Binding of Cephalothin and Cefotaxime to D-Ala-D-Ala-Peptidase Reveals a Functional Basis of a Natural Mutation in a Low-Affinity Penicillin-Binding Protein and in Extended-Spectrum β-Lactamases[†,‡]

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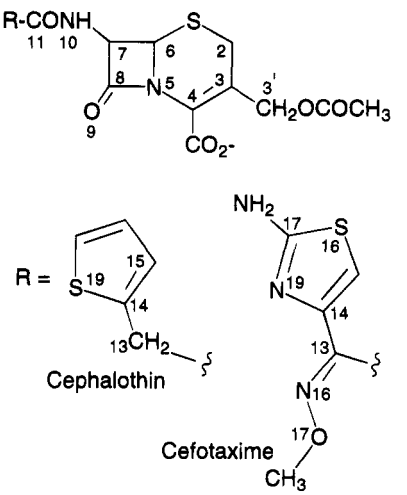
ABSTRACT: Two clinically-important β-lactam antibiotics, cephalothin and cefotaxime, have been observed by X-ray crystallography bound to the reactive Ser62 of the D-alanyl-D-alanine carboxypeptidase/transpeptidase of *Streptomyces* sp. R61. Refinement of the two crystal structures produced *R* factors for 3σ (*F*) data of 0.166 (to 1.8 Å) and 0.170 (to 2.0 Å) for the cephalothin and cefotaxime complexes, respectively. In each complex, a water molecule is within 3.1 and 3.6 Å of the acylated β-lactam carbonyl carbon atom, but is poorly activated by active site residues for nucleophilic attack and deacylation. This apparent lack of good stereochemistry for facile hydrolysis is in accord with the long half-lives of cephalosporin intermediates in solution (20−40 h) and the efficacy of these β-lactams as inhibitors of bacterial cell wall synthesis. Different hydrogen binding patterns of the two cephalosporins to Thr301 are consistent with the low cefotaxime affinity of an altered penicillin-binding protein, PBP-2x, reported in cefotaxime-resistant strains of *Streptococcus pneumoniae*, and with the ability of mutant class A β-lactamases to hydrolyze third-generation cephalosporins.

The enzymic targets of penicillin and cephalosporin antibiotics (the β-lactams) are D-alanyl-D-alanine carboxypeptidase/transpeptidases (DD-peptidase, EC 3.4.16.−) that catalyze the final step in the cross-linking of bacterial cell wall peptidoglycan (Frere et al., 1992; Ghuysen, 1994; Waxman & Strominger, 1983). The effectiveness of β-lactams in inhibiting the DD-peptidases is due to the formation of a serine-bound acyl intermediate, the stability of which made possible the crystallographic observation of this important intermediate in β-lactam hydrolysis (Kelly et al., 1982). That 2.8 Å analysis of a cephalosporin C complex with the DD-peptidase of *Streptomyces* sp. R61 and later crystallographic binding studies of other β-lactam complexes (Kelly et al., 1985, 1989) were hindered by the lack of a refined model for the native DD-peptidase. A very high resolution (1.6 Å) structure for the *S.* R61 enzyme is now available (Kelly & Kuzin, 1995) that allows improved mapping and refinement of its complexes with β-lactams. In this report, two acyl intermediates with the clinically-important β-lactams cephalothin and cefotaxime are described. In the case of reaction with penicillin, the breakdown pathway of such intermediates is known to involve cleavage of the C5−C6 bond (Frere & Joris, 1985), but for the longer-lived cephalosporin intermediates, the pathway is not well characterized. In these cases, a direct hydrolytic pathway might be involved in the regain of enzyme activity. Therefore, in this study we seek the water molecule(s) possibly functioning in a deacylation step of β-lactam turnover, and any candidate residues which may be able to activate the water for the hydrolysis (Wilkin et al., 1993).



Using this enzyme as a model of bacterial penicillin-binding proteins (PBPs) (Ghuysen, 1994; Jamin et al., 1995), we speculate on the role of a spontaneous Thr-to-Ala mutation in PBP-2x of a strain of *Streptococcus pneumoniae* able to grow in the presence of cefotaxime (Laible & Hakenbeck, 1991; Spratt, 1994). Interestingly, the *reverse* mutation, Ala-to-Thr, is found in a homologous position in certain TEM-type β-lactamase mutants able to bind and hydrolyze cefotaxime.

EXPERIMENTAL PROCEDURES

Crystallographic Methods. The native DD-peptidase of *Streptomyces* sp. R61, provided by J.-M. Ghuysen and J.-M. Frere (Université de Liège, Belgium), was crystallized at room temperature from 20% (w/v) PEG 8000 (Sigma) in 50 mM phosphate buffer at pH 6.8 as previously described (Kelly et al., 1989). Crystals measured approximately 0.2

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‡ Atomic coordinates for native DD-peptidase and the cephalothin and cefotaxime complexes have been deposited in the Brookhaven Protein Data Bank under Accession Numbers 3PTE, 1CEG, and 1CEF, respectively.

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Table 1: Preparation of β-Lactam Complexes with DD-Peptidase

	cephalothin	cefotaxime
concn (mM)	20	25
soak time at 20 °C (h)	37	32
half-life of complex in solution (h)[a]	20	40

[a] Estimated error is 10 h.

Table 2: X-ray Data Collection and Reduction

	cephalothin	cefotaxime
elapsed time of data collection (h)	16	22
temp (°C)	7	6
d_{min} (Å)	1.76	2.04
obsd reflections	49740	38280
unique reflections	28901	19106
ave $I/\sigma(I)$	25.4	15.9
% of possible	72.0	67.1
$R_{sym}(I)$[a]	0.051	0.057
$R_{isom}(I)$[b]	0.136	0.152
max change of cell dimensions (%)	0.6	0.4

[a] $R_{sym} = \Sigma|I_{ave} - I_i|/\Sigma I_i$, where I_{ave} is the average of all individual observations, I_i. [b] $R_{isom} = \Sigma|I_{PI} - I_P|/\Sigma|I_{PI} + I_P|$, where I_{PI} and I_P are the intensities of the inhibited and native crystals, respectively.

