Theoretical Study on Tetrahedral Intermediate Formation by Class A β-Lactamase: the Effects of the Oxyanion Hole and Substrate Specificity

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Received July 21, 1998

Bacterial D-Ala-D-Ala-transpeptidase which is responsible for synthesis of bacterial cell-walls with D-Ala-D-Ala substrates is inhibited by β-lactam antibiotics, forming the stable esters of the active serine with the antibiotics.[1] On the other hand, β-lactamases (class A) hydrolyze the antibiotics to protect bacteria against their lethal effect, but do not hyrolyze natural D-Ala-D-Ala substrates.[2] Because of the similar enzymatic reaction it is not surprising that active site structures of D-Ala-D-Ala-transpeptidases and β-lactamases are very similar and highly conserved by the comparison of their crystal structures.[2] The mechanism of these enzymes with active site residues has been actively studied by crystal structure determination,[2-4] theoretical studies,[5,6] solution kinetics studies,[7] and site-directed mutagenesis.[8-11] The chemical function of the well-known triad (Asp--His--Ser)[12] and the oxyanion hole in stabilization of tetrahedral intermediates[13-15] of the general serine protease families are clearly explained. However, in the transpeptidases and β-lactamases, these two factors are not yet understood. We describe the interaction between the oxyanion of the intermediate and the oxyanion hole of the penicillin binding proteins (PBPs) with semiempirical PM3 method implemented in MOPAC and *ab initio* quantum mechanical method,[16] and with the crystal structure of a β-lactamase (3BLM) in the Brookhaven Protein Data Bank.[3]

Active Site Sequence. Active sites of both transpeptidases and β-lactamases (Class A) are known to be similar and have a β-sheet and an α-helix as described in Figure 1 and Table 1; the junction between the β-sheet and the α-helix generates a bonding pocket for substrates. The hydroxy of the α-helical serine reacts with an amide of substrates from the rear side to form a tetrahedral intermediate, and is acylated.[2] The acylated Ser is further hydrolyzed with water in β-lactamases, or synthesizes a new amide bond (a peptidoglycan cross-linkage) with an new amine in the transpeptidases.[1] Sequences of the active site β-sheets and of the transpeptidases are highly conserved as shown in Table 1 except one major difference; the S4 amino acids are threonines in thr former but alanines in the latter. The first lysine is well-conserved with a few exception; its function is known to be an electrostatic anchor, *i.e.* recognizing the carboxylic-terminal of substrates.[3] The second thereonine was switched to a serine in some cases, but they have an equivalent hydroxy group, which was explained to interact with the carboxyl group of the esterified antibiotics to form stable complexes and delay the hydrolysis of the serine ester of the antibiotics in the transpeptidases.[2]

Our semiempirical PM3 calculation[17] verifies that the complex of the substrate and the model β-sheet have three interactions;[2-5] an electrostatic interaction between the C-terminal of the substrates and the Lys (S1) and two hydrogen bonds with backbone amide bonds (Figure 1), where the N-H proton of the Ser (S4) and the amido oxygens of the model β-lactam antibiotic and the D-Ala-D-Ala model are aparted by 1.86 and 2.40 Å, respectively. In this conformation, the active α-helical serine is located just below the substrate amide bond.[19]

Oxyanion Hole. The complex **A** and **B** show the interaction between the β-sheet and the tetrahedral intermediates of the model antibiotic and the D-Ala-D-Ala substrate (Figure 2). Because the narrow distance between the ethoxy C_β of the Ser (α-helix) and the C_α of the Gly (S 3) are 3.716 and 3.667 Å in **A** and **B**, respectively, it is

Table 1. Conserved sequences in binding-site β-strand of penicillin-binding proteins. (Ref 2 and 4)

