Catalytic Mechanism of Class A β-Lactamase. I. The Role of Glu166 and Ser130 in the Deacylation Reaction

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The tetrahedral intermediate formation process, which is the first step in the deacylation reaction by class A β-lactamase, was investigated by the *ab initio* molecular orbital method. In this study, benzyl penicillin was used as the substrate. From the results of our molecular dynamics study of the structure of β-lactam antibiotics–β-lactamase complex, the substrate, Ser70, Lys73, Ser130, Glu166 and a water molecule for the deacylation reaction were considered for construction of a model for calculation. The calculation results indicated that Glu166 plays a role in holding a water molecule, which is necessary for the deacylation reaction, and that the hydrogen bond network among Lys73Nζ, Ser130Oγ, and the carboxyl group of the β-lactam antibiotics was formed by the uptake of β-lactam antibiotics by β-lactamase. The activation energy for this reaction was 33.3 kcal/mol, and it is very likely that the reaction occurred at body temperature. Subsequent calculation results obtained by using the model excluding Ser130 and the carboxyl group of the substrate indicated that the activation energy for this reaction was 40.8 kcal/mol, which is 7.5 kcal/mol higher than that of the previous reaction. It was found that the hydrogen bond network plays an important role in decreasing the activation energy for the tetrahedral intermediate formation reaction. Lys73Nζ, which is located at the edge of the hydrogen bond network, played a role in forming a hydrogen bond with Glu166Oε in order to help the deacylation reaction. The role of amino acid residues around the active site of class A β-lactamase was also discussed.

Key words β-lactamase; tetrahedral intermediate; *ab initio* molecular orbital method; hydrogen bond network; Lys73; deacylation reaction

β-Lactamases are produced by pathogenic bacteria and contribute to a resistance to β-lactam antibiotics by deteriorating the pharmacological effect of catalyzing the hydrolysis of the β-lactam ring.[1] β-Lactam antibiotics react with β-lactamase in the same way as penicillin-binding protein (PBP); *i.e.*, an acylation reaction is initiated by the opening reaction of the β-lactam ring. In the case of β-lactamase, however, the deacylation reaction occurs after the acylation reaction and its activity is recovered, which differs from PBP. We investigated the deacylation reaction in order to elucidate the catalytic mechanism of class A β-lactamase by quantum chemical calculations.

Since the report by Knox *et al*. suggested that the E166A mutant of β-lactamase from *Bacillus licheniformis* induces the accumulation of an acyl-enzyme intermediate,[2] 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) study on the structure of β-lactam antibiotics–β-lactamase complex (Fig. 1). It is thought 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). Furthermore, it is thought that a hydrogen bond network among Lys73Nζ, Ser130Oγ and the carboxyl group of the substrate (β-lactam antibiotics) should be taken into account. Ishiguro *et al*. also discussed the importance of the hydrogen bond network based on the results of their molecular mechanics calculations.[3] In the present study, we investigated the tetrahedral intermediate formation process, which is the first step of the deacylation reaction by β-lactamase and is caused by the interaction between a water molecule and β-lactam antibiotics (benzyl penicillin)–β-lactamase complex, by means of the *ab initio* molecular orbital (MO) method, and we clarified the activation energy using the structure and potential energy changes for the reaction and the function of the hydrogen bond network binding by Glu166Oε–Lys73Nζ–Ser70Oγ–Ser130Oγ–the carboxyl group of benzyl penicillin.

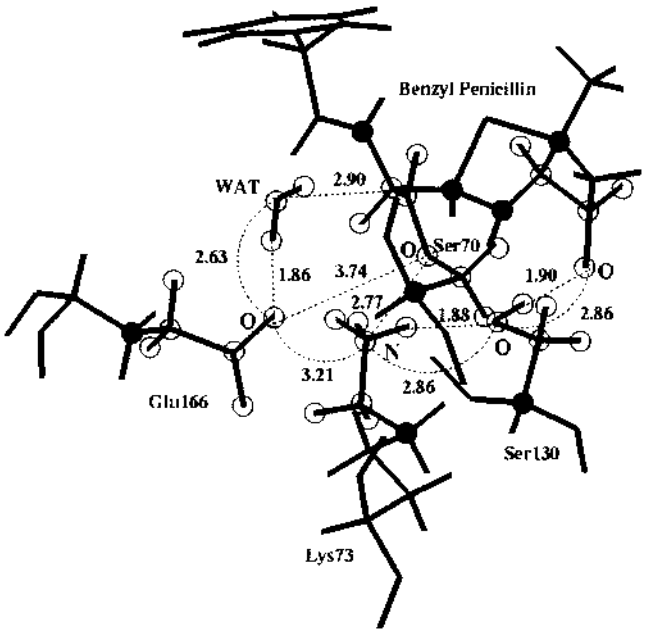

Fig. 1. Active Site of Benzyl Penicillin-Bound Acyl-Enzyme Intermediate Obtained by MD Calculation

Important residues for the deacylation reaction by β-lactamase are extracted and are shown in this figure. Numerals are distance between each atom in Å. WAT is a water molecule for the deacylation reaction by class A β-lactamase.