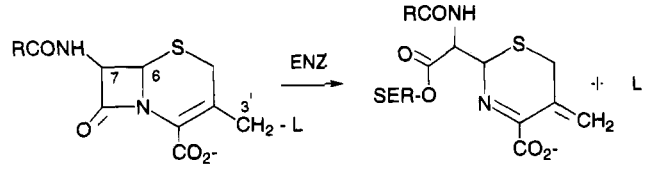
× 0.3 × 0.6 mm and were stored in 30% PEG 8000. Native cell dimensions are $a = 51.1$, $b = 67.3$, and $c = 102.4$ Å, and the space group is $P2_12_12_1$ with $Z = 4$. Crystals of the DD-peptidase exhibit catalytic activity (Kelly et al., 1992). Complexes were prepared by soaking native crystals in solutions of cephalothin or cefotaxime (Lilly). Antibiotic concentrations were increased from 1 mM to the final concentrations (Table 1) over a 12-h period.

The unwashed crystal complex was sealed in a 1 mm glass capillary and gradually cooled to about 7 °C by flowing compressed air through a cold bath. Excess β-lactam in the crystal interstices regenerated the complex as turnover occurred. Data were collected on a Siemens three-axis area detector with graphite-monochromated CuKα radiation (λ = 1.54 Å, 40 kV, and 180 mA) from a Rigaku RU-200 rotating anode generator. An omega scan of width 0.2° was used with count times ranging from 60 to 90 s per frame. Intensities were reduced and scaled with the Xengen programs (Howard et al., 1987). Data collection parameters are summarized in Table 2.

Native Enzyme and β-Lactam Models. The crystallographic structure of the native DD-peptidase, with 347 amino acid residues and 254 water molecules having $B <$ 50 Å^2, has been refined to an R factor of 0.149 at 1.6 Å resolution (Kelly & Kuzin, 1995). Atomic coordinates are deposited in the Protein Data Bank, Department of Chemistry, Brookhaven National Laboratory, Upton, NY, as Entry 3PTE. Structures of cephalothin and cefotaxime are available in the crystallographic literature (Laurent et al., 1982; Spry et al., 1984), but structures with an open, acylated β-lactam ring are not reported.

RESULTS

Initial Mapping of the Complexes. Maps were examined with FRODO (Jones, 1985) on an Evans & Sutherland PS330 graphics system or with CHAIN (Sack, 1988) on a Silicon Graphics IRIS 4D Crimson/Elan. Fourier difference maps calculated with native multiple isomorphous replacement (MIR) phases at 2.25 Å resolution with coefficients $|F_{obs,complex}| - |F_{obs,native}|$ contained the highest density in the β-lactam-binding site and indicated that the reactive Oγ atom of Ser62 had been acylated by the β-lactam carbonyl group (Liu, 1990). A cephalosporin with an open β-lactam ring could be fit to the density after a 50−60° rotation of the amido substituent around the C6−C7 bond. In each intermediate, the maps showed that the acetate substituent at position C3′ had departed upon opening of the β-lactam ring, leaving a terminal 3′-methylene group as expected (Faraci & Pratt, 1986).



Refinement of the Complexes. The maps based on unbiased native MIR phases showed recognizable density for the entire β-lactam moiety in each complex. Since protein conformational changes are possible upon binding of the ligand (Kochkina et al., 1994), the MIR phases were hereafter replaced with model phases. The initial model for each complex was built from coordinates of the native DD-peptidase refined to 1.6 Å resolution. Ligand and water oxygen atoms were excluded. The models were refined by simulated annealing and energy minimization using $|F_{obs,complex}|$ with the program XPLOR (Brunger, 1990), in which the initial temperature for the cooling procedure varied from 1000 to 3000 K. After this protocol, the standard R factor for the protein model was 0.18, and the free R factor (Brunger, 1992) was 0.22.

These interim phases were used to calculate difference maps with coefficients $2|F_{obs,complex}| - |F_{calc,protein}|$ so that the β-lactam and water atoms could be fit. An intermediate model now consisted of protein, covalently-bound ligand, and approximately 250 water molecules. Refinement of each complex by XPLOR required a definition of the chemical topology of the acylated ring-opened form of the β-lactam. Due to the instability of this form in the absence of the enzyme, no crystallographic data are available, so that idealized bond lengths and angles had to be estimated from the structures of the parent β-lactams (Laurent et al., 1982; Spry et al., 1984). The C4−N5 bond was defined as a heterocyclic partial double bond and the C3−C4 bond as an aromatic partial double bond, and the C3−C3′ distance was assumed to have bond order 1.5. The bond angles at C3, C4, and N5 were made 120° to impose planarity at these positions. Partial atomic charges of ligand atoms, needed for the energy refinement, were set to zero. The charge for Oγ of Ser62 was set to −0.55e (Engh & Huber, 1991).