PBPs	S1-S2-S3-S4
DD-Transpeptidases	
Escherichia coli PBP 1A	Lys-Thr-Gly-Thr
Escherichia coli PBP 1B	Lys-Thr-Gly-Thr
Escherichia coli PBP 2	Lys-Ser-Gly-Thr
Escherichia coli PBP 5	Lys-Thr-Gly-His
Bacillus subtilis PBP 5	Lys-Thr-Gly-Ser
Streptomyces R61	His-Thr-Gly-Thr
β-Lactamases	
Bacillus licheniformis 749/C	Lys-Thr-Gly-Ala
Bacillus sereus 1	Lys-Ser-Gly-Ala
Escherichia coli TEM pBR322	Lys-Ser-Gly-Ala
Staphylococus aureus PCI	Lys-Ser-Gly-Gln
Streptomyces albus G	Lys-Thr-Gly-Ala
Citrobacter freundii 1203	Lys-Thr-Gly-Ser

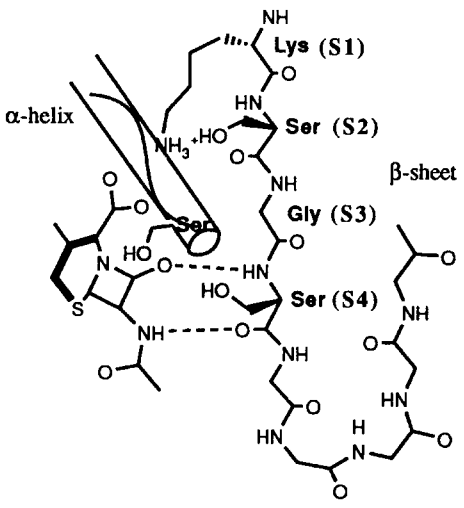

Figure 1. A sketch of the active site of a PBP with a β-lactam.

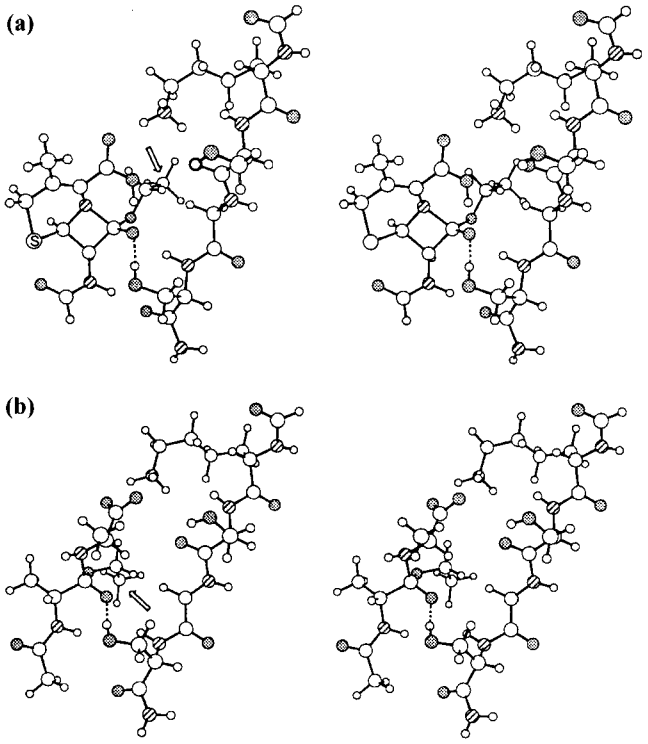

Figure 2. Stereoviews of (a) the complex **A** between the model β-sheet (Lys-Ser-Gly-Ser) and the tetrahedral intermediate of an antibiotics, and (b) the complex **B** between the model β-sheet and the tetrahedral intermediate of the N-acetyl D-Ala-D-Ala. The model C_α of the active α-helical serine is indicated with arrows, and the hydrogen bond with the oxyanion hole hydroxy is indicated with dotted lines. (See the text and ref 17.)

predicted that the α-helical ethoxy group does not allow any bulky residues amino acids at the S3 of the β-sheet, where the Gly (S3) is invariant in all known protein-binding proteins (Table 1).