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Method

Construction of the Model for Calculations In order to obtain a model compound for quantum chemical calculations, the MD simulation was performed using the full structure of clavulanic acid-bound β-lactamase from *Staphylococcus aureus* PC1[4] registered in the Protein Data Bank (PDB).[5—7] Energy minimization and MD calculation were performed with the program package AMBER, Version 4.1.[8] The united-atom force field[9] was applied except for a substrate (clavulanic acid and benzyl penicillin), Ser70, Lys73, Ser130, Asn132, Glu166, Lys234 and Gln237, for which the all-atom force field[10] was used. The solvent was the TIP3P water model,[11] and a rectangular parallelepiped of about 4000 water molecules was generated by the Monte Carlo method.[8] The size is 67.4×54.7×47.4 Å. The center of the mass corresponded to that of the solute. To simplify this calculation, the SHAKE procedure[12] was used. The calculations of the non-bonded term were accelerated with a hardware accelerator, called MD-engine.[13] The MD simulation was performed starting from the energy minimized structure. The temperature was gradually increased by heating up to 310 K for the first 20 ps, then it was kept at 310 K for the next 100 ps. The trajectory at that temperature (310 K for 100 ps) was considered to be the most probable structure under physiologic conditions, and the average structure in the 100 ps MD simulation was obtained.

Since the β-lactam ring and the adjacent five-membered ring of clavulanic acid are cleaved in this data, the inhibitor was regenerated by closing the two rings, and a balanced structure was obtained by means of MD calculation for 100 ps at 310 K. A benzyl penicillin–β-lactamase complex as an acyl-enzyme intermediate was obtained by superimposing the β-lactam ring of benzyl penicillin on that of clavulanic acid in the above-mentioned complex. The complex was fully stabilized under an aqueous condition by MD calculation for 100 ps at 310 K, and the change in the distance of the hydrogen bond (below 3 Å) is not more than 0.1 Å.[14] The active site of the complex is shown in Fig. 1. The model for calculation was constructed by extracting the atoms represented by open and closed circles of benzyl penicillin, Ser70, Lys73, Glu166 and a water molecule (WAT) from the active site of the structure, all of which were considered to be involved in the reaction, and by replacing the atoms represented by closed circles with hydrogen atoms. In actual class A β-lactamase, the movement of residues is restricted by other residues around them. Movement of the carbonyl oxygen of benzyl penicillin binding with the enzyme is also restricted by the oxy-anion hole formed by two main-chain nitrogen atoms of Ser70 and Gln237.[15] To illustrate this fact in the constructed model, some of the atoms of the model were fixed to their initial positions in performing quantum chemical calculations. The fixed atoms are shown by asterisks in Figs. 4(a) and 7(A).

Quantum Chemical Calculation The Schrödinger equations of the model reaction system were solved by the *ab initio* MO method at the Hartree–Fock (HF) level using the 6-31G** basis set. The transition-state (TS) structure in the tetrahedral intermediate formation process of the deacylation reaction by class A β-lactamase was determined by geometry optimization using the energy gradient method, and it was confirmed by normal vibrational analysis that only one normal vibrational mode with imaginary frequency existed. The steepest path on the potential energy hypersurface from the TS structure in the forward and reverse directions of this normal vibrational mode was solved because the direction of the normal vibrational mode matches that of the reaction coordinate. The minimum structures on the potential energy hypersurface, which are located at the last point of the steepest path, are the initial and final states of the reaction, which were obtained theoretically. The computational program used was Gaussian 94.[16]

Results

The potential energy change following the steepest reaction path of the tetrahedral intermediate formation reaction in the deacylation reaction by class A β-lactamase and the structures that appeared on the reaction path are shown in Figs. 2 to 4, respectively.

TS Structure Starting from the model reaction system shown in Fig. 1, TS structure (c) was determined by geometry optimization. It was confirmed by normal vibrational analysis that only one normal vibrational mode with imaginary frequency existed. This is shown by arrows in Fig. 3. In the largest vibration, OH^- from H_2O approached the carbonyl carbon of β-lactam antibiotics binding with Ser70Oγ (Cs), and the proton from H_2O approached Glu166 in the second largest vibration.

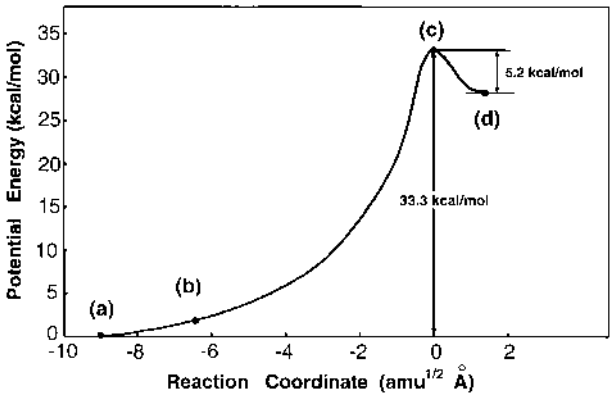

Fig. 2. The Lowest Potential Energy Change Following the Tetrahedral Intermediate Formation Reaction in the Deacylation Step by Class A β-Lactamase

Horizontal and vertical axes represent the reaction coordinates along the steepest path (amu$^{1/2}$ Å) and the potential energy change (kcal/mol), respectively.