With a complete model of each hydrated complex, a final XPLOR refinement with simulated annealing was begun at an initial temperature of 500 K. Resulting crystallographic and free R factors for 3σ data are 0.166 and 0.208 for the cephalothin complex and 0.170 and 0.231 for the cefotaxime complex, respectively. Average temperature factors and other parameters from the refinement are shown in Table 3. Deviations of covalent bonds and angles from ideal values are less than 0.01 Å and 2° for both protein structures. Deviations from ideality are less than 0.025 Å and 4° for both β-lactam species, selected bonds and angles of which are listed in Table 4. Coordinate errors estimated by the Luzzati method (Luzzati, 1952) are 0.20−0.23 Å (Figure 1). Portions of the resulting electron density maps with coefficients $|F_{obs,complex}| - |F_{calc,model}|$ are shown in Figures 2

Table 3: XPLOR Refinement Results

	cephalothin	cefotaxime
resolution range (Å)	20−1.76	20−2.04
no. of reflections used [$F > 3\sigma(F)$]	27098	16066
R_{free} factor	0.212	0.240
R factor	0.166	0.172
mean temp factor (Å^2)		
protein	7.7	4.9
drug	33.4	13.8
water	25.1	22.2
all atoms	9.4	6.5
rms deviations from ideality (protein)		
bond lengths (Å)	0.010	0.009
bond angles (deg)	1.9	1.8
rms deviations from ideality (drug)		
bond lengths (Å)	0.026	0.022
bond angles (deg)	1.9	3.7

and 3. Atomic coordinates for the cephalothin and cefotaxime complexes have been deposited in the Protein Data Bank as entries 1CEG and 1CEF.

Structure of the Complexes. The overall structure of the DD-peptidase and the location of the β-lactam-binding site at the inner edge of a β-sheet are seen in Figure 4. The enzyme is an α + β protein with an α domain and the major five-stranded antiparallel β-sheet protected on both faces with

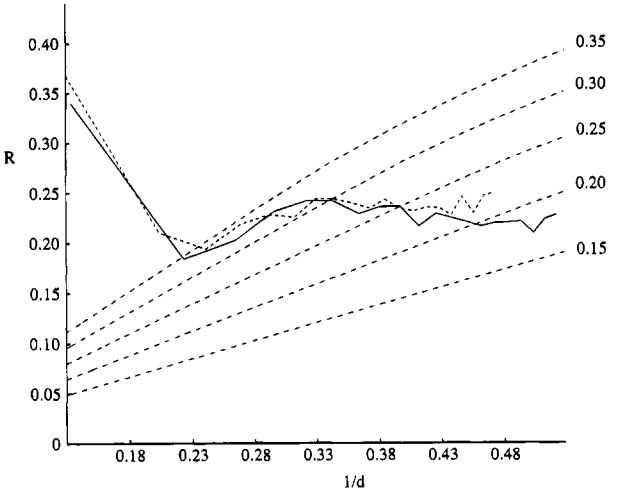


FIGURE 1: Luzzati plot (Luzzati, 1952) for cephalothin and cefotaxime (dashed) complexes. Coordinate errors are indicated in angstroms.

α-helices. Two minor β-sheets exist at the top and bottom of the molecule.

A very stable covalently-bound intermediate results from nucleophilic attack of the reactive Ser62 on the β-lactam

Table 4: Bonds and Angles in the β-Lactam Intermediates

bond (Å)	cephalothin	cefotaxime	bond (Å)	cephalothin	cefotaxime
S1−C2	1.88	1.78	C11−O12	1.24	1.23
S1−C6	1.83	1.81	C11−C13	1.51	1.43
C2−C3	1.56	1.42	C13−C14	1.49	1.48
C3−C3′	1.38	1.34	C14−C15	1.39	1.43
C3−C4	1.57	1.50	C15−C16	1.46	
C4−C4′	1.55	1.58	C16−C17	1.29	
C4′−O4A	1.26	1.25	C17−S19	1.68	
C4′−O4B	1.26	1.26	S19−C14	1.63	
C4−N5	1.40	1.36	C14−N19		1.37
N5−C6	1.48	1.48	C15−S16		1.67
C6−C7	1.55	1.61	S16−C17		1.68
C7−C8	1.53	1.52	C17−N18		1.40
C8−Oγ	1.38	1.40	C17−N19		1.33
C8−O9	1.23	1.23	C13−N16		1.33
C7−N10	1.46	1.46	N16−O17		1.44
N10−C11	1.32	1.33	O17−C18		1.41

angle (deg)	cephalothin	cefotaxime	angle (deg)	cephalothin	cefotaxime
C2−S1−C6	99.8	96.3	O9−C8−Oγ	118.8	121.1
S1−C2−C3	112.5	117.4	C8−C7−C6	106.8	107.6
C2−C3−C4	120.2	120.3	C8−C7−N10	107.0	111.7
N5−C4−C3	120.4	117.6	C6−C7−N10	112.3	111.2
N5−C4−C4′	116.0	117.0	S1−C6−N5	111.7	117.2
C4′−C4−C3	123.5	125.0	S1−C6−C7	112.1	112.7
C4−N5−C6	124.0	125.9	N5−C6−C7	107.2	108.8
C7−C8−O9	119.8	121.2	C7−N10−C11	121.8	121.3
C7−C8−Oγ	118.0	117.5	N10−C11−C13	116.3	112.2
C8−Oγ−Cβ	111.0	111.6	C11−C13−C14	117.7	123.8

dihedral angle (deg)	cephalothin	cefotaxime	dihedral angle (deg)	cephalothin	cefotaxime
N5−C6−S1−C2	57.7	32.4	Ser62 Cβ−Oγ−C8−O9	−1.9	−29.9
C6−S1−C2−C3	−41.8	−52.0	Ser62 Cβ−Oγ−C8−C7	157.3	155.6
S1−C2−C3−C4	3.7	36.6	Ser62 Oγ−C8−C7−N10	−179.9	179.6
C2−C3−C4−N5	31.0	9.5	Ser62 Oγ−C8−C7−C6	−59.6	−58.2
C3′−C3−C4−N5	−145.1	−168.3	C8−C7−N10−C11	−143.0	−139.7
C3−C4−N5−C6	−14.4	−31.5	C6-C7-N10-C11	100.0	100.1
C4−N5−C6−C7	−157.1	−124.6	N10-C11-C13-C14	106.1	53.6
N5−C6−C7−C8	−43.8	−45.2	C11-C13-C14-C15	−85.7	27.4
S1−C6−C7−C8	−167.7	−176.9	C11-C13-N16-O17		25.6
Ser62 N−Cα−Cβ−Oγ[a]	−86.9	−81.3	C13-N16-O17-C18		167.8

[a] −16.3° in native structure.

