Acyl-intermediate Structures of the Extended-spectrum Class A β-Lactamase, Toho-1, in Complex with Cefotaxime, Cephalothin, and Benzylpenicillin*

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Bacterial resistance to β-lactam antibiotics is a serious problem limiting current clinical therapy. The most common form of resistance is the production of β-lactamasess that inactivate β-lactam antibiotics. Toho-1 is an extended-spectrum β-lactamase that has acquired efficient activity not only to penicillins but also to cephalosporins including the expanded-spectrum cephalosporins that were developed to be stable in former β-lactamasess. We present the acyl-intermediate structures of Toho-1 in complex with cefotaxime (expanded-spectrum cephalosporin), cefalothin (non-expanded-spectrum cephalosporin), and benzylpenicillin at 1.8-, 2.0-, and 2.1-A resolutions, respectively. These structures reveal distinct features that can explain the ability of Toho-1 to hydrolyze expanded-spectrum cephalosporins. First, the Ω-loop of Toho-1 is displaced to avoid the steric contacts with the bulky side chain of cefotaxime. Second, the conserved residues Asn190 and Asp246 form unique interactions with the bulky side chain of cefotaxime to fix tightly. Finally, the unique interaction between the conserved Ser237 and cephalosporins probably helps to bring the β-lactam carbonyl group to the suitable position in the oxyanion hole, thus increasing the cephalosporinase activity.

β-Lactam antibiotics are effectively used against a wide range of bacterial infectious diseases (1). The antibiotics form stable acyl-enzymes with penicillin-binding proteins (PBP)s in the membrane of the bacterial cell. PBP function in the biosynthesis and repair of the peptidoglycan of the cell wall; thus, the inhibition of these enzymes induces cell death. However, resistant bacteria escape from the lethal action of β-lactam antibiotics mainly by producing β-lactamasess that hydrolyze the antibiotics. β-Lactamasess are acylated by β-lactam antibiotics in a similar manner as PBP; however, they are rapidly deacylated. β-Lactamasess are classified into four groups (classes A, B, C, and D) according to their amino acid sequences and substrate profiles (1). Classes A, C, and D β-lactamasess are serine β-lactamasess, whereas class B β-lactamasess are zinc-containing β-lactamasess. Among these four classes of β-lactamasess, class A β-lactamasess are especially important, because they exhibit highly variable substrate profiles and in general are encoded by plasmids and are easily transferable between cells, thereby threatening clinical antibiotic therapy.

The expanded-spectrum cephalosporins (oxyimino-cephalosporins) including cefotaxime were developed to be stable in the former class A β-lactamasess such as TEM-1 and SHV-1 (1, 2). These compounds are characterized by a bulky acylamide side chain containing an oxyimino group. Many of these compounds also contain an aminothiazole ring. However, after extensive and sometimes abusive clinical use of these antibiotics, resistant bacteria began to produce new class A β-lactamasess capable of hydrolyzing oxyimino-cephalosporins called extended-spectrum β-lactamasess (ESBLs) (1, 2). ESBLs are classified into two groups. The first of these groups (type I) consists of variants of TEM-1 or SHV-1 that differ by a few amino acid substitutions. The second group (type II) includes enzymes that are not related to TEM-1 or SHV-1 that differ by a few amino acid substitutions. The plasmid encoded CTX-M-type ESBLs are the most widespread family of type II ESBLs and are increasingly on the rise. Contrary to what was originally thought of class A β-lactamasess, CTX-M-type ESBLs have an efficient hydrolytic activity toward oxyimino-cephalosporins but exhibit lower activity toward penicillins than non-ESBLs (3).

