothesis and for inhibitor design. *Biochemistry* 37:16082–16092. <https://doi.org/10.1021/bi981210f>.
 23. Powers RA, Blázquez J, Weston GS, Shoichet BK, Morosini M-I, Baquero F. 1999. The complexed structure and antimicrobial activity of a non- β -lactam inhibitor of AmpC β -lactamase. *Protein Sci* 8:2330–2337. <https://doi.org/10.1110/ps.8.11.2330>.
 24. Patera A, Blaszcak LC, Shoichet BK. 2000. Crystal structures of substrate and inhibitor complexes with AmpC β -lactamase: possible implications for substrate-assisted catalysis. *J Am Chem Soc* 122:10504–10512. <https://doi.org/10.1021/ja001676x>.
 25. Bulychiev A, Massova I, Miyashita K, Mobashery S. 1997. Nuances of mechanisms and their implications for evolution of the versatile β -lactamase activity: from biosynthetic enzymes to drug resistance factors. *J Am Chem Soc* 119:7619–7625. <https://doi.org/10.1021/ja963708f>.
 26. Chen Y, Minasov G, Roth TA, Prati F, Shoichet BK. 2006. The deacylation mechanism of AmpC β -lactamase at ultrahigh resolution. *J Am Chem Soc* 128:2970–2976. <https://doi.org/10.1021/ja056806m>.
 27. Burgi HB, Dunitz JD, Shefter E. 1973. Geometrical reaction coordinates. II. Nucleophilic addition to a carbonyl group. *J Am Chem Soc* 95:5065–5067. <https://doi.org/10.1021/ja00796a058>.
 28. B:Urgi HB, Dunitz JD, Lehn JM, Wipff G. 1974. Stereochemistry of reaction paths at carbonyl centres. *Tetrahedron* 30:1563–1572. [https://doi.org/10.1016/S0040-4020\(01\)90678-7](https://doi.org/10.1016/S0040-4020(01)90678-7).
 29. Radisky ES, Koshland DE. 2002. A clogged gutter mechanism for protease inhibitors. *Proc Natl Acad Sci U S A* 99:10316. <https://doi.org/10.1073/pnas.112332899>.
 30. An YJ, Ahn B-E, Roe J-H, Cha S-S. 2008. Crystallization and preliminary X-ray crystallographic analyses of Nur, a nickel-responsive transcription regulator from *Streptomyces coelicolor*. *Acta Crystallogr F Struct Biol Cryst Commun* 64:130–132. <https://doi.org/10.1107/S1744309108001760>.
 31. An YJ, Kim M-K, Song JM, Kang MH, Lee Y-H, Cha S-S. 2015. Vapor batch crystallization and preliminary X-ray crystallographic analysis of a cold-active endo- β -1,4-glucanase that was produced through the cold temperature protein expression. *Biodesign* 3:138–142.
 32. Kabsch W. 2010. Integration, scaling, space-group assignment and post-refinement. *Acta Crystallogr D Biol Crystallogr* 66:133–144. <https://doi.org/10.1107/S0907444909047374>.
 33. Vagin A, Teplyakov A. 2010. Molecular replacement with MOLREP. *Acta Crystallogr D Biol Crystallogr* 66:22–25. <https://doi.org/10.1107/S0907444909042589>.
 34. Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung L-W, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH. 2010. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* 66:213–221. <https://doi.org/10.1107/S0907444909052925>.
 35. Emsley P, Cowtan K. 2004. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60:2126–2132. <https://doi.org/10.1107/S0907444904019158>.