Modeling Study on a Hydrolytic Mechanism of Class A β-Lactamases

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Comparison of the hydrogen-bond networks at the active site in the crystallographic structures reported for class A β-lactamases revealed an importance of a switch of the hydrogen-bond network for the catalytic process. Taking account of the conformational mobility of the Lys73 residue, we have constructed putative complex models for β-lactam antibiotics and the enzymes in the multistep hydrolysis which consists of a Michaelis complex, an acyl−enzyme, and a tetrahedral oxyanion for deacylation. In the acylation, the C_3 carboxylate of penicillin derivatives would participate in activation of the Ser130 hydroxyl group and then the oxyanion of the Ser130 residue would deprotonate the ammonium group of the Lys73 residue which will act as a general base for activation of the Ser70 residue. In the deacylation, the deacylating water molecule would be accommodated during a conformational change of the acyl moiety without a structural change of the active-site residues and the unprotonated N_4 atom of the penicillins would act as a general base to activate the water molecule. This catalytic process provided a new account for the stability of the acyl−enzyme complexes. This substrate-assisted mechanism would also be extended to a hydrolytic mechanism of class C enzymes.

Introduction

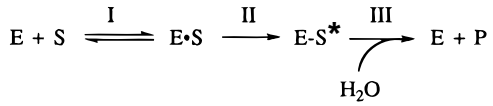
β-Lactamases, which are classified in four main classes, A−D, on the basis of their primary structures, play a major role in bacterial resistance to β-lactam antibiotics. Serine β-lactamases of class A, C, and D hydrolyze the β-lactam antibiotics by an acylation−deacylation mechanism. Among those, class A β-lactamases are the most commonly encountered and best characterized enzymes. Understanding of the catalytic mechanism of the enzymes of this class is crucial for the design of β-lactams having an improved activity against β-lactamase-producing bacteria in view of an increasing clinical importance of β-lactam-resistant strains, thus being a major medicinal interest in antibiotics field.

Class A β-lactamases react with β-lactam antibiotics under a three-step pathway: Michaelis complex formation (I), acyl−enzyme formation (II), and deacylation of the acyl−enzyme (III) as shown in Scheme 1, where E is the enzyme, S the β-lactam, E·S the Michaelis complex, E−S* the acyl−enzyme, and P the product of hydrolysis.

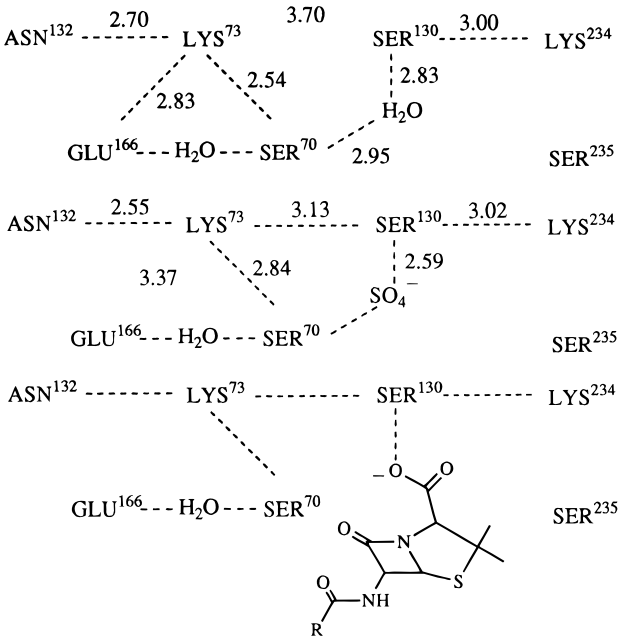
Sequence alignment of class A β-lactamases revealed highly conserved motifs, consisting of three polar regions: the tetrad Ser70-Xaa-Xaa-Lys73, the triad Ser130-Asp131-Asn132, and the triad, Lys234-Thr(Ser)235-Gly236 (Scheme 2).[1] Recent X-ray crystallographic studies of different β-lactamases of class A have shown that those polar residues commonly consist of the active site of the enzymes.[2,3] Besides these motifs, Glu166 has been proposed to be an important residue in the catalysis.[4]

Site-directed mutagenesis has provided further evidence for the importance of those residues in the catalysis. Mutation of Lys73 to Arg (K73R) caused about a 100-fold decrease of a rate of acylation by a penicillin derivative.[5] In S130A and S130G mutants, the enzyme activity decreased about 40-fold and 5-fold, respectively.[6] Participation of a water molecule as a substitute for the hydroxyl group of Ser130 has been suggested for the latter mutant through molecular modeling.[7] A similar role for a water molecule has been

Scheme 1



Scheme 2. Schematic Presentation of Hydrogen-Bond Networks of β-Lactamases Observed by X-ray Crystallography[a]



[a] Top, *S. aureus* PC1; middle, *B. licheniformis* 749/C; bottom, a putative Michaelis complex. Major interatomic distances (Å) between amino acid residues are given.

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Table 1. Selected Atomic Distances (Å) within the Active Site of the Enzymes[a]

atom of amino acid				4BLMA[b]	4BLMB[b]	1MBLA[b]	1MBLB[b]	3BLM	1BLC	1BLP[c]	1BTL	$RTEM_a$	$RTEM_b$
		K^{73}	N^{ζ}	2.84	2.52	2.76	2.79	2.54	3.43 (2.94)[d]	3.26	2.90	2.7	3.3
S^{70}	O^{γ}	S^{130}	O^{γ}	3.32	3.09	3.19	3.35	3.28	3.24 (2.85)[d]	3.36	3.54		
		E^{166}	$O^{\epsilon 1}$	3.58	3.52	–	–	3.97	6.02 (5.72)[d]	–	4.19		
		S^{130}	O^{γ}	3.13	3.24	3.15	2.97	3.70	3.03	3.25	4.17	3.7	2.8
K^{73}	N^{ζ}	S^{130}	O	3.23	3.36	3.55	2.99	3.75	3.40	3.38	3.28	3.1	*e*
		N^{132}	O^{δ}	2.55	2.57	2.71	2.73	2.70	2.70	3.07	2.97	2.9	*e*
		E^{166}	$O^{\epsilon 1}$	3.37	3.07	–	–	2.83	3.84	–	3.42	3.4	
		E^{166}	$O^{\epsilon 2}$	4.79	4.52	–	–	4.25	3.98	–	5.01		
S^{130}	O^{γ}	K^{234}	N^{ζ}	3.02	2.80	3.20	2.96	3.00	3.40	3.79	2.90	2.8	2.8
E^{166}	$O^{\epsilon 1}$	N^{132}	O^{δ}	3.35	3.41	–	–	3.20	4.09	–	3.20		
E^{166}	$O^{\epsilon 2}$	N^{170}	N^{δ}	2.86	3.04	–	–	2.94	3.16	–	2.97	3.1	
K^{234}	N^{ζ}	$T(S)^{235}$	O^{γ}	3.62	3.65	3.62	3.67	4.67	4.57	4.50	4.50		
		$T(S)^{235}$	O	2.82	2.83	2.65	2.84	3.17	2.64	2.81	3.02	3.1	*e*