Toho-1 belongs to the CTX-M-type ESBLs (4). We have previously reported the structure of the Toho-1 mutant E166A whose overall fold was shown to be similar to non-ESBLs (5). Toho-1 has some variations in hydrogen-bonding patterns and an increase in flexibility of the β-strand B3 as well as the Ω-loop. Yet, it was still unclear how these differences increase oxyimino-cephalosporinase activity of Toho-1, because a complex structure with an oxyimino-cephalosporin was not determined. In addition to Toho-1, there have been seven other β-lactamases solved (TEM-52 (6), TEM-64 (7), TEM-1 G238A mutant (7), PER-1 (8), a β-lactamase from Proteus vulgaris K1 (9), NMC-A (10), and a class C β-lactamase from Enterobacter cloacae GC1 (11)), but none of these β-lactamase structures has been determined in complex with an oxyimino-cephalosporin. Here, we report the acyl-intermediate structures of a Toho-1 mutant.
Acyl-intermediate Structures of Toho-1 β-Lactamase

### RESULTS AND DISCUSSION

**Acyl-intermediate Structures of Toho-1—β-Lactamases hydrolyze β-lactam antibiotics so rapidly that it is difficult to trap the acyl-intermediate.** In class A β-lactamases, the conserved residue Glu166 positions and activates the hydrolytic water for the deacylation (1, 18). Therefore, we used a Glu166 mutant of Toho-1 to trap the acyl-intermediate in a similar manner as was done for the acyl-intermediate structures of non-ESBLs (16, 17). In a class A β-lactamase from *Bacillus licheniformis*, the structure of E166A is known to display few differences with the wild type enzyme (19).

The acyl-intermediate structures of the mutant E166A with cefotaxime, cefalothin, and benzylpenicillin were determined at 1.8-, 2.0-, and 2.1-Å resolutions, respectively (Table I). The overall fold of these acyl-intermediate structures is the same as the previously reported unbound Toho-1 E166A (5) with root mean square deviations among the Cα positions of 0.25, 0.21, and 0.25 Å for the cefotaxime-, cefalothin- and benzylpenicillin-acyl-intermediate structures, respectively. The active sites of these acyl-intermediate structures are shown in Fig. 1, A–C, with $2\chi_0 - F_0$ electron density maps around the bound substrates. In the cephalosporin-acyl-intermediate structures (Fig. 1, A and B), the C3′-leaving group has been removed as observed in other acyl-enzyme structures in complex with cephalosporins (15, 16, 20–22). In the cefotaxime-acyl-intermediate structure, residues Pro207, Asn170, Ser237, Asp240, and Arg274 surround the bulky side chain of cefotaxime. In addition, both side chain oxygens of Asp240 interact with the amino group in the aminothiazole ring, which may be involved in the binding of cefotaxime (Fig. 1A). The thiopeptide substituent of cefalothin in the cefalothin-acyl-intermediate and the benzyl group of benzylpenicillin in the benzylpenicillin-acyl-intermediate are seen in a nearly identical position with the aminothiazole ring and the methoxyimino group of cefotaxime, respectively (Fig. 1, B and C).

**Differences among Three Acyl-intermediate and Unbound Toho-1 Structures—Substrate binding does not induce conformational changes in the Ω-loop and the β-strand B3 but rather causes a structural rearrangement of residues Arg274, Ser237, and Asn170 (Fig. 2, A and B).** Arg274 is a unique residue in Toho-1 not found in any other CTX-M-type ESBLs (Fig. 3). In the unbound Toho-1 structure, Arg274 is obstructing the substrate binding pocket, but upon substrate binding, Arg274 is forced out of the active site by the side chain of the substrate (Fig. 2A).

A comparison of the acyl-intermediate structures with the unbound Toho-1 structure shows the side chain of Ser237 in two