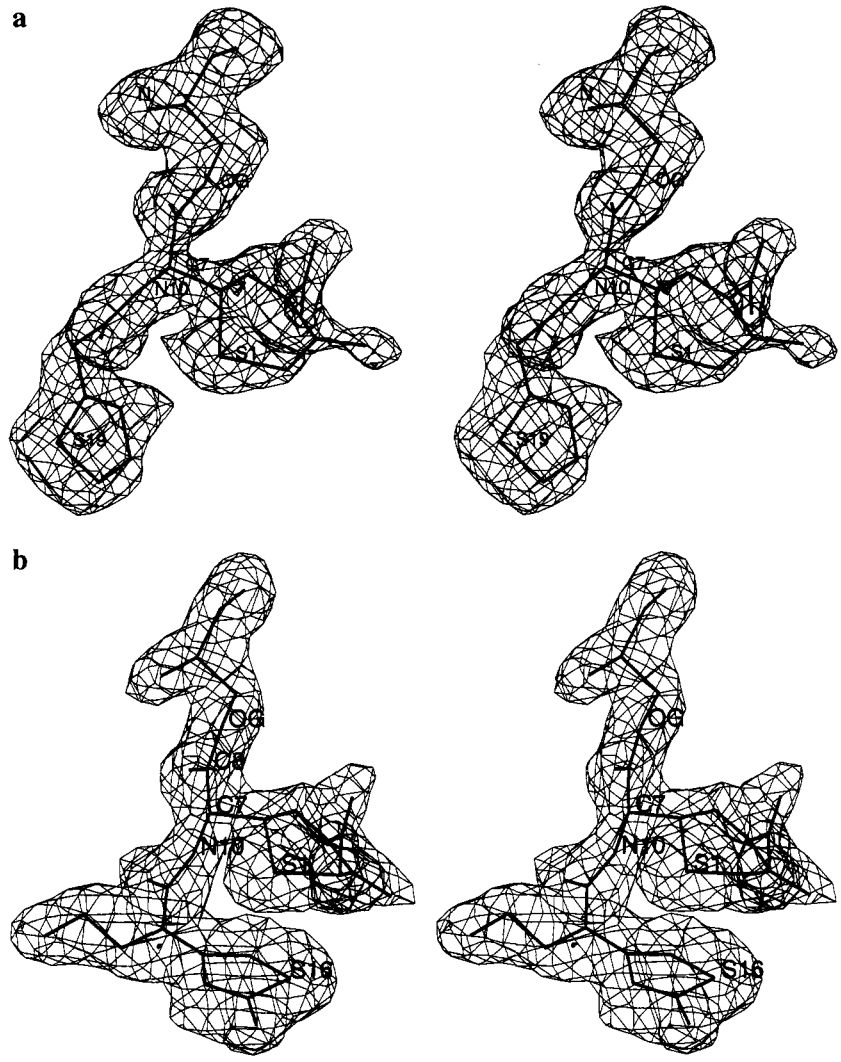


FIGURE 2: Stereoviews of the electron density of the acylated (a) cephalothin and (b) cefotaxime moieties bound to Oγ of Ser62. Coefficients used are $F_{obs} - F_{calc}$. Ser62 and ligand were omitted from the phase calculation. The contour level in each map is 2.5σ.

carbonyl carbon atom C8. Figures 5 and 6 show the β-lactamoyl moieties and the amino acid residues in the binding site. Two binding site residues, Lys65 and Tyr159, having multiple conformations in the native structure, were now found in a single conformation.

Important interatom distances are listed in Table 5 and shown schematically in Figure 7. Navia and co-workers (Navia et al., 1987) have shown that the reactive exocylic 3′-methylene group, generated during β-lactam ring-opening in cephalosporins having a suitable leaving group L, promotes inhibition of classical serine proteases such as elastase. Besides the Ser195 ester bond, a second covalent linkage is formed between C3′ and His57 of elastase. In the DD-peptidase, the His298 in the binding site is structurally unrelated to that in elastase, and given its 7−8 Å distance from C3′, it is not surprising that His298 does not form a 3′ linkage with either intermediate. Though PBPs have a longer lysine side chain in this position, it is unlikely lysine could span the distance to C3′. The His298 does not bind the C4 carboxyl group as might be expected but instead hydrogen bonds to Tyr280 and Thr299. Its ring nitrogen atoms are more than 4 Å from the OH group of Tyr159. Site-directed mutagenesis has shown that His298 contributes a factor of 10−100 to the rate of acylation by β-lactams, but plays only a minor role in deacylation by water (Hadonou et al., 1992). While the exact role of this imidazole remains unclear, our finding that His298 does not directly interact with the acyl intermediate confirms the mutagenesis and kinetic studies.

The nearest contacts between the Oγ atom of Ser62 and the potential general bases in the catalytic reaction (Wilkin et al., 1993), Lys65 and Tyr159, are about 3 Å in each complex (distances A and C) but are slightly shorter in the cephalothin case. In each complex, the residue closer to the Oγ atom is Tyr159. The hydrogen bonding of the serine-bound carbonyl group with the two amide groups in the oxyanion pocket (Murphy & Pratt, 1988) is somewhat asymmetric (L1 and L2), with the CO group closer to the NH group of Ser62.