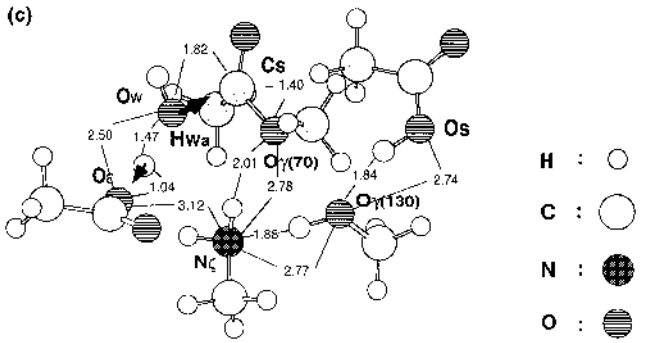

Fig. 3. TS Structure (c) of the Tetrahedral Intermediate Formation Reaction in the Deacylation Step by Class A β-Lactamase

Arrows represent the only normal vibrational mode with an imaginary frequency obtained by normal vibrational analysis.

ES Complex Structure A minimum structure (a) on the potential energy hypersurface was obtained by calculating the steepest descent path along the reverse direction to the only normal vibrational mode with the imaginary frequency of the TS structure (c). Structure (a), which was obtained theoretically, was almost the same as that shown in Fig. 1, which was obtained experimentally, except for the positions of protons in the hydrogen bond network Lys73Nζ–Ser130Oγ–the carboxyl group of the substrate. In structure (a), a water molecule involved in the deacylation reaction formed a hydrogen bond with Glu166Oε (2.72 Å) and interacted with the carbonyl carbon atom of the substrate binding with Ser70Oγ (3.03 Å); *i.e.*, Glu166Oε holds a water molecule in an ES complex. Glu166Oε interacted with Lys73Nζ (3.18 Å), and Lys73Nζ formed hydrogen bonds with Ser70Oγ (2.77 Å) and Ser130Oγ (2.81 Å). Ser130Oγ formed a hydrogen bond with the carboxyl oxygen of the substrate (2.73 Å). These results indicated that a hydrogen bond network was constructed in the ES complex.

Tetrahedral Intermediate Structure Structure (d) was obtained by calculating the steepest descent path along the only normal vibrational mode with the imaginary frequency of the TS structure (c). This structure (d) is the tetrahedral intermediate for the deacylation reaction by class A β-lacta-

