Invited Review

Microbiology

Substrate Deacylation Mechanisms of Serine-β-lactamases

Masayuki Hata,* Yasuyuki Fujii, Yoshikazu Tanaka, Hidenori Ishikawa, Miho Ishii, Saburo Neya, Minoru Tsuda and Tyuji Hoshino

Department of Physical Chemistry, Graduate School of Pharmaceutical Sciences, Chiba University; 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

hata@faculty.chiba-u.jp
Summary
The substrate deacylation mechanisms of serine-β-lactamases (classes A, C and D) were investigated by theoretical calculations. The deacylation of class A proceeds via four elementary reactions. The rate-determining process is the tetrahedral intermediate (TI) formation and the activation energy is 24.6 kcal/mol at the DFT level. The deacylation does not proceed only by Glu166, which acts as a general base, but Lys73 also participates in the reaction. The C3-carboxyl group of the substrate reduces the barrier height at the TI formation (substrate-assisted catalysis). In the case of class C, the deacylation consists of two elementary processes. The activation energy of the TI formation has been estimated to be 30.5 kcal/mol. Tyr150O$_{η}$ is stabilized in the deprotonated state in the acyl-enzyme complex and works as a general base. This situation can exist due to the interaction with two positively charged side chains of lysine (Lys67 and Lys315). The deacylation of class D also consists of two elementary reaction processes. The activation energy of the TI formation is ca. 30 kcal/mol. It is thought that the side chain of Lys70 is deprotonated and acts as a general base. When Lys70 is carbamylated, the activation energy is reduced to less than 20 kcal/mol. This suggests that the high hydrolysis activity of class D with carbamylated Lys70 is due to the reduction of activation energy for deacylation. From these results, it is concluded that the contribution of the lysine residue adjacent to the serine residue is indispensable for the enzymatic reactions by serine-β-lactamases.

Keywords
β-lactamase; tetrahedral intermediate; deacylation reaction; 

ab initio molecular orbital method; density functional theory; molecular dynamics simulation
INTRODUCTION

β-Lactamases are produced by pathogenic bacteria and are enzymes that cause resistance to β-lactam antibiotics by hydrolyzing their β-lactam rings.1-3) β-lactamases are classified into two major types on the basis of the main component of the active site: serine-β-lactamases and metallo-β-lactamases.4,5) Serine-β-lactamases are further classified into three classes: classes A, C, and D; i.e., metallo-β-lactamase is classified into class B.

As is well known, the catalytic mechanism of serine-β-lactamases involves acylation (Fig. 1(a) to (c) via (b)) and deacylation (Fig. 1(c) to (d)). The acylation mechanism has been examined in many experimental6-14) and theoretical works,11,15-22) and acylation is common to serine-β-lactamase and penicillin-binding protein (PBP). Deacylation, on the other hand, is only seen in serine-β-lactamase. This means that the essence of antibiotic resistance by serine-β-lactamase arises from the deacylation reaction. Hence, an understanding of the reaction mechanism of deacylation is important for developing novel β-lactam antibiotics and potent β-lactamase inhibitors. We have investigated the deacylation mechanisms of serine-β-lactamases by using theoretical calculation. In this paper, the results of our recent computational analyses for all classes of serine-β-lactamases are presented.

DEACYLATION MECHANISM OF CLASS A β-LACTAMASE

Class A β-lactamase is more frequently detected in a clinical setting than are other classes of β-lactamase. Since the study by Knox et al. suggested that the E166A mutant of β-lactamase from Bacillus licheniformis induced the accumulation of an acyl-enzyme intermediate;23) the catalytic residue involved in the deacylation reaction has been presumed to be only Glu166. However, it is difficult to conclude that the deacylation reaction occurs only due to Glu166 because the distance between Ser70Oγ and Glu166Oε is too far to interact (ca. 3.74 Å) according to the results of our molecular dynamics (MD) simulation of the structure of β-lactam antibiotics-β-lactamase complex.24) We have speculated that not only Glu166 but also Lys73 is involved in the deacylation reaction because Lys73Nζ is located between Ser70 and Glu166 and interacts with Ser70Oγ and Glu166Oε, nearly forming hydrogen bonds (2.77 and 3.21 Å, respectively).24) Furthermore, a hydrogen bond network among Lys73Nζ, Ser130Oγ and the carboxyl group of the substrate (β-lactam antibiotics)
should be taken into account to clearly explain the reaction mechanism. Ishiguro et al. also discussed the importance of the hydrogen bond network based on the results of their molecular mechanics calculations. Accordingly, we investigated the whole mechanism of the deacylation reaction by theoretical calculation.