[a] 4BLM: *B. licheniformis* 749/C, Knox *et al.* (1991). 1MBL: *B. licheniformis* 749/C (mutant E166A), Knox *et al.* (1993). 3BLM: *S. aureus* PC1, Herzberg (1991). 1BLC: *S. aureus* PC1 (with degradation products of clavulanate), Chen *et al.* (1992). 1BLP: *S. aureus* PC1 P54 mutant ($D^{179}N$), Herzberg *et al.* (1991). 1BTL: *E. coli* TEM1, Jelsch et al. (1993). $RTEM_a$: *E. coli* RTEM-1, Strynadka *et al.* (1992). $RTEM_b$: *E. coli* RTEM-1 (mutant $E^{166}N$), Strynadka *et al.* (1992). [b] The asymmetric unit contains two molecules. [c] There are no coordinate data between R164 and N170. [d] Two conformations are observed for clavulanate. [e] Hydrogen bond has been reported without distance value.

found in a recent crystallographic structure of the S235A mutant of TEM1 β-lactamase.[8] The Glu166 residue mainly participates in deacylation as exemplified by E166N,[9] E166A,[10] and $E166Y$[11] mutants. Since the corresponding acidic residue has not been found in class C enzymes,[12,13] Glu166 should be a specific residue for class A enzymes. Kinetic experiments for a K234E mutant and the wild-type enzyme have shown that Lys234 is involved in both acylation and deacylation in the hydrolysis of penicillins.[14] Although an interaction of the C_3 carboxylate of penicillins with Lys234 is important for the catalysis, changing the carboxylate to a hydroxymethyl group led to little difference in kinetic properties with the K234E or K234A enzyme, although the analog is a poor substrate for the native enzymes. Kinetic studies on the K234R mutant indicated a considerable importance of the basic residue for inhibition by clavulanic acid as well as for the hydrolysis of penicillins and cephalosporins.[15] Results of mutation experiment on class A β-lactamases has been reviewed by Matagne and Frère.[16]

Crystal structures of the Gram-positive β-lactamases from *Streptmyces albus* G,[17] *Staphylococcus aureus* PC1,[2] and from *Bacillus licheniformis* 749/C[3] are known at 1.7−2.0 Å resolution. Recently, the 1.7 and 1.8 Å resolution crystal structures of the Gram-negative β-lactamase from *Escherichia coli* have been published.[18,19] Molecular structures of the acyl−enzyme of the E166N mutant of TEM1 at 1.7 Å resolution[18] and the acyl−enzyme of *S. aureus* PC1 by clavulanate at 2.2 Å resolution[20] have been reported. More recently, Chen *et al.*[21] have reported a structure of the phosphonylated enzyme of *S. aureus* PC1 at 2.3 Å resolution. Also reported were two other mutant structures, E166A[22] and D179N.[23] Those wild and mutant structures show a quite similar structure at the active site, except for the D179N mutant. However, those structures have different hydrogen-bond networks for the residues involved in the catalysis as summarized in the Table 1. The low values (less than 10 $Å^2$) of the temperature factors for the residues in the active site indicate more rigid structures and accuracy for the atomic distances of the residues higher than those of other residues at the protein surface.[2] The atomic distances observed at 1.7−2.0 Å resolution will be significant for evaluation of the hydrogen-bond networks in the active site.

Ser70 has a tight hydrogen bond with Lys73 in the all free enzymes, but not in the acyl−enzymes. The Lys73 residue formed a strong hydrogen bond with the Glu166 residue in the crystal structure of the *S. aureus* PC1 enzyme and a weak hydrogen bond with the Glu166 residue in one of the two crystal isoforms from *B. licheniformis* 749/C enzyme. The Lys73 residue also hydrogen bonded to the Asn132 residue in the all enzymes. Of those residues listed in the Table 1, the Lys234 residue is rigidly maintained at the active site. The hydrophobic part of the side chain was buried in the protein, and the ammonium group tethered a structurally conserved water molecule (Wat101 according to the numbering scheme in 3BLM provided in Brookhaven Protein Data Bank).[3] The Glu166 residue made hydrogen bonds with the Asn170 residue and two water molecules (Wat81 and Wat62). The Lys73 residue would be mobile, switching the hydrogen-bond network depending upon the electrostatic nature of ligands such as water molecule, sulfate ion, or the carboxylate of β-lactam antibiotics. The conformational mobility of the Lys73 residue suggests an important role in acylation or deacylation varying the hydrogen-bond network.

It has been controversial whether $Glu166^{24}$ or unprotonated $Lys73^{16}$ is a general base in the acylation. Taking account that penicillins acylate $E166N$[9] and $E166A$[10] mutants, it is more likely for Lys73 to be the general base for activation of the Ser70 residue. However, the presence of the unprotonated Lys73 residue is dubious in the uncomplexed native enzyme under physiological conditions.[25] Therefore, a more reasonable mechanism for the activation is required, or a process leading to generation of the unprotonated Lys residue should be found. In the deacylation, it has been suggested that a water molecule (Wat81) hydrogen bonded to Glu166 acts as a deacylating water, based upon the crystallographic structure.[3] However, the recent crystallographic structure of the acyl−enzyme of *S. aureus* PC1 by clavulanate[20] indicates that the water (Wat81) will not be involved in the deacylation. It is also noted that class C enzymes do not have the carboxylate group nor an alternative general base

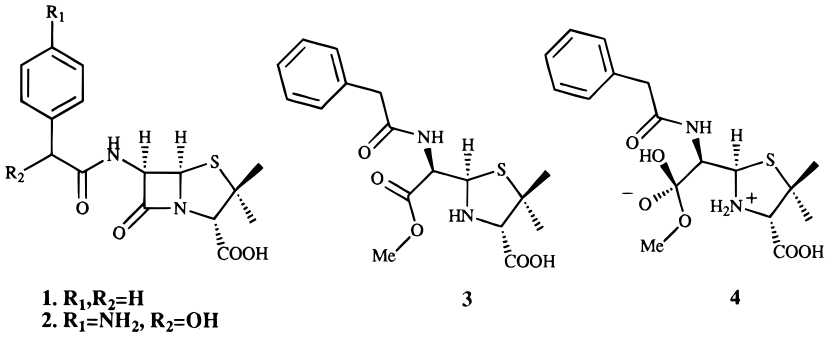
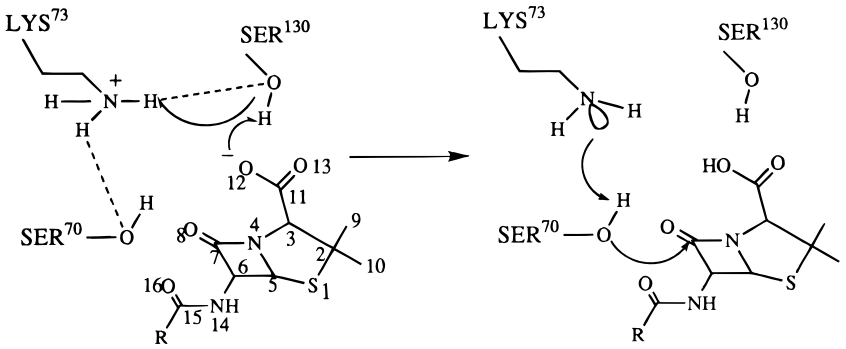


Figure 1.

Scheme 3. Scheme of the Activation of the Ser70 Residue by the Unprotonated Lys Residue[a]



[a] Broken lines, hydrogen bonds. Atom numbering of penicilline is given in penicillin G structure.

corresponding to the Glu166 residue.[12] Hence, we have examined another water molecule as the deacylating water through construction of acyl−enzyme models.