### Table I

| Substrate          | Cefotaxime | Cephalothin | Benzylpenicillin |
|--------------------|------------|-------------|------------------|
| Data collection    |            |             |                  |
| Wavelength (Å)     | 1.0        | 1.0         | 1.0              |
| Resolution (Å)     | 1.8        | 1.8         | 1.8              |
| Measured reflections| 96,844     | 117,110     | 70,280           |
| Unique reflections | 26,885     | 28,507      | 26,625           |
| Completeness (%)   | 95.2       | 96.9        | 92.3             |
| $R_{merge}$ (%)    | 7.3        | 5.0         | 12.8             |
| Space group        | P2_12_1    | P2_12_1     | P2_12_1          |
| Unit cell (Å)      | a = b = 72.6| c = 98.2    | a = b = 72.8     |
|                    | γ = 120°   | γ = 120°    | γ = 120°         |
| Refinement         |            |             |                  |
| Resolution (Å)     | 40–1.8     | 40–2.0      | 40–2.1           |
| No. of protein residues | 260 | 260 | 261 |
| No. of water molecules | 218 | 138 | 155 |
| No. of sulfate ions | 3          | 3           | 3                |
| $R_{merge}$ (%)    | 20.6       | 24.5        | 22.7             |
| Average B factor (Å²) | 12.8 | 33.6        | 33.4             |
| Bond lengths (Å)   | 0.005      | 0.007       | 0.007            |
| Bond angles (°)    | 1.33       | 1.37        | 1.40             |

* $R_{merge} = \sum_{i} \langle h\rangle - \langle h\rangle/\sum_{i} \langle h\rangle$, where $\langle h\rangle$ is the mean intensity of equivalent reflections.

* $R = \sum_{i} F_{o} - F_{c}/\sum_{i} F_{o}$, where $F_{o}$ and $F_{c}$ are the observed and calculated structure factor amplitudes, respectively.

* $R_{free} = \sum_{i} F_{o} - F_{c}/\sum_{i} F_{o}$, calculated using a test data set, 5% of total data randomly selected from the observed reflections.
different conformations (Fig. 2A). In the cephalothin-intermediate structure, Ser$_{237}$ has rotated $-30^\circ$, whereas in the cefotaxime-intermediate and benzylpenicillin-intermediate, there
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FIG. 3. Sequence alignments of class A β-lactamases. The eight β-lactamases above the black line are ESBLs, and the others are non-ESBLs. The CTX-M-type and the chromosomal ESBLs are colored blue and green, respectively. The three conserved regions and Glu106 are highlighted in red. The residues mentioned in the text are highlighted in green. Arrows above the sequences indicate the Ω-loop and the β-strand B3. KLEOX, the β-lactamase from K. oxytoca (4); CITDI, β-lactamase from C. diversus (4); PROVU, β-lactamase from P. vulgaris (4); PC-1, β-lactamase from Streptomyces albus G (4); STRAL, β-lactamase from B. licheniformis (4); YEREN, β-lactamase from Y. enterocolitica (36).

is an ~150° rotation of the side chain. This rotation prevents steric clashes with the methoxyimino (cefoxime) and the methyl group (benzylpenicillin) of the substrate. Asn104 is positioned at a bend in the binding site formed by Val103, Asn106, Tyr105, and Asn106 (Fig. 2B). This VNYN sequence is conserved in the CTX-M-type ESBLs, whereas a VYYS sequence is most common in the non-ESBLs in this region (Fig. 3). In the unbound structure, the side chain of Asn106 makes hydrogen bonds with the backbone groups of Val103 to maintain this bent conformation. However, in the acyl-intermediate structures, the N\text{\textsubscript{2}} of Asn106 changes its hydrogen bond acceptor from backbone oxygen of Val103 to that of Asn106 by causing a slight rotation of the peptide bond between Asn104 and Tyr105, which forces Val103 and Asn106 to be held in unfavorable conformations. This exchange is induced by the side chain movement of Arg244 to interact with the substrate. The movement of Asn104 also induces the movement of Asn132 whose side chain interacts with Asn104 and the substrate (Fig. 2B).