MD simulation of class A β-lactamase binding penicillin G showed that Ser130, Asn132, Ser235, Gly237, and Arg244 cooperatively restricted the mobility of the penicillin G moiety by making salt bridges among the side chains of these residues and the C3-carboxyl or C6-amide group of the substrate. The oxyanion hole composed of N atoms in the main chain of Ser70 and Gly237 was properly reproduced.

We have found by the density functional theory (DFT) that the deacylation reaction proceeds via four elementary reactions. The potential energy profile is shown in Fig. 2 and the reaction scheme is shown in Fig. 3. The model compound was constructed from the active site structure of the penicillin G-class A β-lactamase acyl-enzyme intermediate and was composed of a substrate penicillin G, four catalytic residues (Ser70, Lys73, Ser130, and Glu166), and a water molecule.

First, Lys73 is deprotonated by a concerted double proton transfer from Lys73Nζ to Ser130Oγ and from Ser130Oγ to C3-carboxylate in the substrate because a hydrogen bond network among Lys73Nζ-Ser130Oγ-C3-carboxylate was formed in the acyl-enzyme intermediate (A1→TSA1→A2). The activation energy for this first reaction was calculated to be 7.9 kcal/mol (Fig. 2). Second, the acyl-enzyme tetrahedral intermediate (TI) is formed by the assistance of Glu166, which acts as a general base catalyst (A2→TSA2→A3). The activation energy for this reaction was found to be 24.6 kcal/mol, which is the highest in the deacylation reaction. From the obtained mechanisms of the first and second elementary reactions, we hypothesized that reduction of the ionic interaction between Glu166Oε and Lys73Nζ enhances the general base activity of Glu166 and that the reduction is caused by the deprotonation of Lys73. Furthermore, the deprotonation of Lys73 was confirmed to be induced by the C3-carboxyl group of the substrate penicillin G. When the model excluding Ser130 and the carboxyl group of the substrate was used for the calculation, the activation energy level for TI formation was much higher. Hence, the hydrogen bond network plays an important role in decreasing the activation energy for the TI formation reaction. Lys73Nζ, which is located at
the terminus of the hydrogen bond network, played a role in the formation of a hydrogen bond with Glu166Oε in order to help the deacylation reaction.

Third, Lys73 is protonated by the concerted double proton transfer from C3-carboxylate to Ser130Oγ and from Ser130Oγ to Lys73Nζ (A3→TSA3→A4). For detachment of the degraded substrate from the active site, it is necessary to donate a proton to Ser70Oγ to cleave the C7-Ser70Oγ bond. The catalytic residue that plays this role has been presumed to be Glu166.7,27 According to the structure of A3, however, the distance between Glu166Oε and Ser70Oγ is too far for direct proton migration to occur (4.07 Å).26 Another mechanism that involves proton migration from Glu166Oε to Ser70Oγ via a water molecule located between Glu166Oε and Ser70Oγ has been also proposed.28-31 However, no water molecule was observed between Glu166Oε and Ser70Oγ during the MD calculation.26 Judging from the facts above, Glu166 is not thought to be a candidate for the catalytic residue. For these reasons, the two previous hypothetical mechanisms seem incorrect. It is notable that Lys73 must be reprotonated because the amino group is in the neutral state in A3. We speculate that a hydrogen atom migrates from Ser130Oγ to Lys73Nζ because a lone pair of Lys73Nζ is oriented toward the hydrogen atom. Hence, the probable reaction mechanism of the reprotonation of Lys73 will be a concerted double proton transfer from C3-carboxylate to Ser130Oγ and from Ser130Oγ to Lys73Nζ. The activation energy for this reaction has been calculated to be 9.0 kcal/mol (Fig. 2).

Finally, the degraded substrate is detached from the enzyme in concert with a single proton transfer from Lys73Nζ to Ser70Oγ. The activation energy for this reaction has been estimated to be 3.9 kcal/mol.

If the C3-carboxyl group does not participate in the deacylation step, the mechanism will be based on a simple ester bond cleavage. First, the TI is formed by nucleophillic attack on C7. Second, the C7-Ser70Oγ bond is dissociated in concert with a single proton migration from Lys73Nζ to Ser70Oγ. The potential energy curve of this deacylation path was successfully determined as shown in Fig. 4. This mechanism is summarized in Fig. 5. The activation energies for the TI formation and for the proton transfer from Lys73 to Ser70 were found to be 26.8 kcal/mol and 2.4 kcal/mol, respectively. It is notable that the deacylation does not proceed only by Glu166 but that Lys73 also participates in the reaction. Moreover, it
has been found that the presence of the C3-carboxyl group of the substrate reduces the barrier height at the TI formation, suggesting that the deacylation is a substrate-assisted catalysis (SAC).