Other interactions (J and K) which bind the 7-amido linkage of the β-lactam to the enzyme involve Thr301 and the side chain groups of Thr116 and Asn161. The only strong interaction of the six-membered dihydrothiazine ring with the enzyme occurs between the C4 carboxylic acid group and the side chain hydroxyl groups of Thr299 and Thr301 (distances M). The N5 atom in the ring does not interact with the enzyme; the OH of Tyr159 is at least 3.8 Å away in each complex. As seen in other β-lactamoyl complexes with the DD-peptidase (Kelly et al., 1985, 1989), the hydrophobic thiophene substituent of cephalothin is rather exposed and is not restrained by the enzyme. The rotational conformation about the C11−C13 bond of the branched cefotaxime side chain, with the methoxy group buried in the

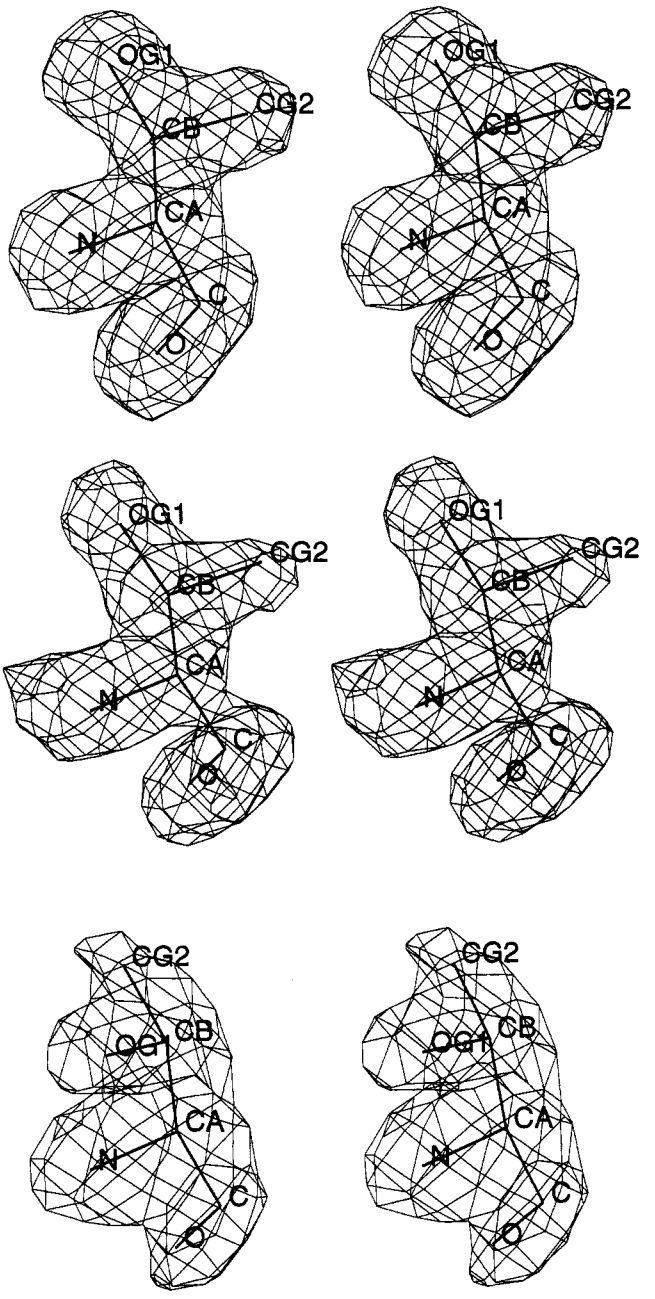


FIGURE 3: Stereoviews of the electron density of residue Thr301 in (a, top) native enzyme, (b, middle) cephalothin complex, and (c, bottom) cefotaxime complex. Coefficients used are $F_{obs} - F_{calc}$. Thr301 was omitted from the phase calculation. The contour level in each map is 3.5σ.

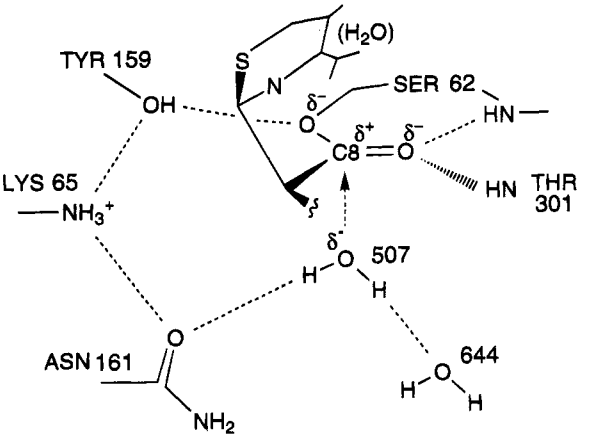
binding site and the aminothiazole ring more exposed, is opposite to the conformation observed in the side chain of the monobactam aztreonam bound to a class C β-lactamase (Oefner et al., 1990) because aztreonam has a larger, hydrophilic *tert*-carbon group in the oxime.

Upon formation of the cephalothin intermediate, five water molecules in the native enzyme (554, 620, 624, 645, 650) were displaced from the catalytic site, and three additional molecules (455, 579, and 584) were displaced by the larger cefotaxime. Water molecule 554 had been in the vicinity of the C4 carboxylic acid group of the dihydrothiazene ring. Water 650, which had been bonded between Thr116 and Asn161, was displaced by the 7-amido C−O group of each β-lactam. Notably, no water was displaced by the C8 carbonyl group from the oxyanion pocket, as none is present here in the native enzyme. Two native water molecules (507 and 644) remain near the ester linkage, hydrogen bonded to each other (2.6 Å), but now rather buried by the β-lactamoyl moiety. Each has shifted 0.5−0.8 Å from its native position. Of the two, 507 is closer to the acyl carbon atom C8 (distances S1) of each intermediate.

Comparison of the Two Complexes. The rigidity of the branched side chain of cefotaxime, and the contact of its methoxy group with Phe120, Trp233, and the B3 β-strand, causes a marked tilt of this ligand relative to cephalothin (Figure 6). The difference in side chain size and rigidity results in a significant difference in the way the two ligands hydrogen bond to the B3 β-strand. While cephalothin bonds to the backbone CO of Thr301 (J1 distance), the tilted cefotaxime is unable to form this hydrogen bond and instead bonds to the side chain OH group (J2). The lengths of the J1 and J2 hydrogen bonds in the two complexes are equivalent (2.9 Å).