The main differences between the benzylpenicillin-intermediate and the cephalosporin-intermediates are observed around Ser237 and the oxyanion hole. In the benzylpenicillin-intermediate, the carbonyl group of the β-lactam ring does not position suitably for the hydrolysis in the oxyanion hole (Fig. 2C). This is caused by van der Waals contact between the methyl group of the thiazolidine ring, and the C\text{\textsubscript{\beta}} and the O\text{\textsubscript{\gamma}} of Ser237. An oxyanion hole formed by the backbone amides of Ser70 and residue 237 interacts with the carbonyl oxygen of the β-lactam ring in the substrate (1). These interactions play essential roles for the hydrolysis by stabilizing the negative charge appeared on the β-lactam carbonyl oxygen when the tetrahedral intermediates are formed during both acylation and deacylation. These interactions also help to polarize the carbonyl group of the β-lactam ring, thus favoring the nucleophilic attack to the carbonyl carbon by Ser70 in acylation and by the hydrolytic water in deacylation. In the cephalosporin-intermediate structures of Toho-1, the carbonyl oxygens of the β-lactam ring are located at similar distances from both backbone nitrogens (2.6–2.9 Å) to those in other class A β-lactamase acyl-intermediate structures with substrate (2.7–3.0 Å) (16, 17, 20). In the benzylpenicillin-intermediate structure, however, the carbonyl oxygen exists at a distance of 2.6 Å from the backbone nitrogen of Ser70, but 3.2 Å from the backbone nitrogen of Ser237, meaning that the polarization of the carbonyl group of benzylpenicillin is less efficient for deacylation than that of cephalosporins. This finding indicates that the lower penicillinae activity of CTX-M-type ESBLs compared with non-ESBLs is due to the conserved residue Ser237 (Fig. 3). Actually, in CTX-M-4 and a chromosomal β-lactamase from P. vulgaris K1 sharing high sequence homologies with CTX-M-type ESBLs, the S237A mutants exhibited increased $k_{\text{cat}}/K_m$ value toward benzylpenicillin (23, 24). This information may be useful for the development of new type inhibitors against CTX-M-type ESBLs.

Comparison with the Acyl-intermediate Structures of Non-ESBLs—The acyl-intermediate structures of Toho-1 and non-ESBLs show similar interactions with the substrates (16, 17) (Figs. 1, A–C, and 4, black dotted lines), but there are some key differences that can explain their specificities. First, Toho-1 lacks two hydrogen bonds formed by Arg244 in non-ESBLs, because CTX-M-type ESBLs do not contain Arg244 present in most non-ESBLs (Fig. 3). In the acyl-intermediate structures of non-ESBLs, Arg244 interacts with the carboxylate oxygen of the substrate and the backbone oxygen of Gly206 (Fig. 4). Site-directed mutagenesis analyses indicate that Arg244 is critical for the catalysis of TEM-1 (25, 26). In Toho-1, Arg276 was predicted to be a substitute for Arg244 (4); however, this residue has no interactions with the substrates (Figs. 1, A–C, and 2A). Instead, in Toho-1, Ser237 forms a hydrogen bond with the carboxylate oxygen of the substrate (Fig. 4). This unique interaction induces the rotation of the carboxylate group in the acyl-intermediate structures of Toho-1 compared with those of non-ESBLs. The residue Ser237 is conserved in all CTX-M-type ESBLs (Fig. 3), suggesting that this interaction plays a key role in the hydrolysis of oxyimino-cephalosporins. Interestingly, in the cefotaxime-intermediate structure of a PBP from Streptomyces sp. R61, Thr206, the corresponding residue to Ser237 of Toho-1, interacts with the amide group of acylamide side chain in cefotaxime to stabilize substrate binding, whereas in the cephalothin-intermediate structure (15), Thr206 interacts with the carboxylate oxygen as is seen in Toho-1 acyl-intermediate structures. This difference is brought about by steric hindrance between the bulky side chain of the substrates and the active site residues causing a tilt in cefotaxime binding compared with that of cephalothin.