SAC is an enzymatic reaction that is accelerated by a functional group of a substrate. It has been proposed that SAC can be observed in the catalytic mechanisms of many different types of enzymes\textsuperscript{32} as well as class A β-lactamase. The SAC found in this study is similar to the mechanism proposed by Ishiguro and Imajo\textsuperscript{15} because the deprotonation of Lys73 is induced by the C3-carboxyl group in both Ishiguro's mechanism and ours. Furthermore, Zawadzke et al. suggested that the $pK_a$ of Lys73 was dramatically reduced upon substrate binding,\textsuperscript{33} which is also compatible with our mechanism. The substrate molecule examined in this study was only penicillin G. However, our SAC may be applicable to the enzymatic hydrolysis of other penicillins because penicillins generally have a C3-carboxyl group in their five-membered ring. It is interesting that several β-lactams that have no functional group at C3 work as β-lactamase inhibitors.\textsuperscript{34} This suggests that the inhibition ability of the compounds arises from an absence of the C3-carboxyl group. As is well known, cepharosporin antibiotics have a carboxyl group at C4. The role of the C4-carboxyl group seems to be similar to that of the C3-carboxyl group of penicillins. According to the results of a quantum mechanics calculation study by Vaterro et al., however, the orientation of the C4-carboxyl group differed from that of the C3-carboxyl group of penicillins.\textsuperscript{35} Moreover, structural analysis of the cepharoridine-acylated double mutant (Glu166Asp:Asn170Gln) showed that the C4-carboxyl group made a weak hydrogen bond with Ser130O$_γ$ (The C4-carboxyl oxygen-Ser130O$_γ$ distance was found to be 3.78 Å.).\textsuperscript{36} From these findings, the C4-carboxyl group seems to be inappropriate for a proton abstractor from the hydroxyl group of Ser130. Hence, the reaction path shown in this study will be impossible in the case of cephalosporins because the C4-carboxyl group cannot participate in the deacylation process. This is the reason why cepharosporins show better resistance to the β-lactamase than do penicillins. An experimental study by Christensen et al. has shown that the deacylation rate of cephalosporin (nitrocefin) is considerably smaller than that of penicillin G.\textsuperscript{37} This result is compatible with our suggestion stated above.
The deacylation ability of the Lys73 mutant was lower than that of the wild-type enzyme, suggesting that Lys73 plays some role in the deacylation.\textsuperscript{6,38} Our calculations demonstrated that Lys73 served as a proton donor to Ser70O\textsubscript{γ} in the detachment of the degraded substrate from the active site of the enzyme.

Ser130 made a bridge between Lys73 and the C3-carboxyl group. It has been reported that Ser130 mutants have less substrate affinity.\textsuperscript{39, 40} Several structural studies on the acyl-enzyme intermediate analogues indicated that Ser130 made a hydrogen bond with the carboxyl group.\textsuperscript{7,41-43} From these experimental findings, the function of Ser130 was thought to be binding a substrate. Dietz et al. suggested that Ser130 did not only take part in substrate binding but also enzymatic catalysis.\textsuperscript{16} Our study suggests that Ser130 is a residue assisting substrate binding and the deacylation mechanism.

The results of this study indicated that the function of Glu166 in deacylation was as a general base catalyst for the TI formation reaction. The TI formation mechanism obtained in this work is consistent with results of several experimental studies showing that the deacylation ability of the Glu166 mutant was greatly impaired compared to that of the wild-type enzyme.\textsuperscript{6,23,27,29,44,45} In our study, the activation energy for the TI formation in pathway A (Fig. 2) was 24.6 kcal/mol, and the reaction appeared to be the rate-determining step of the deacylation step. Wladkowski et al. found that the rate-determining step in the acylation half was TI formation, and the activation energy was calculated to be 25.9 kcal/mol at the DFT level.\textsuperscript{46} Thus, results of some theoretical investigations are also consistent with our results. Pitarch et al. clarified by \textit{ab initio} MO calculations the mechanism of the induction of nonenzymatic hydrolysis of a β-lactam compound by a water molecule. They suggested that the rate-determining step in the nonenzymatic hydrolysis was TI formation, and the activation energy of the reaction was estimated to be 53.77 kcal/mol.\textsuperscript{47} The TI formation in the present study is energetically more favorable than the nonenzymatic TI formation. This demonstrates the catalytic efficiency of the enzyme.