Hence, we report the model building of the complex structures of the β-lactamase with penicillin G based on the recent structural information and discuss a hydrolytic mechanism of class A β-lactamases through the modeling study. Michaelis complex and acyl−enzyme models have been previously built to discuss the hydrolytic mechanism with lack of those substantial structure information.[7] Our model would provide a new view of the formation of the complex structures.

Methods

Modeling of Hydrogen-Bond Networks at the Active Site. The three-dimensional structure of the *S. aureus* PC1 β-lactamase at 2.0 Å (accession number 3BML in the PDB)[2] was used for the present model-building study. During the network model building, all the coordinates for the protein and water molecules except the side chains of Lys73 and Ser130 were kept unchanged. Hydrogen-bond networks were generated with a DGEOM distance−geometry calculation.[26] In each distance−geometry (DG) calculation, 50 conformers were generated randomly. The conformations of side chains of Lys73 and Ser130 were optimized using the distance constraints with AMBER molecular mechanics V3[27] and a conformation having the closest structure to the original crystal structure (i.e., the lowest rms value) was selected for modeling of the complex structure at each step.

Modeling of Putative Complexes in the Catalytic Process. The crystal structure of the penicillin nucleus shows two typical conformations of which an open structure (amoxycillin-type) of the thiazoline ring was deduced to be an active conformer.[28] Thus, penicillin G structure **1** (Figure 1) was generated from the crystal structure of amoxycillin (**2**, Cambridge Structural Database: AMOXCT10),[29] and its structure was optimized by MM2 force field implemented in MACROMODEL V3.[30] The structures of the penicilloate (**3**, unprotonated form at N_4; see Scheme 3 for atomic numbering), a β-lactam bond-cleaved structure, and the tetrahedral oxyanion (**4**, protonated form at N_4) were optimized by AM1 semiempirical method.[31] Each ligand was docked into the active site of the corresponding step, substituting C_7-OMe group with the Ser70 residue using the DG calculation to fulfill the expected hydrogen bond between the acyl moiety and the enzyme. Water molecules which were located in a proximity (within 2.5 Å) to the ligand molecule at the active site were omitted in the modeling of the complex model structures. During the calculation, the thiazoline and the β-lactam rings were kept rigid. Among the 50 generated structures, a few conformers were arbitrarily selected for energy minimization. The initial complex structures were minimized stepwisely as described by Juteau *et al.*[32] in a Monte Carlo water bath until the root-mean-square gradient was less than 0.1 kcal/mol per Å with the AMBER force field.

Results

The optimized structures at each step of the hydrolytic process were essentially the same. Tables 2 and 3 show the atomic distance constraints for the modeling of each complex and the atomic distances (hydrogen-bond lengths) obtained from the energy optimized complex models, respectively.

Figure 2 shows a Michaelis complex model. The β-lactam carbonyl oxygen was located at the oxyanion hole where the carbonyl oxygen made hydrogen bonds with the amide protons of the Ser70 and Gln237 peptide bonds. The acylamide at C_6 was located to form hydrogen bonds with Asn132N^{δ} and the main chain carbonyl oxygen of Gln237. When a carboxylate oxygen at C_3 was located at a hydrogen-bond distance to Ser130O^{γ}, both of the carboxylate oxygens were disposed at similar sites for two water molecules (Wat64 and Wat111) observed in the crystal structure. The distance between the O_{12} atom and Lys234N^{ζ} was 3.36 Å and the O_{13} atom was located within a hydrogen-bond distance to Ser235O^{γ} (2.74 Å) and Arg244N^{ζ} (2.63 Å).

Table 2. Distance Constraints Used for the Generation of the Complex Models

atom of amino acid				Michaelis complex	acyl−enzyme	acyl−enzyme[a]	tetrahedral oxyanion
S^{70}	O^{γ}	K^{73}	N^{ζ}	2.7	3.4	3.4	2.8
		S^{130}	O^{γ}	3.3	3.3	3.3	3.3
K^{73}	N^{ζ}	S^{130}	O^{γ}	2.8	3.75	3.75	3.75
		E^{166}	$O^{\epsilon 1}$	3.4	2.8	2.8	2.8
S^{130}	O^{γ}	K^{234}	N^{ζ}	2.8	2.8	2.8	2.8
PNG	O_{12}[b]	S^{130}	O^{γ}	2.8	–	–	–
		K^{234}	N^{ζ}	3.4	4.0	–	–
		S^{235}	O^{γ}	–	2.8	–	–
		R^{244}	$N^{\eta 1}$	–	–	4.0	–
		Wat_B	O	–	–	2.8	2.8
PNG	O_{13}	S^{130}	O^{γ}	–	–	–	–
		K^{234}	N^{ζ}	–	–	–	–
		S^{235}	O^{γ}	–	–	–	–
		R^{244}	$N^{\eta 1}$	–	2.8	2.8	2.8
		R^{244}	$N^{\eta 2}$	–	–	2.8	–
PNG	N_4	S^{130}	O^{γ}	–	–	–	–
PNG	O_8	S^{70}	NH	2.8	2.8	2.8	2.8
		Q^{237}	NH	2.8	2.8	2.8	2.8
PNG	C_7	S^{130}	O^{γ}	3.1	–	–	–
PNG	N_{14}	Q^{237}	O	3.2	3.2	3.2	3.2
PNG	O_{16}	N^{132}	$N^{\delta 2}$	2.8	2.8	2.8	2.8
Wat_A	O	S^{130}	O^{γ}	–	–	2.8	2.8[c]
		PNG	N_4	–	–	2.8	2.8[c]
		PNG	C_7	–	–	2.6	–
Wat_B	O	K^{234}	N^{ζ}	–	–	2.8	2.8
		S^{235}	O^{γ}	–	–	2.8	2.8
		Wat_A	O	–	–	2.8	2.8[c]

[a] Complex with the deacylating water molecule (see text). [b] See Figure 5 for atomic numbering. [c] Hydroxy group.

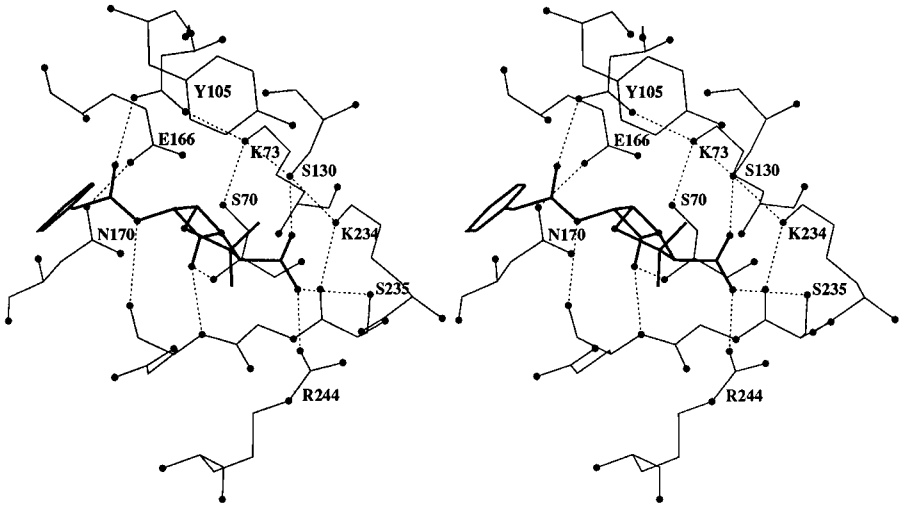


Figure 2. Stereoview of the Michaelis complex model. Heavy lines, penicillin G moiety; narrow lines, main residues at the active site; broken lines, hydrogen bonds;closed circles, heteroatoms (O, N, and S). For clarity, water molecules are omitted.