Other differences are observed on the interactions with the acylamide side chain of the substrate in Toho-1. In the acyl-intermediate structures of class A β-lactamases, the acylamide side chain of the substrate interacts with the N\text{\textsubscript{\alpha}} of Asn132 and the backbone oxygen of residue 237 (Fig. 4). The residue Asn132 is the third residue of the conserved Ser130-Asp131-Asn132 motif in class A β-lactamases, and the hydrogen bond between the N\text{\textsubscript{\alpha}} of Asn132 and the carbonyl oxygen of the substrate is proposed to be important for the recognition of cephalosporins (27). The residue 237 exists just after the conserved Lys234-Thr235-Gly236 (Figs. 1, D–F), but there are some key differences between these non-ESBLs. Considering that the oxyimino group and the aminothiazole ring are introduced in the acylamide side chain of oxyimino-cephalosporins, the different positions of Asn132 and Ser237 would contribute to the oxyimino-cephalosporinase activity of Toho-1. In non-ESBLs, the position of Asn132 is fixed by the interactions between Asp131 and Thr/Ser109 as well as the interaction between the N\text{\textsubscript{\alpha}} of Asn132 and the backbone oxygen of residue 104 (Fig. 4) (28, 29). However, CTX-M-type ESBLs lack these interactions because these enzymes have an alanine at position 109 (Fig. 3), and the peptide bond between residues 104 and 105 is flipped compared with non-ESBLs (Fig. 4). Instead, the O\text{\textsubscript{\gamma}} of Asn104 interacts with the N\text{\textsubscript{\alpha}} of Asn132 in Toho-1. This unique peptide...
Gly236 and Arg244 present in non-ESBLs and the flexibility of tives lose important salt bridges that stabilize from S. aureus. 69 is variable in class A of residue 69 in class A between the backbone amide groups of Ser 70 and Ser237 con-

allows Asn170 to flip almost 180, which unwinds a short helix in the region and leaves a large cavity in the binding site. A type II ESBL PER-1 also has changed the conformation of the Ω-loop to enlarge the binding site by the unique sequence of the loop (8). Furthermore, it has been shown that an Ω-loop deletion mutant of the β-lactamase from S. aureus can bind oxyimino-cephalosporins (34).

A comparison between non-ESBLs and Toho-1 shows the Ω-loop in a slightly different position (Fig. 5A). In contrast to the other ESBLs mentioned above, the Ω-loop in Toho-1 has shifted to the helix H5. As a result, the positions of Pro167 and Asn170 are ~1 Å closer to the active site of Ser104 in Toho-1 than TEM-1. This shift shortens the depth of the binding site, and thereby, the binding position of the aminothiazole ring of ceftaxime is more exposed to solvents. In Fig. 5A, the aminothiazole ring of ceftaxime is positioned within van der Waals distances from the Ω-loops of non-ESBLs. For example, in TEM-1, the sulfur atom in the aminothiazole ring is located at distances of 3.0 Å from the backbone oxygen and the Cβ of Pro167. In contrast, in Toho-1, the closest distance is observed between a carbon atom in the aminothiazole ring of ceftaxime and the backbone oxygen of Pro167 (3.3 Å) (Fig. 1A). This means that the shift of the Ω-loop in Toho-1 keeps the loop away from the aminothiazole ring of ceftaxime and prevents the unfavorable contacts. Thus, Toho-1 has high affinities for oxyimino-cephalosporins. We believe that the different position of the Ω-loop is the main reason why Toho-1 can hydrolyze oxyimino-cephalosporins and non-ESBLs cannot.

We investigated the probable causes of this shift of the Ω-loop by comparing Toho-1 with TEM-1. Van der Waals contacts between Trp165 on the Ω-loop and residues Leu139, Thr140, and Pro145 on the α-helix H5 restrict the position of the Ω-loop in TEM-1 (Fig. 5B). In Toho-1, these restrictions are removed by replacing Trp165 with a threonine (Fig. 3). Therefore, the Ω-loop of Toho-1 is positioned closer to the α-helix H5 relative to TEM-1 (Fig. 5, A and B). However, the fact that a W165S mutant of TEM-1 does not hydrolyze ceftaxime (35) indicates the likelihood of multiple factors influencing the unfavorable disposition of the Ω-loop in non-ESBLs.