**DEACYLATION MECHANISM OF CLASS C β-LACTAMASE**

A deacylation mechanism similar to that of class A is expected for class C β-lactamase because class C β-lactamase is also a serine-β-lactamase. However, as seen in X-ray
crystallographic analysis structures of class C β-lactamase, there is no acidic amino acid residue corresponding to Glu166 in class A in the active site, while a residue corresponding to Lys73 in class A is seen (Lys67). In place of Glu166 in class A, a tyrosine residue (Tyr150) exists in the active site of class C. Some studies have suggested that the side chain of Tyr150 is in a deprotonated state due to the special surrounding environment of class C and plays a role as a general base. We aimed to clarify the deacylation mechanism of class C β-lactamase at the atomic level through theoretical calculations to confirm that the deprotonated state can exist stably in the active site in the process of deacylation.

The X-ray crystallographic structure of the inhibitor moxalactam-bound class C β-lactamase was utilized for the computational model and moxalactam was changed to the substrate cefaclor. A model for quantum chemical calculations was constructed using an energy-minimized structure of the substrate-bound enzyme obtained by molecular mechanics calculation, in which the enzyme was soaked in thousands of TIP3P water molecules.

It was found that substrate deacylation of class C β-lactamase consisted of two elementary processes. The potential energy change is shown in Fig. 6, and the reaction scheme is shown in Fig. 7. The first elementary process is the acyl-enzyme TI formation, which is initiated by the activation of catalytic water by Tyr150 (Fig. 7(a)→(b)). Tyr150Oη is stabilized in the deprotonated state in the structure of the acyl-enzyme complex. The side chain of Tyr150 is usually neutral, but in this case the side chain can be in the negatively charged state due to the interaction with two positively charged side chains (two lysine residues, Lys67 and Lys315). The oxygen anion has the ability to abstract a proton from a water molecule. For this reason, Tyr150 of class C enzyme is thought to function as a general base instead of an acidic residue like Glu166 of class A. In the TI structure, a water molecule is dissociated into a proton and a hydroxyl ion. The oxygen atom of the water molecule interacts with Tyr150Oη by a hydrogen bond. The activation energy of this process is 39.8 kcal/mol at the HF level (Fig. 6).

The second elementary process is the detachment of a hydroxylated substrate assisted by the movement of a hydrogen atom from Lys67 to Ser64 (Fig. 7(b)→(c)). There are two TI structures (TI1 and TI2). The major difference between TI1 and TI2 structures is that in the TI2 structure: Lys67 is slightly away from Tyr150 and, consequently, Lys67Hζ approaches Ser64Oγ, while Lys315 approaches Tyr150 (Fig. 8 and Table 1). Moreover, a hydrogen bond
between Lys67Nζ and Ser64Oγ is formed in the TI2 structure but not in the TI1 structure. These structural differences suggest that the second elementary process occurs more easily from TI2 than from TI1. No noticeable potential barrier was observed between the two structures. The potential energy of TI2 is about 5 kcal/mol lower than that of TI1. For this reason, the structural change from TI1 to TI2 will occur spontaneously and TI2 is thought to be the tetrahedral intermediate structure detectable in experiments.

In the S2 structure, a covalent bond between a carbonyl carbon atom of the substrate and Ser64Oγ is broken. The activation energy of this process is 2.5 kcal/mol at the HF level (Fig. 6). The proton transfer from Tyr150 to Lys67 does not occur at the last stage of the deacylation reaction due to large activation energy (25.96 kcal/mol).

Dubus et al. suggested from the results of site-directed mutagenesis experiments^{8, 57} and measurements of enzymatic stability and activity^{8} that Tyr150 plays an important role in the deacylation process. Lamotte-Brasseur et al. also suggested from the measurement of pK_a of Tyr150 that Tyr150 works as a general base because Tyr150 showed a lower pK_a value than those of other tyrosine residues.^{54} These speculations based on experimental results are compatible with our theoretical results.

Recently, Gherman et al. proposed a Tyr150/Lys67 general base mechanism for the tetrahedral intermediate formation in the deacylation of class C β-lactamase: i.e., the side chains of Tyr150 and Lys67 are neutral in the initial state of deacylation and a proton relay from the water molecule for deacylation to Lys67 via Tyr150 occurs in the tetrahedral intermediate formation.^{58} We also calculated the initial structure of the Tyr150/Lys67 general base mechanism. The potential energy of the structure was 0.76 kcal/mol higher than that of the Tyr150 general base mechanism (this study) at the DFT level. Calculation using another basis set and a higher-order calculation method also showed that the structure had a higher potential energy than that of the Tyr150 general base mechanism.^{59} It is necessary to confirm whether both Tyr150 and Lys67 are neutral or not in the structure produced as a result of the acylation process. For this reason, only the Tyr150 general base mechanism was considered in this study.