The N_4 atom of the β-lactam occupied a close site for a water molecule (Wat71). Thus, four water molecules (Wat22, Wat64, Wat71, and Wat111) observed in the crystal structure were excluded by the heteroatoms in the β-lactam.

Docking the penicilloate moiety into the active site by the use of the hydrogen-bond information shown in the Table 2, we constructed an acyl−enzyme structure (Figure 3). Amino acid residues at the binding site of the β-lactamase did not change their conformation, except for the residues which are directly involved in the catalysis. The ester carbonyl oxygen was located at the same position as that of the β-lactam carbonyl oxygen for the Michaelis complex. The carboxylate adapted the hydrogen bond to Ser235O^{γ} (2.77 Å) and Arg244N^{η} (2.68 Å). The torsion angles for the C_5-C_6, C_6-C_7, and C_6-N_{14} bonds had changes of 60.4°, 30.3°, and 35.9°, respectively, from the β-lactam structure in the Michaelis complex (Table 4). Through the torsion angle changes, the C_3 carboxylate broke the hydrogen bond with the Ser130 residue and moved toward Arg244 at a surface of the active site in the acyl−enzyme model. The most significant difference of the structure from previously reported acyl−enzyme models is that the N_4 atom is located far from the hydrogen-bond distance to Ser130O^{γ} (4.79 Å), while the previous models have hydrogen-bond distances (2.4−2.5 Å). This might come from a difference of the conditions used for building of an initial acyl−enzyme model. However, since details of the modeling conditions for the previous models are

Table 3. Selected Atomic Distances within the Active Site of the Refined Complex Models (Å)

atom of amino acid				Michaelis complex	acyl−enzyme	acyl−enzyme[a]	tetrahedral oxyanion
		K^{73}	N^{ζ}	2.65	3.38	3.38	2.94
S^{70}	O^{γ}	S^{130}	O^{γ}	3.30	3.24	3.32	3.25
		E^{166}	$O^{\epsilon 1}$	4.07	5.13	4.94	4.78
		Wat^{81}	O	3.00	3.26	3.42	3.56
		S^{130}	O^{γ}	2.80	3.76	3.87	3.81
K^{73}	N^{ζ}	S^{130}	O	2.92	3.67	3.75	3.83
		N^{132}	$O^{\delta 1}$	2.70	2.68	2.63	2.63
		E^{166}	$O^{\epsilon 1}$	3.39	2.62	2.64	2.62
S^{130}	O^{γ}	K^{234}	N^{ζ}	2.80	2.77	2.79	2.79
		Wat_A	O	–	–	2.79	2.81
E^{166}	$O^{\epsilon 1}$	N^{132}	$O^{\delta 1}$	3.70	3.52	3.66	3.18
		Wat_{81}	O	2.80	2.80	2.80	2.76
E^{166}	$O^{\epsilon 2}$	N^{170}	$N^{\delta 2}$	2.70	2.69	2.74	2.70
N^{170}	$O^{\delta 1}$	Wat_{81}	O	2.77	2.76	2.76	2.76
		S^{235}	O^{γ}	4.45	4.27	4.90	4.72
K^{234}	N^{ζ}	S^{235}	O	2.71	2.71	2.71	2.73
		Wat_B	O	–	–	2.90	2.87
		Wat_{101}	O	3.26	3.64	3.12	2.97
S^{235}	O^{γ}	Wat_B	O	–	–	2.82	2.73
O_{12}		S^{130}	O^{γ}	2.76	4.19	5.81	4.98
		K^{234}	N^{ζ}	3.36	4.00	5.57	5.47
		S^{235}	O^{γ}	3.72	2.77	2.91	3.85
		Wat_B	O	–	–	2.90	2.82
		R^{244}	$N^{\eta 1}$	4.81	3.72	3.91	2.78
		R^{244}	Nh2	6.55	5.21	2.68	4.27
O_{13}		S^{130}	O^{γ}	4.93	5.95	5.53	6.94
		K^{234}	N^{ζ}	4.85	5.87	5.06	7.56
		S^{235}	O^{γ}	2.74	2.94	2.79	5.04
		Wat_B	O	–	–	3.28	4.77
		R^{244}	$N^{\eta 1}$	2.63	2.68	2.67	2.81
		R^{244}	$N^{\eta 2}$	4.43	3.32	2.99	3.16
N_4		S^{130}	O^{γ}	4.03	4.79	4.96	5.29
		Wat_A	O	–	–	3.05	2.86
O_8		S^{70}	NH	2.80	2.74	2.80	2.87
		Q^{237}	NH	2.80	2.80	2.79	2.73
C_7		S^{70}	O^{γ}	3.09	1.36 bonded	1.36 bonded	1.39 bonded
		Wat_A	O	–	–	3.07	1.41 bonded
		Wat_{81}	O	5.08	3.64	4.37	4.64
Wat_A	O	Wat_B	O	–	–	2.43	2.80

[a] Complex with the deacylating water molecule (see text).

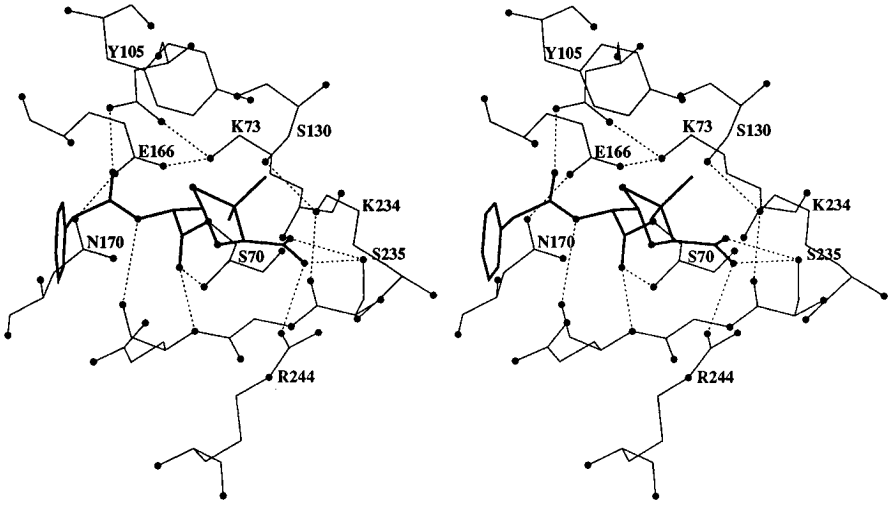


Figure 3. Stereoview of the acyl−enzyme model. Lines and circles, same as those in Figure 2.

not available,[33] it is difficult to explain the distinction. The present model indicates that the acyl moiety loosely bound to the active site and there is a space for accommodation of a few water molecules in the active site. Thus, we examined the size of the space, introducing a few water molecules.

It was possible to introduce one or two water molecules in the active site but not three water molecules.

Table 4. Torsion Angles for the Penicilloyl Moiety in the Refined Complex Models

	amoxycillin X-ray	Michaelis complex	acyl−enzyme	acyl−enzyme[a]	tetrahedral oxyanion
N4−C5−C6−C7	8.8	12.3	−48.1[b]	−51.1	−47.1
C5−C6−C7−$O^{\gamma}S^{70}(N_4)$	(−9.3)	−103.1(−13.4)	−72.8	−120.3	−160.9
C5−C6−N14−C15	133.8	101.0	136.9	104.4	102.4

[a] Complex with the deacylating water molecule (see text). [b] About −30 ° in the mutant acylenzyme reported by Strynadka *et al.* (1992).