In TEM-1, Leu162 and Leu169 on the Ω-loop form van der Waals contacts with Phe72 on the α-helix H2 in a hydrophobic core (Fig. 5B). However, Toho-1 has a serine at position 72 (Fig. 3) and lacks these van der Waals contacts. This residue substitution permits the Ω-loop of Toho-1 to approach the α-helix H2. A decrease in hydrophobicity in this core region along with the replacement of a large hydrophobic side chain at position 72 is compensated for by Met135 and Phe165 in Toho-1 (Fig. 5B). These interpretations are supported by the fact that Trp165 and Phe165 are highly conserved in non-ESBLs, whereas Thr140, Ser72, Met135, and Phe165 are conserved in CTX-M-type ESBLs (Fig. 3). Further support is obtained by comparing a β-lacta-
mase from \textit{Yersinia enterocolitica}. This $\beta$-lactamase has a high sequence homology with Toho-1 (36) but still does not hydrolyze oxyimino-cephalosporins because of the existence of the residues Phe$^{72}$ and Trp$^{165}$ as in non-ESBLs (Fig. 3). CTX-M-type ESBLs share high sequence similarity with the chromosomal $\beta$-lactamases from \textit{Klebsiella oxytoca}, \textit{Citrobacter diversus}, and \textit{P. vulgaris} (Fig. 3). However, CTX-M-type ESBLs exhibit more efficient oxyimino-cephalosporinase activity than these chromosomal ESBLs (1). Recently, the structure of a chromosomal ESBL from \textit{P. vulgaris} K1 was determined (9). Interestingly, the position of the $\Omega$-loop in this ESBL shows higher similarity to those of non-ESBLs than that of Toho-1 (Fig. 5A), indicating that the affinities for oxyimino-cephalosporins are lower than Toho-1 in this ESBL. Actually, the $K_m$ value for cefuroxime, an oxyimino-cephalosporin, was $\approx 8 \times$ larger than that for cephaplatin in a $\beta$-lactamase from \textit{P. vulgaris} K1 (24), whereas the $K_m$ values for cefotaxime and cephaplatin are almost identical in Toho-1 (4). Thus, together with the lack of Asn$^{193}$ (Fig. 3), the unfavorable position of the $\Omega$-loop for the binding of oxyimino-cephalosporins could be one of the reasons for the lower activity in a $\beta$-lactamase from \textit{P. vulgaris} K1 than CTX-M-type ESBLs. More specifically, this unfavorable position of the $\Omega$-loop may be attributed to the different conformations of the $\beta$-strand B3 between these enzymes. In class A $\beta$-lactamases, the $\beta$-strand B3 has contacts with residue 69 and the $\Omega$-loop (30). In a $\beta$-lactamase from \textit{P. vulgaris} K1, the shorter side chain of Ala$^{69}$ compared with that of Cys$^{69}$ in Toho-1 (Fig. 3) allows the $\beta$-strand B3 to extend.
inward toward the binding site (Fig. 5A), which may hinder the shift of the β-strand as observed in Toho-1. In addition, the interaction between Arg238 and Gly238 in Toho-1 may also contribute to the difference of the β-strand conformation, although this is lost in the cephalothin-intermediate structure (Fig. 2A). This interpretation is not applicable to chromosomal ESBLs from K. oxytoca and C. diversus, because they possess Cys87 (Fig. 3). The lack of Asn104 and/or Ser237 may be the main cause of lower oxyimino-cephalosporinase activity in these enzymes.

Mechanism for the Extended-spectrum of Toho-1—In addition to Toho-1, there have been several oxyimino-cephalosporin-hydrolyzing β-lactamase structures solved (TEM-52, 6). TEM-64 (7), TEM-1 G238A mutant (7), PER-1 (8), a β-lactamase from P. vulgaris K1 (9), NMC-A (10), and a class C β-lactamase from E. cloacae GC1 (11). Compared with the former β-lactamases, these enzymes have sufficient space to accommodate the bulky side chain of oxyimino-cephalosporins. TEM-64 and PER-1 have each changed the conformation of the β-loop to generate broad binding cavities as mentioned above (7, 8). TEM-52 is an E104K/M128T/G238S mutant of TEM-1 (6). In TEM-52, Ser238 forms two new hydrogen bonds with the main and side chains of Ser243 that alter the conformation of loop 238–243 and widen the active site cavity. In TEM-1 G238A mutant, the steric contact between the side chain of Ala238 and the backbone oxygen of Asn170 enlarges the active site (7). In NMC-A, the unique disulfide bond between Cys69 and Cys238 induces distortion in the β-strand B3, which increases the space between residue 170 and this strand (10). GC1 β-lactamase has a three-residue insertion in the β-loop, which produces the wider binding cavity (11). The complex structure of class C β-lactamase AmpC with ceftazidime (22) proposes the importance of this insertion for the improved activity of GC1. In the case of a β-lactamase from P. vulgaris K1, such conformational changes are not reported, but replacements of glutamates at positions 104 and 240 with shorter alanine and aspartate, respectively, are thought to be more accommodating to aztreonam and ceftazidime with negatively charged oxyimino groups (9).