In the first elementary process (S1 → TS1 → TI1), both Lys67Nζ and Lys315Nζ move away from Tyr150Oη in accordance with the abstraction of a proton from Wat1 by Tyr150Oη, but
Lys315Nζ approaches Tyr150Oη again when Lys67Nζ approaches Ser64Oγ and goes further away from Tyr150Oη in the second elementary process (Table 2). For this reason, the amino groups of the side chains of Lys67 and Lys315 cooperatively fix the location of the side chain of Tyr150. Monnaie et al. suggested from the results of a site-directed mutagenesis experiment that Lys67 plays an important role in the enzymatic activity, particularly in control of the electrostatic environment.60 They also performed another site-directed mutagenesis experiment on Lys315 and reported that this residue mainly enhanced nucleophilic attack.61 Their conclusion is also consistent with our theoretical results.

The activation energy of the rate-determining process (the first elementary process) was estimated to be 30.5 kcal/mol from the DFT calculations considering electron correlation. Since calculations by other higher-order methods also gave the same results, this value is reliable. The value obtained in this study is the same level as other activation energies of the rate-determining process of enzymatic reactions that proceed via a tetrahedral intermediate.26, 58, 62, 63

DEACYLATION MECHANISM OF CLASS D β-LACTAMASE

Although the catalytic mechanisms of class A and C have been studied in detail, little is known about the catalytic mechanism of class D. Despite the similar folding structures5, 64, 65 and the similar acylation-deacylation processes13, 14 of class D and the other two classes of serine-β-lactamases,66 the homology in amino acid sequence is not high between them. The crystal structure of OXA-13 β-lactamase,67 one of the class D β-lactamases, revealed that the hydrogen bonding network at the catalytic center was different from those of class A and C, suggesting that there exists a particular reaction scheme in class D β-lactamases.49, 64, 67 In class A and C β-lactamases, there are two important residues that initiate the acylation-deacylation process for hydrolysis (Lys73 and Glu166 in class A,24 Lys73 and Tyr150 in Class C8, 48, 54). In class D, however, there seems to be only one residue, Lys70, that can be involved in the catalytic reaction.14, 64, 67 A recent study suggested that this key residue Lys70 was carbamylated during the hydrolysis reaction.68 In order to investigate the hydrolysis reaction of class D β-lactamases, we focused on the deacylation mechanism of OXA-13 β-lactamase.
First, we executed four different 1-ns MD simulations. OXA-13 β-lactamase and substrate (amoxicillin)/inhibitor (meropenem) complexes were modeled under the conditions in which the catalytic residue Lys70 was in the unprotonated state and in the carbamylated state. Snapshots of these MD simulations are shown in Figs. 9 to 12. In the unprotonated Lys70 model (Fig. 9), the substrate (amoxicillin: Axl) interacts with Arg250 through hydrogen bondings and the catalytic water molecule (Wat) is held at the catalytic center by two interactions with Lys70 (2.89 Å) and Trp154 (3.02 Å). Similarly, the inhibitor (meropenem: Mer) interacts with Arg250, and Wat is held by the same residues (2.83 Å with Lys70, 2.94 Å with Trp154) (Fig. 10). However, the distance between the oxygen atom of Wat and the carbon atom of the carbonyl group of the substrate is approximately 1 Å shorter (3.91 Å) than that of the inhibitor (4.93 Å). In the carbamylated Lys70 model (Fig. 11), although the interactions between the substrate and Arg250 disappears, Wat is kept at the catalytic center due to the strong negative charge of the carbamoyl moiety of Lys70 (2.59 Å). Wat is also restrained by the negative charge of the carbamoyl moiety in the case of the inhibitor (2.61 Å) (Fig. 12). The distance between the oxygen atom of Wat and the carbon atom of the carbonyl group of the substrate/inhibitor is shorter in the carbamylated Lys70 model than in the unprotonated Lys70 model. Furthermore, this distance becomes shorter in the case of the substrate than in the case of the inhibitor as well. This suggests that one of the essences of the inhibitor is to keep Wat away from its carbonyl group carbon atom. The ground for the difficulty in the deacylation reaction is that the inhibitor has an alpha conformation for the ethanoyl side chain at the 6th position. In general, substrates to OXA-13 class D β-lactamase such as amoxicillin have a beta conformation at their 6th position. Because a methyl group on the side chain of meropenem at the 6th position is oriented towards the active site of the enzyme, this conformation makes Lys70 and Wat more difficult to engage in the reaction. Furthermore, the space occupied by the side chain of meropenem is smaller compared to the substrates (Amoxicillin has 6-p-phenyl-amide). That is, a ligand with an alpha conformation at its 6th position can make the side chain enter the catalytic center, while a ligand with a large side chain at its 6th position in the beta conformation cannot make the side chain enter the catalytic center due to the steric hindrance. Furthermore, meropenem has an ethanoyl
group on the side chain at its 6th position. This hydrophilic ethanoyl group heads towards the outer side of the catalytic center, thus keeping the catalytic center more hydrophobic.