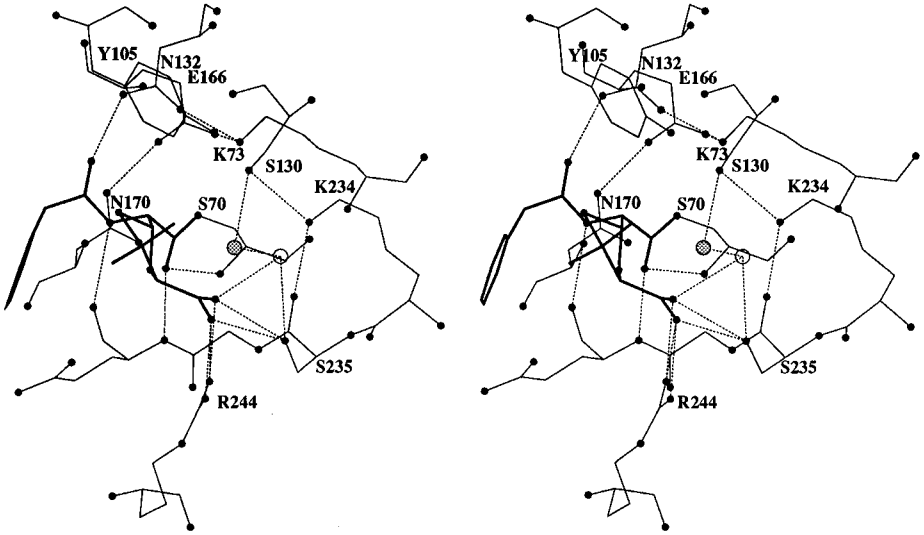


Figure 4. Stereoview of the acyl−enzyme model with two water molecules. Lines and closed circles, same as those in Figure 2; shaded circle, water molecule (Wat_A); open circle, water molecule (Wat_B).

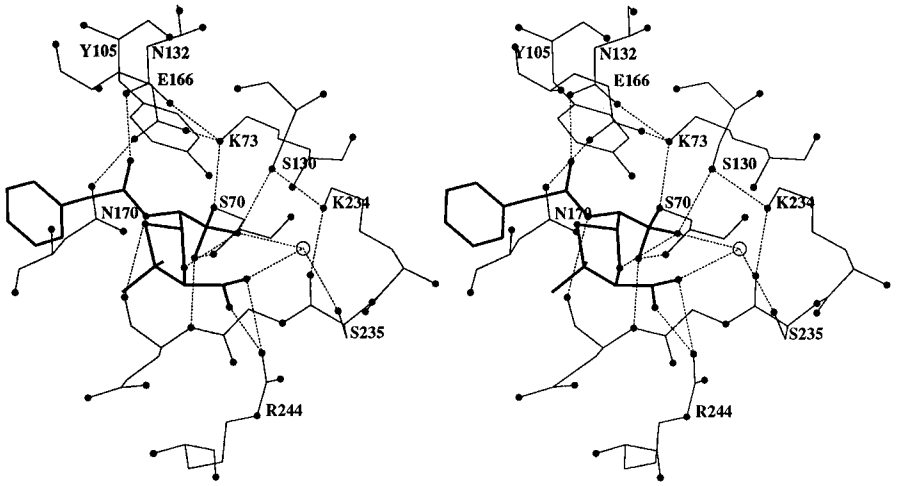


Figure 5. Stereoview of the tetrahedral oxyanion model. Lines and closed circles, same as those in Figure 2; open circle, water molecule (Wat_B).

The complex with one water molecule, however, did not provide any structural significance. Table 2 shows the distance constraints for modeling of the acyl−enzyme with two water molecules at the active site. One of the two water molecules in the optimized complex structure (Figure 4) was located above the plane of the ester carbonyl group in a proximity to the ester carbonyl carbon (3.05 Å) within hydrogen-bond distances to Ser130O^{γ}, the N_4 atom, and the carboxylate oxygen (O_{12}). Thus, the water was in a suitable position to directly participate in the deacylation. The other water formed hydrogen bonds with the deacylating water, the residues of Lys234 and Ser235, and the carboxylate oxygen atom (O_{12}). The carboxylate moved up to a position where the two carboxylate oxygen atoms could interact with the basic residue of Arg244, with large changes (47.5° and 32.5°) of the torsion angles for C_6−C_7 and C_6−N_{14}. The C_5−C_6 bond changed its torsional angle in a small degree (3.0°) (Table 4). The carbonyl oxygen of the ester and the acylamide side chain at C_6 stayed at the almost unchanged position. The water (Wat81) hydrogen bonding to the Glu166 residue was located at a far distance from the ester carbonyl carbon (4.22 Å). Thus, this acyl−enzyme model suggested that the newly introduced water was located at a more suitable site for the deacylation than the crystallographically observed water molecule (Wat81).

An oxyanion formed by attack of the deacylating water stayed at the same position (Figure 5). The other remaining part of the acyl moiety was also located at the almost same positions as those of the acyl−enzyme (Figure 4), although a large change (40.6°) of the torsion angle for the C_6−C_7 bond occurred due to formation of the tetrahedral carbon at C_7 (Table 4). An N_4 am-

monium proton made a hydrogen bond with the hydroxyl group of the tetrahedral oxyanion moiety. The hydroxyl group was also located within a hydrogen-bond distance to Ser130O$^{\gamma}$.

Discussion

Model Building. In macromolecule modeling study, energy-optimized structures depend on their initial structures generated by a docking method, showing local energy minima. We have generated 50 initial structures of the penicillin derivatives in the rigid active site at each step using the atomic distance constraints by the distance−geometry calculation (see Table 2). Those atomic distance constraints for the docking provided similar structures of the ligands in each reaction. This result indicates that the constraints confine the ligand structures in the active site although it does not imply that the present models have the global energy minimum. Provided that the residues consisting of the active site do not dramatically change their conformations to affect the ligand conformations, our present complex models would afford a significant view of the catalytic process. For further studies, molecular dynamics simulations would be suitable for finding the global energy minimum by sampling initial structures during the dynamics simulation, although there is no way to ascertain the global minimum structure.

Acylation. Site-directed mutation experiments have shown that the Lys73 and Lys234 residues are involved in the acylation of penicillins.[5,14] The experiment on K234A mutant enzyme indicated that an interaction of the carboxylate group of penicillins with Lys234N$^{\zeta}$ is important for enhancement of the hydrolytic activity of the β-lactamases.