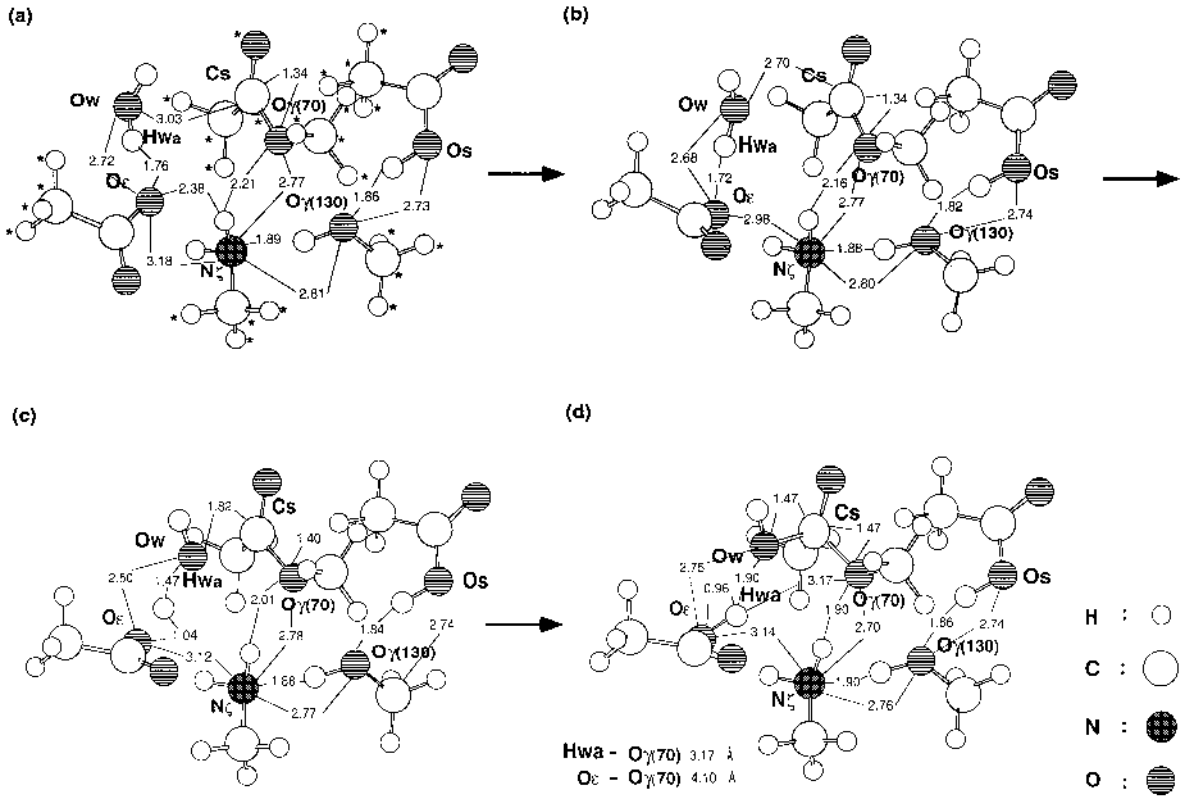

Fig. 4. Structural Changes Following the Tetrahedral Intermediate Formation Reaction in the Deacylation Step by Class A β-Lactamase
(a)—(d) correspond to that shown in Fig. 2

mase. The oxygen atom of the water molecule held by Glu166Oε in the ES complex (a) formed a covalent bond with the carbonyl carbon of the β-lactam ring bound with Ser70Oγ (1.47 Å) and a hydrogen bond with Glu166Oε (2.75 Å). The hydrogen bond chain Lys73Nζ–Ser130Oγ–the carboxyl oxygen atom of the substrate, was always maintained in the tetrahedral intermediate formation process.

Water Molecule-Holding Mechanism in the Deacylation Reaction by β-Lactamase In Fig. 4(a), proton migrations from Lys73Nζ to Ser130Oγ and from Ser130Oγ to the carboxyl oxygen of β-lactam antibiotics occurred. This change will be described in Discussion. When the water molecule held by Glu166Oε approached the carbonyl carbon of the β-lactam ring bound with Ser70Oγ, the structure shown in Fig. 3(b) appeared due to rotation of the water molecule and the carboxyl group of Glu166. The distance between the oxygen atom of the water molecule and the carbonyl carbon of the β-lactam ring was 2.70 Å. It should be noted that a hydrogen bond is formed between Glu166Oε and Lys73Nζ in structure (b). This hydrogen bond plays a role in stabilizing the water molecule which is held by Glu166 and is used for the deacylation reaction. When the water molecule approaches the carbonyl carbon of the β-lactam ring bound with Ser70Oγ, it begins to decompose into a proton and OH^-. Hwa, a proton in the water molecule, migrates to Glu166Oε, while OH^- approaches the carbonyl carbon of the substrate, thereby leading to the formation of a TS structure (c). The distance between the proton Hwa and Glu166Oε is 1.04 Å, which is almost equal to the bond length of the O–H bond, and the distance between Ow of OH^- and the carbonyl carbon Cs of the β-lactam ring of the substrate is 1.82 Å. The distance between Cs and Ser70Oγ increases gradually from 1.34 Å in structure (a) and becomes 1.40 Å in structure (c). It was clearly indicated in the vibrational mode shown in structure (c) (Fig. 3), that the proton from the water molecule which was involved in the deacylation reaction by class A β-lactamase connects with Glu166Oε, and OH^- connects with the acyl carbon of the β-lactam ring of the substrate. Figure 2 shows that, starting from the ES complex structure (a), the reaction intermediate (d) was formed *via* structure (b) and the TS structure (c); *i.e.*, (a) was connected with (d) by the steepest path. It was found from the potential energy change shown in Fig. 2, that the activation energy for the tetrahedral intermediate formation reaction was 33.3 kcal/mol. It is very likely that this reaction occurs at body temperature. The role of Glu166 is to hold a water molecule for the deacylation reaction by class A β-lactamase in the reaction system. It is considered that an acyl intermediate accumulates in the Glu166Ala mutant of β-lactamase,[2] because the mutant loses this function.

Discussion

Reaction mechanism There have been many studies on mechanism of substrate inactivation by β-lactamase. Strynadka *et al*. reported from the result of their X-ray crystallographic analysis of RTEM-1 β-lactamase, that Lys73Nζ is neutral because the pK_a of Lys73 decreases due to a positive environment that is produced by residues around Lys73, that Lys73 only takes part in the acylation of the OH group of Ser70, and that only Glu166 takes part in the deacylation reaction of β-lactamase.[17] On the other hand, Damblon *et al*. showed from their NMR experiments that only Glu166, not