The high level of deacylation activity of the carbamylated Lys70 can be explained by the negative charge of the carbamoyl moiety, which attracts a catalytic water close to the carbonyl group carbon atom. From these results, we propose the deacylation scheme of class D β-lactamase in which Lys70 is a key residue primarily dominating the enzymatic reaction (Fig. 14).

In this scheme, Lys70 plays the role of an acidic residue, which deprotonates the catalytic water molecule and subsequently plays the role of a basic residue, which protonates the active serine. Lys70 is required to be neutral at the beginning of deacylation process. Since it has been reported that the active site surrounding OXA-13 class D β-lactamase was in a hydrophobic environment, Lys70 will be in favor of being neutral rather than being protonated as a normal lysine residue in aqueous solutions. We also performed pKₐ calculation of the Lys70 residue using Multi-Conformation Continuum Electrostatics (MCCE program). In both the crystal structure and the equilibrated structure from MD simulations, the pKₐ values of Lys70 were calculated to be less than 0. This supports our assumption that Lys70 is in the neutral form.

According to the suggestion from the MD simulation, the deacylation mechanism of class D β-lactamase with deprotonated Lys70 was investigated by quantum chemical calculation. The influence of carbamylated Lys70 was also elucidated.

Based on the crystal structure of class D β-lactamase OXA-13 (pdb entry code: 1H8Y), a model of the acyl-enzyme intermediate was constructed. Lys70 was deprotonated or carbamylated and the substrate was amoxicillin in the model. Model compounds for quantum chemical calculations was constructed by extracting some important residues, including Lys70, from the structure by MD simulation. Potential energy minima and saddle point structures and the lowest energy path were determined at the Hartree-Fock (HF) level with the 6-31G** basis set. Potential energy calculations for the stationary points were performed at the DFT (B3LYP) level with 6-31G** or 6-31+G** basis set to obtain more accurate potential energy. The self-consistent reaction field (SCRF) method with the Onsager model was used to consider the solvent effect.
It was found that the deacylation of class D β-lactamase with deprotonated Lys70 consisted of two elementary reaction processes (Figs. 13 and 14). The first elementary process is the formation of TI in which the deprotonated Lys70 abstracts a proton from a water molecule and the hydroxyl ion from the water molecule makes a nucleophillic attack on the carbonyl group of amoxicillin, a substrate. The second elementary process is the formation of a Michaelis-Menten complex in which Ser67Oγ abstracts a proton from the ε-amino group of Lys70 and bond dissociation between Ser67 and amoxicillin occurs for elimination of the hydrolyzed substrate. The rate-determining process of the reaction is the first elementary process. The activation energies are 29.8 kcal/mol and 31.1 kcal/mol at the B3LYP level with 6-31G** and 6-31+G**, respectively. The deacylation of class D β-lactamase with carbamylated Lys70 also consisted of two elementary processes (Figs. 15 and 16). The first elementary process is the formation of TI in which the carbamylated Lys70 abstracts a proton from a water molecule and the hydroxyl ion from the water molecule makes a nucleophillic attack on the carbonyl group of amoxicillin. In the second elementary process, Ser67Oγ contributes to the stabilization of the model system by hydrogen bonding with the carbamoyl group of Lys70. The reaction is the formation of a Michaelis-Menten complex in which Ser67Oγ abstracts a proton from the carbamoyl group of Lys70 and bond dissociation between Ser67 and amoxicillin occurs for elimination of the hydrolyzed substrate. Although the potential energy level of the structure of TS2 is higher than that of TS1, the rate-determining process of the reaction is the first elementary process. The activation energies are 16.52 kcal/mol and 19.20 kcal/mol at the B3LYP level with 6-31G** and 6-31+G**, respectively. These theoretical results suggested that the high level of hydrolysis activity of class D β-lactamase with carbamylated Lys70 was due to the reduction of the activation energy for deacylation.

**CONCLUSION**

The deacylation mechanisms of serine β-lactamases were clarified by theoretical calculations. We obtained reasonable activation energies and structural changes for each class of β-lactamase. To facilitate the enzymatic reaction, the contribution of the lysine residue
adjacent to the serine residue is important. In classes A and C, the lysine residue is located between serine and general base residues and stabilizes the active site, whereas lysine has a role of a general base in class D.