The anchoring of the C4 carboxylic acid group to Thr299 and Thr301 differs somewhat in the two complexes because of the tilt discussed above. The COO^- group of cephalothin is held by two strong hydrogen bonds (M1 and M2) while the COO^- of cefotaxime is held by only one to Thr299. If one compares the binding of cephalosporins and penicillins in this binding site (Kelly et al., 1989), it is likely that the M2 hydrogen bond may be weaker in penicillin complexes. This difference may explain results of an early selection study on *Escherichia coli* containing a class A TEM β-lactamase (Hall & Knowles, 1976), and a later site-saturation mutagenesis study (Healey et al., 1989), showing that in the β-lactamase a hydrogen-bonding group (Thr or Asn) introduced at a position equivalent to 301 gave improved cephalosporinase activity.

The bonding of O9 in the ester carbonyl group to the two amide NH groups in the oxyanion hole (L distances) is stronger in the cephalothin complex. The hydrogen bond from the protonated Tyr159 (see below) to the Oγ of Ser62 is also slightly stronger in this complex. Both bondings enhance the nucleophilicity of the two oxygen atoms of the ester linkage in the cephalothin complex. Therefore, the C8 of cephalothin, compared to that in cefotaxime, is likely to be more electrophilic and more susceptible to attack by water molecule 507, as indicated below. Accordingly, the S1 distance is significantly shorter in the cephalothin complex. Furthermore, the two dihedral angles about the Ser62Oγ−C8 bond translate to an out-of-plane deviation of C8 toward 507, 0.15 Å in cephalothin but only 0.04 Å in cefotaxime, which is consistent with the relative hydrolytic instability of the cephalothin intermediate (Table 1).



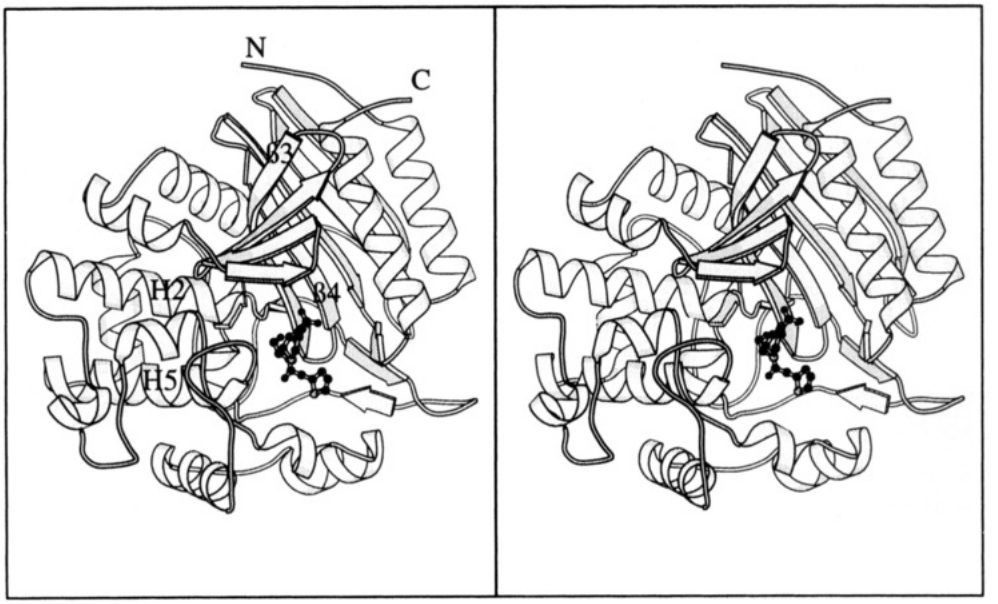


FIGURE 4: MOLSCRIPT (Kraulis, 1991) drawing of DD-peptidase with the Ser62-bound cephalothin intermediate in the β-lactam-binding site.

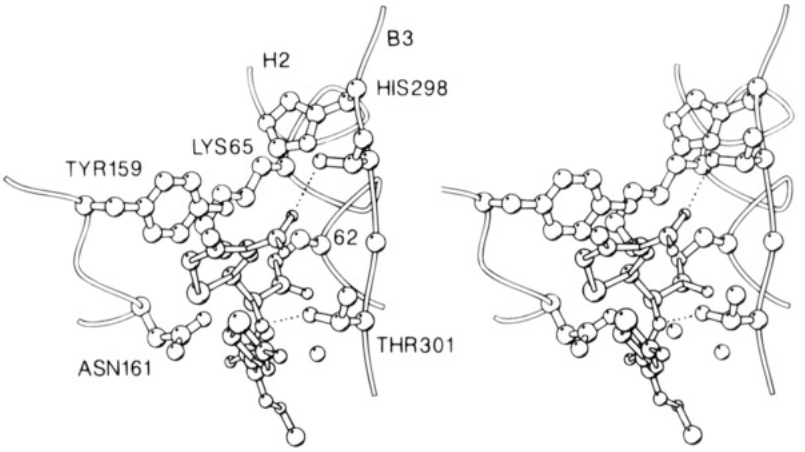


FIGURE 5: MOLSCRIPT (Kraulis, 1991) drawing of the residues surrounding the acylated cefotaxime in the binding site of DD-peptidase. Some hydrogen bonds are indicated by dotted lines (hydrogen atoms are omitted). Two water molecules (507 on left and 664) are shown as spheres.