Lys73, takes part in the acylation reaction of Ser70Oγ and the deacylation reaction, because the pK_a of Lys73 is about 10 where protonated Lys73Nζ is stable.[18] Knox *et al*. reported that only Glu166 takes part in the deacylation reaction of β-lactamase on the basis of their experimental results which showed an accumulation of acyl intermediate in the Glu166Ala mutant of β-lactamase from *Bacillus licheniformis*.[2] Using the *ab initio* MO method, we investigated the tetrahedral intermediate formation reaction, which is the first step of the deacylation reaction of the substrate-bound class A β-lactamase, in the β-lactam antibiotics inactivation mechanism by β-lactamase, and found that Glu166 plays a role in holding the water molecule used for hydrolysis. As the above-mentioned scholars report, if only Glu166 takes part in the deacylation reaction, then Hwa from the water molecule, which connects with Glu166, must migrate to Ser70Oγ (Fig. 4(d)). However, it is reasonable to assume that the proton migration occurs *via* a Glu166Oε–Lys73Nζ–Ser70Oγ hydrogen bond network rather than from Glu166, because the distance between Glu166Oε and Ser70Oγ is too far for proton migration to occur (4.10 Å, Fig. 4(d)). It should be considered that not only Glu166Oε but also Lys73Nζ take part in the deacylation reaction. Lys73Nζ, however, is neutral in structure (d) and it is necessary for proton migration from Lys73Nζ to Ser70Oγ to provide a proton from Glu166Oε or Ser130Oγ. It is thought that the proton might be provided from Ser130Oγ because a lone pair of Lys73Nζ is oriented toward Ser130Oγ. The mechanism is now being investigated.

Importance of the Carboxyl Group of the β-Lactam Antibiotics-Ser130-Lys73 Hydrogen Bond Network In

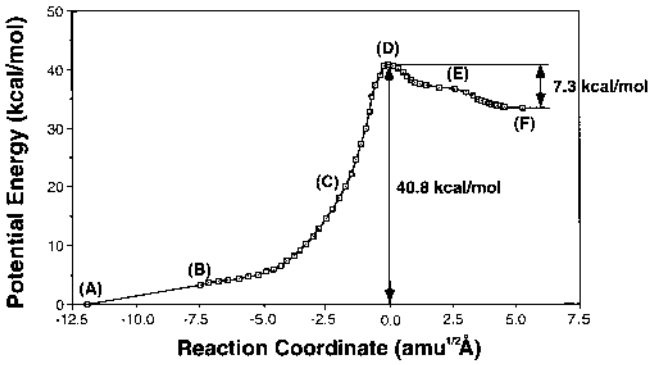

Fig. 5. The Lowest Potential Energy Change Following the Tetrahedral Intermediate Formation Reaction in the Deacylation Step by Class A β-Lactamase, Obtained by Using the Model Excluding Ser130 and a Carboxyl Group of the Substrate from that Shown in Fig. 4

Horizontal and vertical axes represent the reaction coordinate along the steepest path (amu$^{1/2}$ Å) and the potential energy change (kcal/mol), respectively.

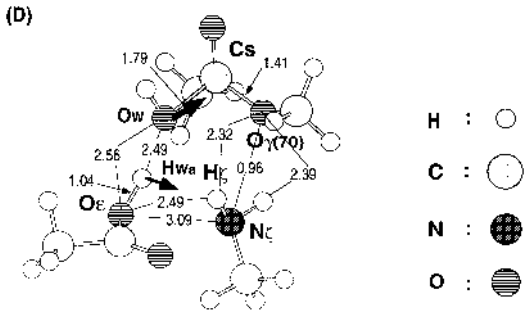

Fig. 6. TS Structure (D) of the Tetrahedral Intermediate Formation Reaction in the Deacylation Step by Class A β-Lactamase, Obtained by Using the Model Excluding Ser130 and a Carboxyl Group of Substrate from That Shown in Fig. 4

Arrows represent the only normal vibrational mode with an imaginary frequency obtained by normal vibrational analysis.