In Toho-1, the shift of the β-loop avoids the steric interactions with the bulky side chain of oxyimino-cephalosporins. Interestingly, this shift narrows the binding site but creates space for the bulky side chain to bind without bad contacts. This shift is attained by a structural rearrangement in the hydrophobic core in the vicinity of the β-loop. In addition, Asn104 and Asp240 interact with the bulky acylamide side chain of oxyimino-cephalosporins, which assist in the binding of the substrate. The characteristic arrangements of Asn132 and Ser237 in Toho-1 may also aid in the binding of a bulky acylamide side chain. Thus, Toho-1 has improved affinities for oxyimino-cephalosporins. Some ESBLs have improved affinities for oxyimino-cephalosporins by changing the conformation of the β-loop as mentioned above (2, 7, 8, 31). However, their kcat values against oxyimino-cephalosporins remain low (7, 31, 37), because the hydrolytic water is positioned and activated by Glu166 and Asn170 on the β-loop in class A β-lactamases. In contrast, Toho-1 retains high kcat values (4), because the shift of the β-loop does not induce the conformational changes observed in other ESBLs. Moreover, Ser237 probably contributes to the high kcat values against oxyimino-cephalosporins. Ser237 forms a unique hydrogen bond with the carbonyl oxygen of the substrate. Ser237 is conserved in all CTX-M-type ESBLs and in some of the chromosomal ESBLs. The S237A mutant of CTX-M-4 decreased the relative hydrolytic efficiencies against oxyimino-cephalosporins (23). The S237A mutant of a chromosomal ESBL from P. vulgaris K1 decreased kcat and kcat/Km values not only toward cephalothin but also toward cefuroxime (24). These results indicate that the interaction formed by Ser237 is important for the acylation of cephalosporins in CTX-M-type ESBLs. Penicillins and cephalosporins differ in the relative positions of the carbonylate group against the β-lactam ring, and the active site residues in class A β-lactamases are thought to be ideally located for hydrolyzing penicillins but not cephalosporins (38). Considering the acyl-intermediate structures of Toho-1 and the kinetic analyses of the mutants of the related enzymes, the unique interaction formed by Ser237 in Toho-1 would assist in bringing the carbonyl group of the β-lactam ring of cephalosporins to the optimal position in the oxyanion hole for the acylation. However, in the case of the hydrolysis of penicillins, this interaction seems to produce undesirable contacts with the substrate, which prevent the proper positioning of the carbonyl group of the β-lactam ring in the oxyanion hole. Similarly, the introduction of A237T mutation into TEM-1 or TEM-derived ESBLs increased cephalosporinase activities (2, 39, 40). In contrast, the cefotaxime-resistant PBP2x from Streptococcus pneumoniae has a threonine to an alanine replacement at the corresponding position to 237 in Toho-1, which caused the 90% reduction of the acylation for cefotaxime, whereas the acylation by benzylpenicillin was not affected (41).

The acyl-intermediate structures of Toho-1 reveal that the mechanism of the extended-spectrum of CTX-M-type ESBLs is characterized by the increased affinity for oxyimino-cephalosporins and the efficient acylation of cephalosporins. These features are accomplished through interactions with the conserved residues Cys69, Ser237, and Asp240, and it is these residues that discriminate CTX-M-type ESBLs from other class A β-lactamases.

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