It has been suggested that Ser130 has a critical role for substrate binding, in particular, through a hydrogen bond with the carboxylate of β-lactam antibiotics.[32] The crystal structures (4BLM; this data involves two distinct structures which are assigned as 4BLMA and 4BLMB) of the enzyme from *B. licheniformis* 749/C and the crystal structure (3BLM) from the *S. aureus* PC1 enzyme supported the role of the Ser130 residue for substrate binding. As shown in Scheme 2, a negatively charged oxygen atom of the sulfate in 4BLMA and a water molecule in 3BLM interact with the Ser130 residue (2.59 and 2.83 Å, respectively). Further inspection of those crystal structures has revealed quite distinct hydrogen-bond networks in the active site. In particular, the Lys73 residue in 3BLM makes a hydrogen bond with the Glu166 residue whereas the ammonium residue in 4BLMA does not have the hydrogen bond but with the Ser130 residue. The other structure (4BLMB) shows a weaker hydrogen bond (3.24 Å) between the residues of Lys73 and Ser130 and a longer distance (2.76 Å) between the Ser130 residue and the oxygen atom of the sulfate. The same relation of the distances can be found in the two structures of the E166A mutant enzyme (1MBLA and 1MBLB) from *B. licheniformis* 749/C (Table 1). These data suggest that an access of a negatively charged atom to the Ser130 residue causes the change of the hydrogen-bond network. Thus, the interaction of the penicillin carboxylate with the Ser130 residue in a Michaelis complex formation would lead to a similar hydrogen-bond network as observed in the sulfate-bound enzyme.

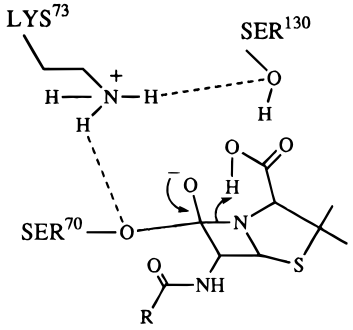


Figure 6. A putative proton transfer process in the cleavage of the four-membered ring.

The Michaelis complex model (Figure 2) suggests that the Lys234 residue has roles in not only hydrogen bond with the Ser130 residue but also polarization of the carboxylate group by a van der Waals contact (3.36 Å). Hence, this carboxylate oxygen will efficiently act as a proton acceptor from the hydroxyl group of Ser130 which would be exceptionally acidic due to the hydrogen bonds with both of the Lys73 and -234 residues. It is also the case for the water molecule, a plausible substitute for the hydroxymethyl group of the serine residue, in the S130G mutant. A preliminary quantum chemical calculation for the native and mutant enzymes confirmed this process (data not shown). Concomitantly, a proton would be transferred to Ser130O$^{\gamma}$ from the ammonium group of Lys73. The resulting unprotonated amine of Lys73 will act as a general base to activate the hydroxyl group of Ser70 (Scheme 3). Strynadka *et al.*[18] suggested a role of the unprotonated Lys73 residue for the activation. The carboxylate-assisted mechanism can account for the generation of the unprotonated Lys73 residue. This would be also the case for the K73R mutant since positively charged arginine residue will become neutral by the proton transfer.

The following activation of the Ser70 residue by the unprotonated Lys73 residue would be assisted by the dipoles of the neighboring α-helix[35] and the polarized carbonyl group of the β-lactam hydrogen bonding in the oxyanion hole. Thus, the change of the hydrogen-bond network induced by the interaction of the penicillin carboxylate with the Ser130 residue would be one of the most important events in the acyl−enzyme formation.

Comparing the catalytic groups in the acylation of class A β-lactamases with a chymotrypsin-like catalytic triad of Ser-His-Asp, Ser130O$^{\gamma}$ would have a similar role to the N$^{\delta}$ atom of the His residue and the carboxylate of penicillins to the Asp residue while Lys73N$^{\zeta}$ would correspond to the N$^{\epsilon}$ atom of the His residue. A recent crystallographic analysis has revealed that an esterase from *Streptomyces scabies* replaces the Asp residue with a main chain carbonyl within the catalytic triad.[35] The main chain carbonyl of the esterase might correspond to carbonyl groups of cephalosporin analogs at C_4.[36] It has been shown that modified cephalosporins bearing a lactone or an aldehyde at C_4 are better substrates for a class A β-lactamase than the original cephalosporin though those analogs are much poorer substrates than penicillins. We assume that the carbonyl oxygen of the cephalosporin analogs forms a resonance-assisted hydrogen bond (RAHB) with the Ser130 hydroxyl group followed by concomitant proton transfer from the Lys73 to Ser130 residues to form a positive charge assisted hydrogen bond ((+)CAHB).[37] This process would not be

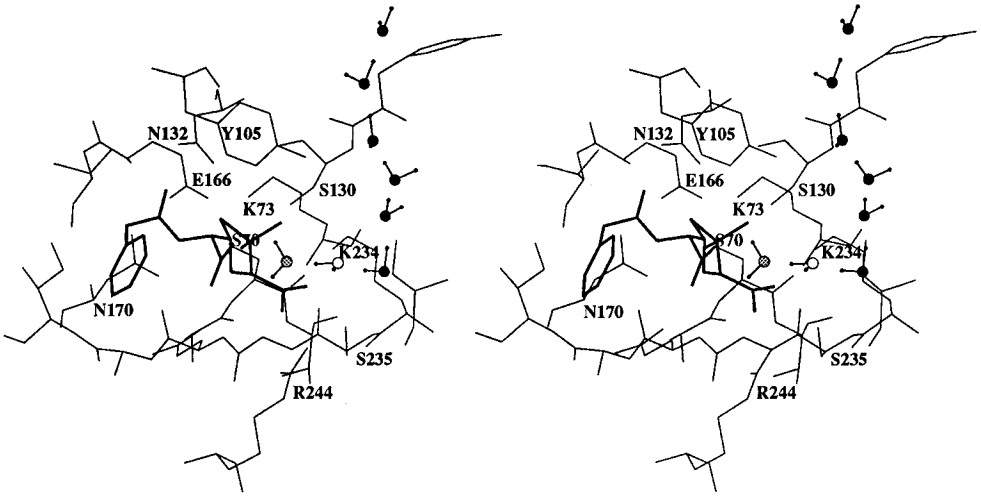


Figure 7. Stereoview of spatial disposition of water molecules at the active site (water path). Heavy lines, penicillin G moiety; narrow lines, main residues at the active site; shaded and open circles, oxygen atoms for Wat_A and Wat_B respectively; closed circles, oxygen atoms for water molecules forming water path; small closed circles, protons of the water molecules.

so efficient as that triggered by the carboxylate. In any case, further studies are required to validate the proton transfer process through the RAHB and the following (+)CAHB.

Perturbation of the hydrogen-bond network for the acylation should lead to a disturbance of the acylation. Thus, the mutation of the Glu166 residue to aspartic acid has been reported to affect the acylation.[9] The E166D mutant would have a more extended conformation of the Lys73 residue to form an effective hydrogen bond with the aspartic acid, and hence, the longer distance between the ammonium group and the Ser130 residue would impede the change of the hydrogen-bond network upon binding of the substrates. Thus, the mutation of the residue would influence the reaction although the Glu residue would not be directly involved in the acylation. Since the K_m value was not largely affected by the substitution of Asp for Glu166,[9] the substrate recognition by Ser130 would be independent of the acylation.

After completion of the first proton-transfer process, an uncleaved tetrahedral oxyanion of the penicillin should develop the second proton-transfer pathway which will lead to a β-lactam ring cleavage. Since the N_4 nitrogen has an amine character in the tetrahedral oxyanion and the further cleavage of the N_4-C_7 bond will develop an anion character of the amine, the proton of the carboxylic acid of the penicillin would be directly transferred to the N_4 (Figure 6). Alternatively, the proton of the hydroxyl group of Ser130 would be transferred to the amine, and subsequently the proton of the carboxylic acid would be transferred to Ser130O$^\gamma$ since the N_4 is disposed in a proximity to the Ser130 residue as well as the carboxylic acid. We, however, need a more precise study on this process.