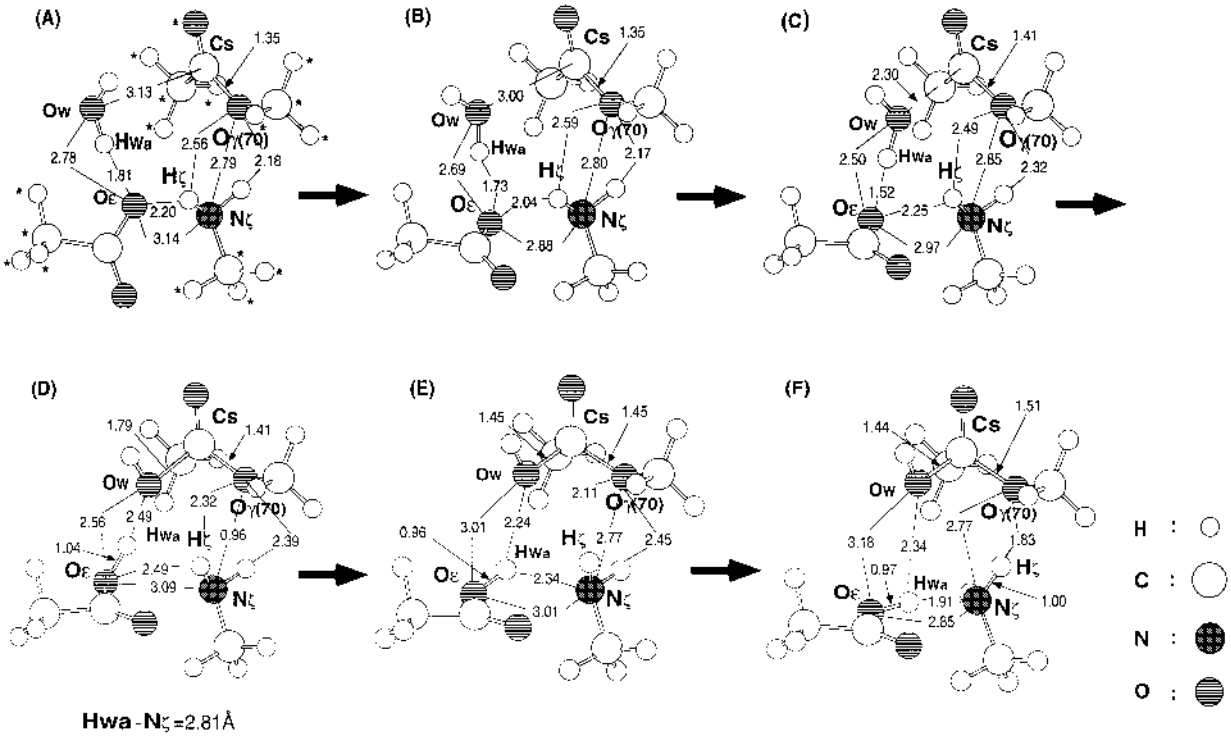

Fig. 7. Structural Changes Following the Tetrahedral Intermediate Formation Reaction in the Deacylation Step by Class A β-Lactamase, Obtained by Using the Model Excluding Ser130 and a Carboxyl Group of Substrate from That Shown in Fig. 4

(A)—(F) correspond to that shown in Fig. 5.

the previous section, it was stated that a hydrogen bond network is produced between Lys73, Ser130 and the carboxyl group of the β-lactam antibiotics, and that the proton relay between the hydrogen bond network is involved in the activity of class A β-lactamase. When the substrate, benzyl penicillin, is caught by β-lactamase, its carboxyl group is incorporated in the hydrogen bond network. The protonated Lys73Nζ is neutralized and is activated by the proton relay *via* Ser130Oγ. The mechanism in which the hydrogen bond network works effectively is shown in Fig. 4. The mechanism shown in this figure is reasonable because the potential energy of the structure, which assumes that the tetrahedral intermediate is produced without proton relay from Lys73Nζ to the carboxyl group of the substrate *via* Ser130Oγ, is 23.5 kcal/mol higher than that of structure (d).

Ser130 and the carboxyl group of the substrate were excluded from the model reaction system to estimate the effect of the Lys73–Ser130–substrate hydrogen bond network. The potential energy change and the structural changes obtained by quantum chemical calculations are shown in Figs. 5 to 7, respectively. A hydrogen atom of protonated Lys73Nζ, which forms a hydrogen bond with Ser130Oγ, was removed from this model structure because Ser130 was not included in the model structure. In Fig. 7, structural changes from an initial structure (A) to a TS structure (D) did not differ greatly from that in Fig. 4. However, it is observed in the only normal vibrational mode with an imaginary frequency of the TS structure (D) that proton Hwa from the water molecule involved in the deacylation reaction is oriented toward Lys73Nζ (Fig. 6). Moreover, after passing the TS structure (D), a point of inflection appeared in the potential energy change shown in Fig. 5, which did not appear in that shown in Fig. 4. The structure is shown in Fig. 7(E). Structure (E) is a point of inflection that appeared as a result of stabilization in which the proton Hwa from the water molecule changes its moving direction naturally in order to break the hydrogen bond with