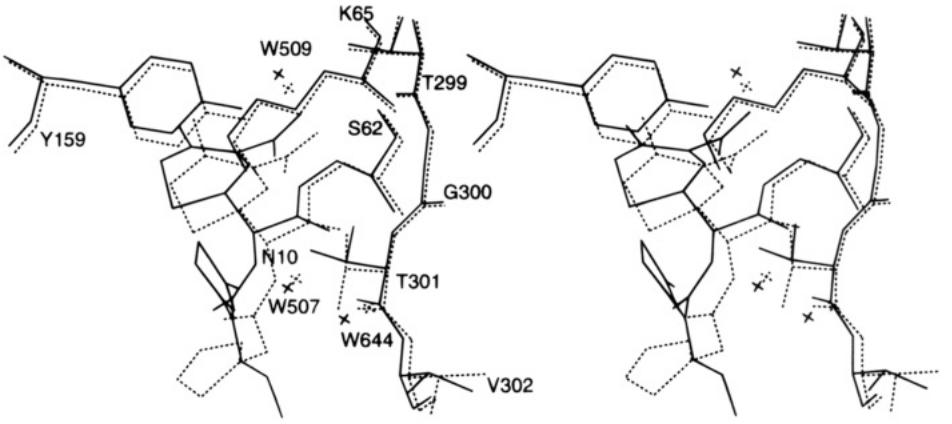


FIGURE 6: Stereoview of serine-bound cephalothin (dotted) and cefotaxime moieties and water molecules (x) in the binding site. View is approximately 90° to Figure 5.

The diagram shows that the dihydrothiazene ring, in effect a tethered leaving amine group, could prevent a water molecule from approaching the acyl ester linkage from the opposite (top) side of the ester plane. Such a water molecule might otherwise be activated by Tyr159, as possibly in the class C β-lactamases (Lobkovsky et al., 1994; Oefner et al., 1990). In fact, we see that the ring has displaced water molecule 554, which was quite near this position in the native enzyme. Thus, water 507 is perhaps not the intended deacylation agent, but only a poor substitute.

Comparison of Complexes with the Native DD-Peptidase Structure. Changes in the protein structure after acylation were evaluated by overlaying the coordinates of the complex and minimizing differences (Nyburg, 1974). The rms

Table 5: Distances (Å) in Complexed and Native DD-Peptidase

distance[a]	cephalothin	cefotaxime	native[b]
A	3.05	3.22	4.13
B	2.71	2.88	2.76
C	2.96	3.12	3.76
D	4.11	3.78	
E	3.05	3.19	2.86
F	3.45	3.26	3.41
J1	2.82	3.38	
J2	5.03	2.89	
J3	2.92	2.69	2.91
K1	2.79	3.07	
K2	4.36	4.42	
L1	2.66	2.83	
L2	2.82	2.99	
M1	2.82	2.65	
M2	2.73	3.42	
S1	3.07	3.59	
S2	3.44	3.70	
P	2.78	2.83	2.61
Q	2.87	2.72	2.59

[a] See Figure 7. [b] Kelly & Kuzin (1995). With Lys65 and Tyr159 in conformation A.

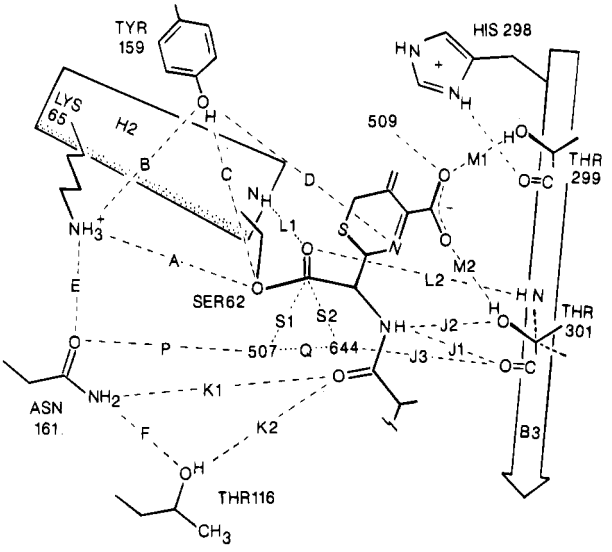


FIGURE 7: Schematic drawing of interactions between the β-lactamoyl intermediate and groups in the binding site. Labeled distances for each complex are given in Table 5.

Table 6: Difference (rms, Å) between Complexes and Native Structure

atoms	cephalothin	cefotaxime
Cα only	0.13	0.18
protein	0.29	0.36
water	0.33	0.46
all	0.30	0.44

deviation in position for Cα atom coordinates in the cepahlothin and cefotaxime complexes is only 0.13 Å and 0.18 Å, respectively (Table 6).

Upon formation of the acyl bond between Ser62 and C8 of the β-lactam ring, the Oγ atom of Ser62 rotates 65−70° away from B3 (Table 4). This rotation is required for the carbonyl oxygen atom O9 to remain bound in the oxyanion pocket. The most important change relative to the native conformation, however, involves Thr301 of the cefotaxime complex. Figure 3 shows the side chain of Thr301 undergoes a 110° rotation about its Cα-Cβ bond to allow hydrogen bonding (J2) with the tilted cefotaxime species. No rotation occurs in the cephalothin complex.

In the native enzyme, active site residues Lys65 and Tyr159 exhibited two conformations A and B, each with about equal occupancy, such that the Nε or OH atoms differed in position by about 2.5 Å. After complexation with either cephalosporin, Lys65 and Tyr159 become fixed in the A conformation, bringing Tyr159 closer to the intermediate than would the B conformation. Based on examination of interresidue distances in a transition state intermediate of a class C β-lactamase having a similar active site (Lobkovsky et al., 1994), we assume here that Tyr159 is unprotonated in the native DD-peptidase and is protonated in these complexes. That a new hydrogen bond is formed in the complexes between Tyr159 and the acylated Oγ atom of Ser62 is supported by the significant shortening of distance C by 0.6−0.8 Å (Table 5).