The oxyanions of the complex **A** and **B**, where the tetrahedral intermediate are presumed to be covalently bound to the α-helical Ser, have an additional interaction compared to the Figure 1; the hydrogen bonding (1.725 and 1.789 Å) between the oxyanion of the tetrahedral intermediates and the gamma hydroxy group the S4 Ser. Direction of the hydroxy hydrogens follows the stereoelectronic control;[20] the oxyanion hole is located in the anti position to the incoming hydroxy group of the α-helical Ser. When the model β-sheet has an S4 Alanine (not the S4 Serine as in most β-lactamases), the oxyanion can not be stabilized by the hydrogen bond with the residue of the S4 amino acid which is located in the ideal oxyanion-hole position. Therefore, the corresponding tetrahedral intermediate of the natural D-Ala-D-Ala substrate will not have oxyanion-stablizing assistance from the S4 Ala of the β-lactamases.

Ab initio calculation estimates that stabilization of the tetrahedral intermediate of N-methyl acetamide and methoxide by the hydrogen bonding with a methanol in gaseous phase is 18.1 kcal/mol at RHF/6-31+G(d) (Figure 3). Previous studies show that the the oxyanion hole backbone amide of class A β-lactamase stabilizes the oxyanion intermediate by 11.8 kcal/mol at the same basis

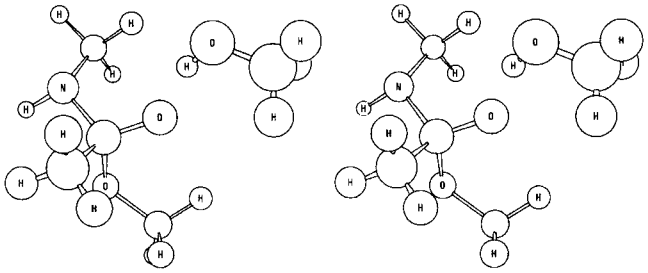

Figure 3. Stereoview of the tetrahedral intermediate of N-methyl acetamide and methoxide with the oxyanion hole methanol (Thr) at RHF/6-31+G(d). The hydrogen bond distance, d(O-H), is 1.725 Å.

set,[5] and that stabilization of a tetrahedral intermediate with an enzymatic oxyanion hole is more efficient by 7 kcal/mol than with solvation.[14,15] Although there are several more energy-stabilizing components in the active site, the hydroxy group of the Thr (S3) must make up an important component for the oxyanion hole.

Rate enhancement of the hydrolysis of the β-lactam antibiotic substrates was estimated to be the 10^4-fold (equivalent to the lower activation energy by 5 kcal/mol) compared to acyclic amides because of the intrinsic strain of the β-lactam ring.[7] Our previous study shows that the difference in the activation energy for the amide bond cleavage of β-lactam/acyclic amide is ~8 kcal/mol.[21] Therefore, it is predicted that the strained β-lactam antibiotics undergo the enzymatic acylation in both the transpeptidases and β-lactamases, but the natural D-Ala-D-Ala substrates (acyclic amide) which have higher activation barrier undergo the acylation only in the presence of the oxyanion-binding assistance as in D-Ala-D-Ala-trans-peptidases.

Acknowlegment. We would like to thank to the financial support of the Korea Research Foundation made in the program year of 1997 and the Yeungnam University Research Fund. We also thank the SERI Supercomputer Center.

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17. The model antibiotic is a cephalosporin having a formylated C-7 amido group and the model natural substrate is N-acetyl D-Ala-D-Ala. The model tetrapeptide β-sheet follows the crystallographic structures as possible and has a sequence of Lys-Ser-Gly-Ser, where the N-terminal was formylated and the C-terminal was blocked with an NH_2 group. Because an antiparallel β-sheet is known to have a repeat of puckered structures at every 7.0 Å with every two peptide bonds, we fixed the model tetrapeptide by applying the torsional angles of $\phi = -140^{\circ}$ and $\psi = +135^{\circ}$, which gives a slightly puckered chain.[18] However, except those two torsional angles, all geometric parameters were released for full optimization. The model α-helical serine was simplified to an ethanol and the relative position of the C-2 carbon of the ethanol was fixed and aparted by 4.08 Å form the C_{α} of the glycine of the β-sheet according to the crystal structure of a β-lactamase (3BLM)[4] deposited in the Brookhaven Protein Data Bank.
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