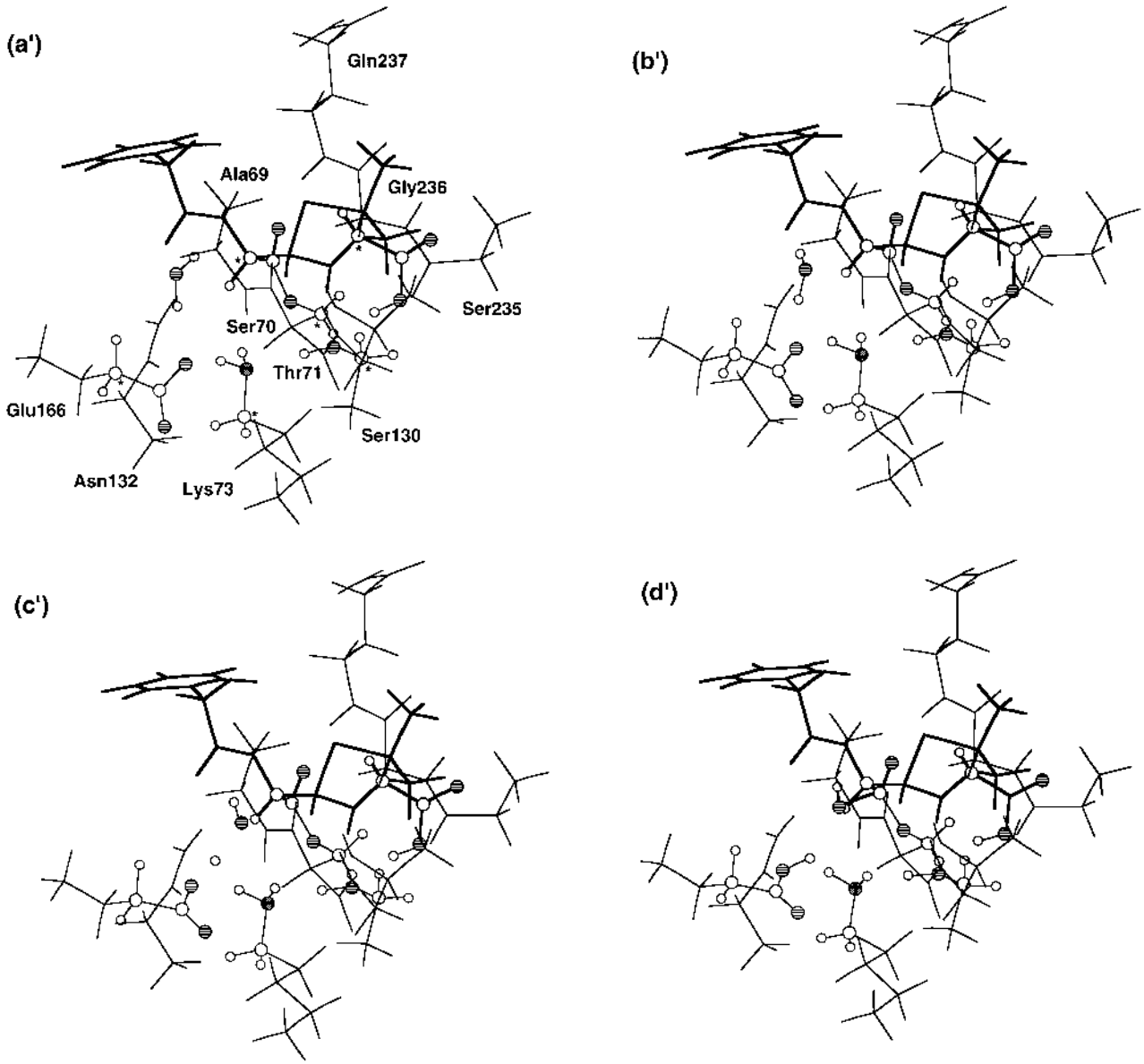

Fig. 8. Structures of Expanded Models

Atoms which are also used for the elucidation of a tetrahedral intermediate formation reaction mechanism (Results) are shown by circles. In Results, atoms marked by asterisks in these figures were always fixed. Residue shown by a thick line is benzyl penicillin.

the Ow oxygen atom of the water molecule and to form a hydrogen bond with Lys73Nζ. From structure (E), the exchange of the hydrogen bond occurred and a stable tetrahedral intermediate (F) was produced; *i.e.*, in the tetrahedral intermediate structure (F), Hζ, which connects with Lys73Nζ, forms a hydrogen bond only with Ser70Oγ (1.83 Å), whereas in the TS structure (D), it interacts with Glu166Oε and Ser70Oγ, whose distances are 2.49 and 2.32 Å, respectively. It is thought that an exclusion of Ser130 and the carboxyl group of the β-lactam antibiotics causes these structural changes. Due to this exclusion, the activation energy of this reaction is 40.8 kcal/mol, which is 7.5 kcal/mol higher than that obtained in Results. For this reason, it is concluded that the hydrogen bond network consisting of Lys73, Ser130 and the carboxyl group of the β-lactam antibiotics plays an important role in remarkably decreasing the activation energy of the tetrahedral intermediate formation reaction, which is the first step of the deacylation reaction, by a proton relay among this network.

Role of Amino Acid Residues around the Active Site of Class A β-Lactamase It is considered that amino acid residues and solvent molecules, which are located around the active site, also affect enzymatic reaction mechanisms and the potential energy change. Though it is quantitatively difficult to take all these factors into computation, we constructed a larger model than that shown in Fig. 4, based on the structure shown in Fig. 1. The model is called expanded model. The expanded model includes four active site residues (Ser70, Lys73, Ser130, Glu166), six residues near the active-site (Ala69, Thr71, Asn132, Ser235, Gly236, Gln237) and the full structure of the benzyl penicilloyl moiety. The water molecule which is involved in the expanded model as a solvent molecule is only WAT, as shown in Fig. 1, because the nearest water molecule from WAT did not interact with the active site, including WAT.

From this structure, the positions of the atoms included in both the expanded model and the model reaction system in Fig. 4 were changed into those of structure (a) to (d) in Fig. 4. (Because atoms marked by asterisks in Fig. 4(a) were fixed through investigation on the deacylation reaction mechanism of class A β-lactamase, the positions of each atom are equal to those shown by asterisks in Fig. 8.) The structures are shown in Fig. 8.