Although the Lys73 residue of the wild-type enzyme would hydrogen bond with the negatively charged Glu166 residue at this stage, the Lys73 residue of the E166N mutant would remain within a hydrogen-bond distance to the Ser130 residue due to the absence of the negative charge of the residue at 166 position. Thus, Ser130O$^\gamma$ of the mutant enzyme[18] forms good hydrogen bonds with the Lys73 and 234 residues. These two hydrogen bonds would reinforce the other hydrogen bond between the Ser130 residue and the N_4 atom of

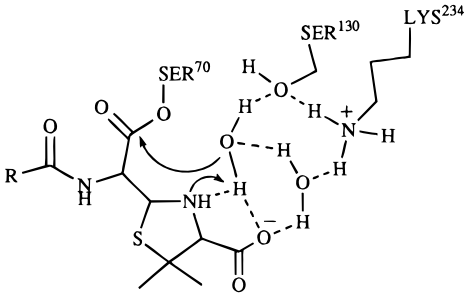
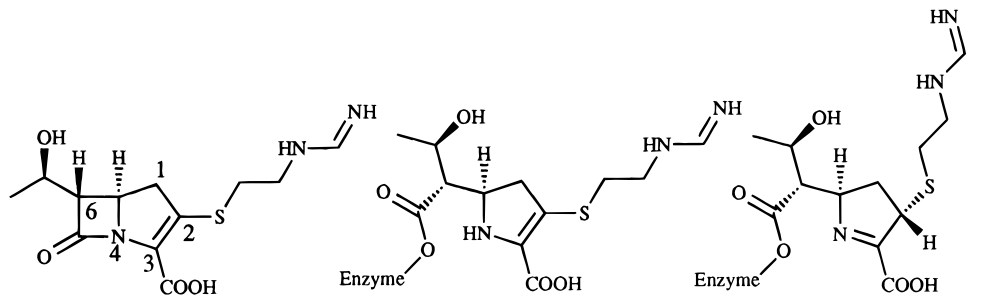


Figure 8. Putative hydrogen-bond network in the activation of the deacylating water molecule by the N_5 atom. Broken lines, hydrogen bonds.

the acyl moiety to enhance the stability of the acyl mutant−enzyme complex. We note that the N_4 atom is an unprotonated amine after the cleavage of the four-membered ring. Hence, we suppose that the Glu166 residue mainly contributes to a switch of the hydrogen bond of the Lys73 residue from Ser130 to Glu166 at the acyl−enzyme stage. Consequently, the Ser130 residue of the wild-type enzyme would offer a possible coordination site to another hydrogen bond donor such as water molecule for the deacylation.

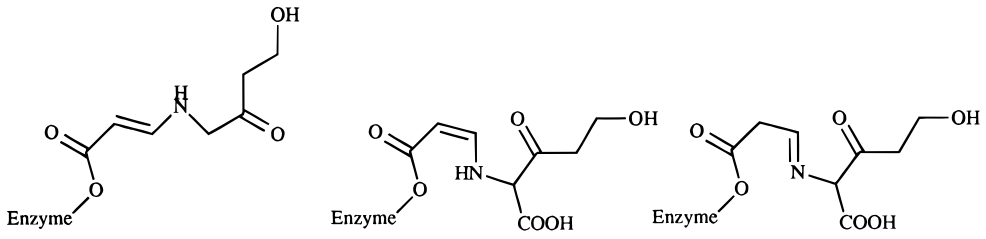
Deacylation. The thiazoline moiety would have a mobile conformation since only the carboxylate group harbors at the active-site residues. Taking account of the available coordination site for hydrogen bond to Ser130O$^\gamma$, a water molecule was introduced at a space for interaction with Ser130O$^\gamma$ and another water was accommodated in a remaining space.

This model (Figure 4) suggests that the water located in a proximity to the ester carbonyl carbon is a better candidate for the deacylation than the previously proposed water (Wat81), since the latter has a longer distance (4.22 Å) to the carbonyl carbon than the former (3.05 Å). We find a water path which is formed by the hydrogen-bonded water molecules as indicated by the closed circles in Figure 7. Thus, the deacylating water molecule will be delivered through the path and will attack the carbonyl carbon from the *re* (β) face with an aid of the unprotonated N_4 atom and the carboxylate oxygen (Figure 8). The limited space and path for the hydrolyzing water molecule (Figure 7) would hardly allow the accommodation of other nucleophiles such as



5 **6** **7**

Figure 9.



8 **9** **10**

Figure 10.

methanol.[38] The importance of a mobile water molecule for deacylation has been suggested through the time-resolved Laue crystallography on catalysis of a serine protease, trypsin.[39] Substrate-assisted ligand activation has been suggested as a key mechanism for aspartate carbamoyl transferase,[40] DNA cleavage by type II restriction endonucleases,[41] aminoacylation of glutamyl- and aspartyl-tRNA synthetases,[42,43] and GTP hydrolysis of $p21^{ras}$.[44]

In the E166N mutant, the acyl−enzyme would resist the hydrolytic attack of the deacylating water by the formation of the hydrogen bond between the N_4 atom and the Ser130 residue. It has been reported that the ester bond of the Δ^3 tautomer **7** (Figure 9) of the acyl−enzyme formed with imipenem (**5**) is kinetically more resistant to the hydrolysis than that of the Δ^2 derivative (**6**).[45−47] The α-substituted moiety at C_2 of **7** would block the water path more efficiently than the moiety of **6**. Thus, blockage of the accommodation of the deacylating water would result in a more stable acyl−enzyme.

The crystal structure of the enzyme from *S. aureus* PC1 acylated by clavulanic acid showed two structures for degraded clavulanates without significant conformational changes of the residues involved in the catalysis.[20] Of the two structures, a structure having a *trans*-enamine moiety **8** (Figure 10) showed a large conformational change resulting in a distinct location of the acyl moiety. The other structure has a *cis*-enamine moiety **9** in which the N_4 atom bearing a vinylogous amide proton made a hydrogen bond (2.8 Å) with Ser130O$^{\gamma}$. Besides, the carboxylate made a strong salt bond (2.33 Å) with the Lys234 residue (Figure 11). This tightly bound moiety would resist the hydrolytic attack of the deacylating water. Although precise investigations are required for understanding the process of the inhibition, it is plausible that Schiff base **10**, a putative intermediate of the stable acyl moieties, is not basic enough for the activation of the deacylating water and then rapidly isomerizes to the less reactive