ACKNOWLEDGEMENT

This work was partly supported by a Grant-in-Aid from The Ministry of Education, Culture, Sports, Science and Technology (MEXT). The authors thank the Computer Center of the Institute for Molecular Science, for the use of Fujitsu VPP5000 computer. The computations were also carried out by DRIA system at Graduate School of Pharmaceutical Sciences, Chiba University.
REFERENCES AND NOTES


FIGURE CAPTIONS

Fig. 1. Substrate inactivation mechanism by serine-β-lactamase. Cited from ref. 59.

Fig. 2. Potential energy profile for the substrate-dependent path. Horizontal and vertical axes represent the reaction coordinate along the steepest reaction path (amu$^{1/2}$Å) and the potential energy (kcal/mol), respectively. Cited from ref. 26.

Fig. 3. Deacylation mechanism by class A β-lactamase proposed in ref. 26.

Fig. 4. Potential energy profile of substrate-independent path. Horizontal and vertical axes represent the reaction coordinate along the steepest reaction path (amu$^{1/2}$Å) and the potential energy (kcal/mol), respectively. Cited from ref. 26.

Fig. 5. Substrate-independent deacylation mechanism by class A β-lactamase proposed in ref. 26.

Fig. 6. Profile of the lowest energy path along the intrinsic reaction coordinate (IRC). Vertical and horizontal axes are the potential energy (kcal/mol) and the reaction coordinate (amu$^{1/2}$Å), respectively. Cited from ref. 59.

Fig. 7. Deacylation mechanism by class C β-lactamase proposed in ref. 59.

Fig. 8. Two TI structures obtained by IRC calculations. Residue names and atom names are shown in (TI1) and (TI2), respectively. Cited from ref. 59 with some modification.

Fig. 9. Active-site structure of enzyme-substrate (amoxicillin) complex. The illustration is a snapshot at 1 ns. Distances (Å) are the averages between 600-1,000 ps of simulation.

Fig. 10. Active-site structure of enzyme-inhibitor (meropenem) complex. The illustration is a snapshot at 1 ns. Distances (Å) are the averages between 600-1,000 ps of simulation.
**Fig. 11.** Active-site structure of enzyme-substrate (amoxicillin) complex with carbamylated lysine residue. The illustration is a snapshot at 1 ns. Distances (Å) are the averages between 600-1,000 ps of simulation.

**Fig. 12.** Active-site structure of enzyme-inhibitor (meropenem) complex with carbamylated lysine residue. The illustration is a snapshot at 1 ns. Distances (Å) are the averages between 600-1,000 ps of simulation.

**Fig. 13.** Profile of the lowest energy path along the IRC at the HF level. Vertical and horizontal axes are the potential energy (kcal/mol) and the reaction coordinate (amu$^{1/2}$Å), respectively. S2 structure is the same as S3 structure.

**Fig. 14.** Deacylation mechanism by class D β-lactamase with deprotonated Lys70 proposed in this paper.

**Fig. 15.** Profile of the lowest energy path along the IRC at the HF level. Vertical and horizontal axes are the potential energy (kcal/mol) and the reaction coordinate (amu$^{1/2}$Å), respectively. S2 structure is the same as S3 structure.

**Fig. 16.** Deacylation mechanism by class D β-lactamase with carbamylated Lys70 proposed in this paper.
**TABLES**

**Table 1.** Important inter-atomic distances in TI1 and TI2 obtained by IRC calculations.

Cited from ref. 59 with some modification.