An expanded model for an acyl-enzyme intermediate was also constructed. First, an acyl-enzyme intermediate (structure (Int.)) was optimized by the same method as described in Method. The geometry was almost the same as that shown in Fig. 1. Second, the optimized data was put through the same process, converting (a′) to (d′). The structure (structure (Int.′)) is shown in Fig. 9. For these expanded models, only potential energy calculations were performed at the HF level using a 6-31G** basis set because of computational limits.

The result is shown in Fig. 10. It should be noted that structure (Int′) is more stable than structure (a′), although the potential energy of structure (Int.) is 6.4 kcal/mol higher than that of structure (a). This result suggests that the amino acid residues around the active site of class A β-lactamase might play an important role in the stabilization of protonated Lys73 and the carboxyl group of the substrate in acyl-enzyme intermediate. It is predicted that structure (Int′) links to structure (a′) by a proton relay from Lys73Nζ to the carboxyl group of the substrate *via* Ser130Oγ, on the potential energy hypersurface. The mechanism is now being investigated.

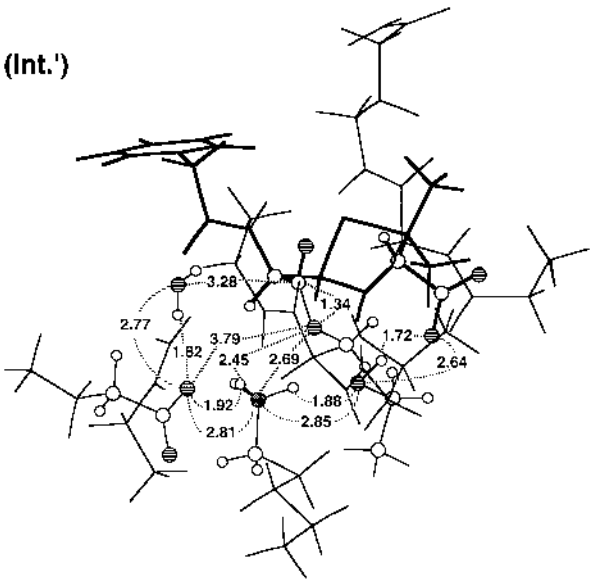

Fig. 9. Structure of Expanded Model (Int′.)

Atoms shown by circles are used for geometry optimization on the structure (Int.) (see text). Residue shown by a thick line is benzyl penicillin. Numerals are inter-atomic distances (Å) obtained by geometry optimization.

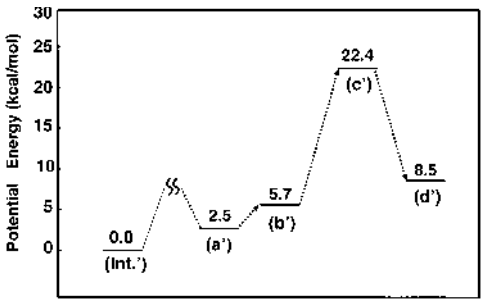

Fig. 10. Potential Energy Diagram on Structures (Int′.) and (a′) to (d′), Which Were Obtained by Potential Energy Calculations at the HF/6-31G** Level

The activation energy for tetrahedral intermediate formation process is 19.9 kcal/mol ((a′)→(c′)). The value is smaller than that shown in Fig. 2 (33.3 kcal/mol). Furthermore, the stabilization energy from the TS structure (c′) to the tetrahedral intermediate (d′) is 13.9 kcal/mol. The value is larger than that shown in Fig. 2 (5.2 kcal/mol, (c)→(d)). It is thought that the amino acid residues around the active-site of class A β-lactamase might also play roles in decreasing the activation energy for tetrahedral intermediate formation reaction and in stabilizing the tetrahedral intermediate.

Conclusions

The conclusions obtained in this investigation are as follows. 1. Glu166 plays a role in holding a water molecule, which is necessary for the deacylation reaction by class A β-lactamase. For this reason, an acyl intermediate accumulates in the Glu166Ala mutant. 2. The hydrogen bond network consisting of Lys73Nζ, Ser130Oγ and the carboxyl group of the β-lactam antibiotics is formed by the uptake of β-lactam antibiotics by β-lactamase, and plays an important role in de-

creasing the activation energy for the tetrahedral intermediate formation reaction, which is the first step of the deacylation reaction, by the proton relay among this network. 3. Lys73Nζ, which is located at the edge of the hydrogen network, forms a hydrogen bond with Glu166Oε in order to help the deacylation reaction by the water molecule held by Glu166. 4. Amino acid residues around the active site of class A β-lactamase might play the roles of stabilizing protonated Lys73 and the carboxyl group of the substrate in the acyl-enzyme intermediate, in stabilizing the tetrahedral intermediate, and in decreasing the activation energy for tetrahedral intermediate formation reaction.

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