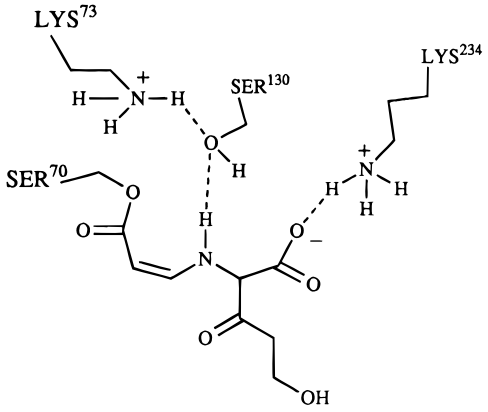


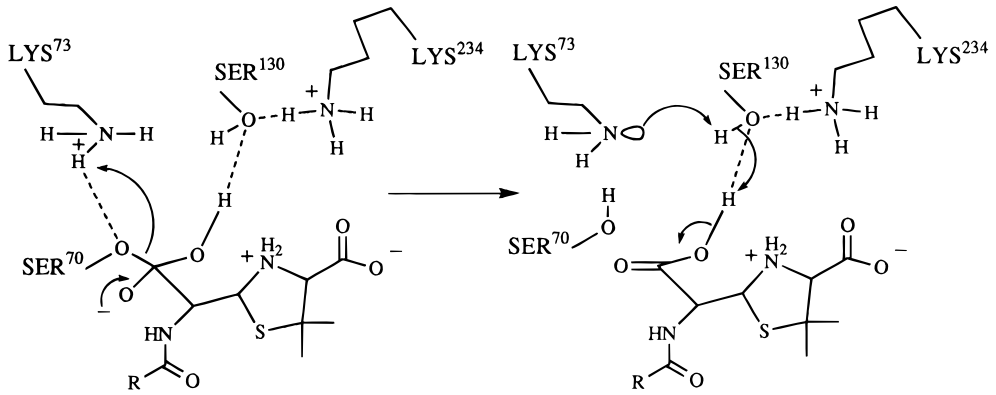
Figure 11. Schematic picture of the hydrogen-bond network of the acyl−enzyme with the degraded *cis*-clavulanate.

vinylogous amide moieties or forms an as yet unidentified covalent bond with a residue at the active site.[48,49]

The large change (32.5°) of the torsion angle for the C_6-N_{14} bond during the conformational change of the acyl moiety (Figures 3 and 4) suggests that substituents at the C_6 position which inhibit the bond rotation would also affect the mobility of the acyl moiety and then the stability of β-lactam antibiotics against the β-lactamases.

After the deacylating water attacked the ester carbonyl carbon, a penicilloic acid derivative will be released from the ester linkage via a tetrahedral oxyanion (Figure 5). After transfer of a proton from the Lys73 residue to Ser70O$^{\gamma}$, the neutral amine would prefer an interaction with the Ser130 residue to that of the Glu166 residue. The concomitant proton transfer will then afford the cleaved carboxylate as described in the Scheme 4. The hydrogen-bond network for the deacylated structure would be similar to the Michaelis complex structure (Scheme 2, bottom) since the generated carboxylate remains at the site close to the Ser130 residue. The replacement of the hydrolyzed compound

Scheme 4. Scheme of Cleavage of the Acyl Bond[a]



[a] The origins of the two protons at N4 are indicated in Figures 6 and 8.

(penicilloic acid derivative) with water molecules will lead to the initial hydrogen-bond structure.

As described above, based on the crystallographic data of the class A β-lactamases listed in the Table 1, we have discussed a process of the hydrolytic mechanism of class A β-lactamases. In the process, the switch of the hydrogen-bond network is important for the catalysis. This switch mainly owes to the mobile conformation of the Lys73 residue. The mobility of the residue has been confirmed by a recent molecular dynamics study on the class A β-lactamase from *S. aureus* PC1.[50] This switch would be triggered by binding of β-lactam antibiotics. In the acylation, the C_3 carboxylate would act as a proton shuttle, carrying the proton at the Ser residue to the N_4 atom, and thus the resulting Ser130 oxyanion would generate the unprotonated Lys73 residue. In the deacylation, the N_4 atom would play a role as a general base to activate the deacylating water.

We have also discussed that the deacylating water would be introduced into the active site during the conformational change of the acyl moiety. Since the water (Wat81) hydrogen bonded to the Glu166 residue receded from the Ser70 residue during the catalytic process, we suppose that it contributes to structural maintenance of residues around the Glu166 residue. The location of the deacylating water at the previously undiscussed site provided the other plausible accounts for the stability of the acyl−enzyme complexes: blockage of the accommodation of the deacylating water by substituents on acyl moieties, generation of less basic N_4 atom in the acyl−enzymes, and inhibition of the conformational change of acyl moiety.

Similarity to Class C Lactamase Catalysis. Two crystal structures of class C β-lactamases, one from *Enterobacter cloacae* P99[13] and the other from *Citrobacter freundii* 1203,[51] have been reported recently. Several experiments revealed important roles of the amino acid residues in the tetrad Ser64-Xaa-Xaa-Lys67, the triad Tyr150-Xaa-Asn152, and the triad Lys315-Thr316-Gly317 at the active site for acylation and deacylation.[52−55] However, a striking difference of class C lactamases from class A lactamases is lack of the acidic residue corresponding to Glu166 of class A enzymes. Site-directed mutation experiments have failed to find the acidic residue.[12]

It has been proposed that the phenoxide anion of Tyr150 would play a role as a general base to directly activate the hydroxyl group of the Ser64 residue in acylation.[51] A recent report[52] has shown that the Tyr150 residue is not unprotonated in the native enzyme. They have also proposed a mechanism similar to that proposed for a class A enzyme by Strynadka *et al.* in which the unprotonated Lys67N$^{\zeta}$ may act as a general base for activation of the Ser64 residue. However, it is unlikely that Lys67N$^{\zeta}$ is unprotonated under physiological conditions in the native enzyme.

Our proposed catalytic mechanism for class A enzymes can give an alternative explanation for the generation of the unprotonated Lys67N$^{\zeta}$. The unprotonated Lys67N$^{\zeta}$ should be generated by the Tyr150 residue which is deprotonated by the carboxylate of β-lactam antibiotics. Due to its lower pK_a, the deprotonation of the Tyr150 residue must be easier than that of the Ser130 residue of the class A enzyme.

Because of the lack of the acidic residue corresponding to Glu166 in the class A enzyme, the Tyr150 residue will keep a hydrogen-bond network with the two basic residues of Lys67 and Lys315 at a deacylation stage as observed in the acyl−enzyme of *C. freundii* 1203.[51] Although this network should be unfavorable in a class A enzyme, deprotonation of the phenol group of the Tyr150 residue is much facilitated by those two hydrogen bonds. Thus, the phenol would be very easily deprotonated by the amine generated by cleavage of a β-lactam ring of cephalosporin. A lifetime of the generated phenoxy anion would be long enough to work as a general base for activation of a deacylating water. Oefner *et al.* (1990) has suggested that the deprotonated Tyr150 residue acts as a general base for activation of the deacylating water, although they have improperly postulated the deprotonated residue all through the catalytic process.

We suppose that basicity of the nitrogen atom at N_5 of cephalosporins is critical for the deacylation. Aztreonam, a monobactam antibiotic, forms a very stable acyl−enzyme whose structure was observed by X-ray crystallography.[51] Since the N_1 atom of the acyl moiety of aztreonam bears sulfonate, the nitrogen atom has a poor basicity for activation of the Tyr150 residue. As a result, the stable acyl−enzyme should be formed in the reaction of aztreonam with the class C β-lactamase.

Although further structural analyses should be required for elucidation of their hydrolytic mechanism, we assume that class C β-lactamases hydrolyze β-lactam antibiotics in a catalytic manner similar to that class A β-lactamases as discussed above.

We have discussed a plausible hydrolytic mechanism for catalysis of class A β-lactamases. This mechanism

was applicable to accounts for the inactivation of the enzymes by the inhibitor and for the results of the mutation experiments on the amino acid residues at the active site. Furthermore, this would be common for the hydrolytic mechanism of the class C β-lactamases. This novel mechanism will lead us to new ideas for design of β-lactamase-resistant β-lactam antibiotics or β-lactamase inhibitors. Design of novel β-lactam derivatives and thus verification of the mechanism are forthcoming in due course.

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