<table>
<thead>
<tr>
<th>Inter-atomic distance (Å)</th>
<th>TI1</th>
<th>TI2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wat1O&lt;sub&gt;ω&lt;/sub&gt;-CclC&lt;sub&gt;c&lt;/sub&gt;</td>
<td>1.48</td>
<td>1.46</td>
</tr>
<tr>
<td>Wat1O&lt;sub&gt;ω&lt;/sub&gt;-Tyr150O&lt;sub&gt;η&lt;/sub&gt;</td>
<td>2.77</td>
<td>2.74</td>
</tr>
<tr>
<td>Wat1O&lt;sub&gt;ω&lt;/sub&gt;-CclO&lt;sub&gt;41&lt;/sub&gt;</td>
<td>3.34</td>
<td>3.28</td>
</tr>
<tr>
<td>Wat1H&lt;sub&gt;ω2&lt;/sub&gt;-CclO&lt;sub&gt;41&lt;/sub&gt;</td>
<td>2.51</td>
<td>2.48</td>
</tr>
<tr>
<td>Wat1H&lt;sub&gt;ω1&lt;/sub&gt;-Tyr150O&lt;sub&gt;η&lt;/sub&gt;</td>
<td>0.97</td>
<td>0.97</td>
</tr>
<tr>
<td>Ser64O&lt;sub&gt;γ&lt;/sub&gt;-Lys67H&lt;sub&gt;ζ&lt;/sub&gt;</td>
<td>2.05</td>
<td>1.68</td>
</tr>
<tr>
<td>Ser64O&lt;sub&gt;γ&lt;/sub&gt;-Lys67N&lt;sub&gt;ζ&lt;/sub&gt;</td>
<td>2.78</td>
<td>2.72</td>
</tr>
<tr>
<td>Ser64O&lt;sub&gt;γ&lt;/sub&gt;-CclC&lt;sub&gt;c&lt;/sub&gt;</td>
<td>1.45</td>
<td>1.49</td>
</tr>
<tr>
<td>Tyr150O&lt;sub&gt;η&lt;/sub&gt;-Lys67N&lt;sub&gt;ζ&lt;/sub&gt;</td>
<td>2.96</td>
<td>3.18</td>
</tr>
<tr>
<td>Tyr150O&lt;sub&gt;η&lt;/sub&gt;-Lys67H&lt;sub&gt;ζ1&lt;/sub&gt;</td>
<td>1.96</td>
<td>2.62</td>
</tr>
<tr>
<td>Tyr150O&lt;sub&gt;η&lt;/sub&gt;-Lys315N&lt;sub&gt;ζ&lt;/sub&gt;</td>
<td>3.23</td>
<td>2.99</td>
</tr>
<tr>
<td>Tyr150O&lt;sub&gt;η&lt;/sub&gt;-Lys315H&lt;sub&gt;ζ2&lt;/sub&gt;</td>
<td>2.24</td>
<td>1.99</td>
</tr>
<tr>
<td>Lys315N&lt;sub&gt;ζ&lt;/sub&gt;-Wat2O&lt;sub&gt;ω&lt;/sub&gt;</td>
<td>2.68</td>
<td>2.69</td>
</tr>
<tr>
<td>Lys315H&lt;sub&gt;ζ1&lt;/sub&gt;-Wat2O&lt;sub&gt;ω&lt;/sub&gt;</td>
<td>1.66</td>
<td>1.66</td>
</tr>
<tr>
<td>Glu272O&lt;sub&gt;ε1&lt;/sub&gt;-Wat2O&lt;sub&gt;ω&lt;/sub&gt;</td>
<td>2.62</td>
<td>2.61</td>
</tr>
<tr>
<td>Glu272O&lt;sub&gt;ε1&lt;/sub&gt;-Wat2H&lt;sub&gt;ω1&lt;/sub&gt;</td>
<td>1.67</td>
<td>1.66</td>
</tr>
<tr>
<td>CclO&lt;sub&gt;c&lt;/sub&gt;-Ser64N</td>
<td>2.93</td>
<td>2.85</td>
</tr>
<tr>
<td>CclO&lt;sub&gt;c&lt;/sub&gt;-Ala318N</td>
<td>2.82</td>
<td>3.06</td>
</tr>
</tbody>
</table>
Table 2. Important inter-atomic distances between Tyr150 and two lysine residues (Lys67 and Lys315) in the potential energy minima structures obtained by IRC calculations. Cited from ref. 59 with some modification.

<table>
<thead>
<tr>
<th>Inter-atomic distance (Å)</th>
<th>S1</th>
<th>TS1</th>
<th>TI1</th>
<th>TI2</th>
<th>TS2</th>
<th>S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr150Oη-Lys67Nζ</td>
<td>2.77</td>
<td>2.90</td>
<td>2.96</td>
<td>3.18</td>
<td>3.20</td>
<td>3.55</td>
</tr>
<tr>
<td>Tyr150Oη-Lys67Hζ1</td>
<td>1.74</td>
<td>1.90</td>
<td>1.96</td>
<td>2.62</td>
<td>2.63</td>
<td>3.31</td>
</tr>
<tr>
<td>Tyr150Oη-Lys315Nζ</td>
<td>2.82</td>
<td>3.17</td>
<td>3.23</td>
<td>2.99</td>
<td>2.95</td>
<td>2.84</td>
</tr>
<tr>
<td>Tyr150Oη-Lys315Hζ2</td>
<td>1.80</td>
<td>2.18</td>
<td>2.24</td>
<td>1.99</td>
<td>1.96</td>
<td>1.85</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
Fig. 3
Fig. 4

Potential Energy (kcal/mol)

TSB1
TSB2
B2
26.76
0.17
2.44
9.07
B3

B1

Reaction Coordinate (amu\(^{1/2}\) Å)

Fig. 4
Fig. 5
Fig. 6
Fig. 8
Fig. 10
Fig. 13
Fig. 14