DISCUSSION

Using the *S.* R61 DD-peptidase as a model of the penicillin-binding proteins has proven invaluable to understanding the structure, kinetics, and mechanism of the homologous multidomain higher molecular weight membrane-bound forms (Frere et al., 1992; Ghuysen, 1994; Jamin et al., 1993; Xu et al., 1994). It has long been known that the inhibition of cell wall synthesis by β-lactams arises from the acylation of the PBPs with a deacylation rate which is relatively slow compared to the growth rate of the microorganism (Tipper, 1985; Waxman & Strominger, 1983).

The conformations of the serine-bound β-lactam moieties in these two complexes, and those with cephalosporin C, (2,3)-α-methylene penicillin, and a monobactam (Kelly et al., 1989), suggest that prior to acylation, the pseudopeptide backbone of the β-lactam aligns antiparallel to β-strand B3 with the C4 carboxylic acid group near the side chain hydroxyl group of Thr299. The carbonyl group of the β-lactam ring binds in the oxyanion pocket formed by the backbone NH groups of Ser62 and Thr301. As a result, the carbonyl group is polarized and made susceptible to nucleophilic attack by the γ-oxygen atom of Ser62 prior to formation of the tetrahedral intermediate, which collapses to the acyl intermediate seen here. Although subsequent steps are not well understood in the case of cephalosporins (Frere & Joris, 1985), it is thought that a slow hydrolysis of the acyl ester bond via a second tetrahedral intermediate regenerates the active enzyme. The search for a general acid/base catalyst in this nonclassical serine-reactive peptidase has focused on Tyr159 (Wilkin et al., 1993). In the present work, and in parallel studies of a transition-state analog of a class C β-lactamase (Lobkovsky et al., 1994), we note that the distance C between the phenolic oxygen atom of Tyr159 (or Tyr150 in the β-lactamase) and the Oγ of the ester bond of the intermediate is always shorter than in the native enzymes, an observation supporting an earlier β-lactamase mechanism (Oefner et al., 1990) with a catalytic phenoxide anion in the native enzyme.

β-Lactamases, which have similar three-dimensional structure to the DD-peptidase and presumably evolved from it (Kelly et al., 1986; Knox & Kelly, 1989; Lobkovsky et al., 1994; Murphy & Pratt, 1991; Samraoui et al., 1986), are distinguished by their more rapid rates of deacylation (Escobar et al., 1994; Waley, 1992), and it has been considerably more difficult to observe by crystallographic methods the acyl complexes between β-lactamases and β-lactams. Mapping of β-lactamase intermediates has re-

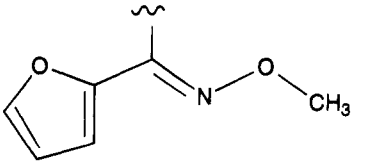
quired the use of either catalytically-impaired mutants (Knox et al., 1993; Strynadka et al., 1992), poor substrates (Oefner et al., 1990), or nonantibiotic inhibitors (Chen & Herzberg, 1992; Chen et al., 1993; Lobkovsky et al., 1994), often in combination with cryocooling and rapid X-ray data collection. In the β-lactamase studies, the orientation of the β-lactamoyl moiety generally resembled that previously reported in the much more stable DD-peptidase complexes (Kelly et al., 1982, 1985, 1988, 1989).

Relative Stability of the Acyl Intermediates. The kinetic rate constant k_3 for turnover of the acyl form of cephalothin or cefotaxime is very low, near 5×10^{-6} s^{-1} (Frere & Joris, 1985). We have observed two buried water molecules likely to be involved in the slow hydrolysis, and we are able to correlate their interactions with the measured turnover rates in Table 1. The less stable intermediate, cephalothin with $t_{1/2} \approx 20$ h, would be expected to display the shorter S1 distance (Table 5), the stronger polarization of the carbonyl bond by the oxyanion pocket (shorter L distances), the stronger polarization of the ester Oγ atom (shorter A and C distances), the greater tetrahedral character of the ester C8 atom, and the stronger activation of the water molecule by the enzyme (shorter P distance). Thus, this degree of enzyme-induced nucleophilicity at C8, the activation of water 507 (albeit weak), its proximity to the C8 carbon atom, and the nonplanarity at C8 are all nicely consistent with the relative instability of the cephalothin intermediate compared to the cefotaxime intermediate.

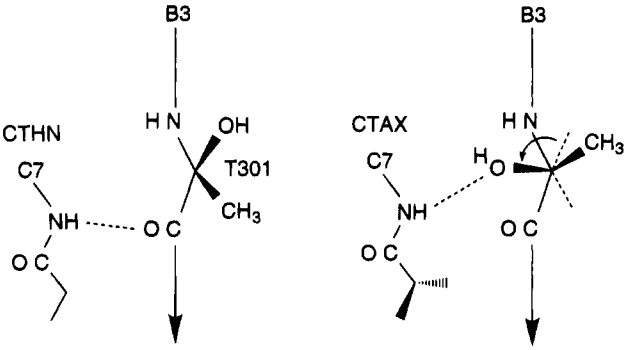
In this DD-peptidase, and probably in the PBPs, the remarkable stability of cephalosporinyl intermediates may be due to two factors: (1) the blocking by the tethered dihydrothiazene ring of the water molecule attempting to attack the acyl bond; and (2) the inability of residues such as Asn161 to effectively activate a substitute water molecule (507) for attack from another direction. In contrast, in the catalytic site of class A β-lactamases, a nucleophilic water is strongly activated by three residues, primarily a conserved glutamic acid (Herzberg, 1991; Jelsch et al., 1993; Knox & Moews, 1991; Lamotte-Brasseur et al., 1991; Strynadka et al., 1992). Interestingly, a well-activated water molecule has not been clearly identified in the catalytic site of a class C β-lactamase (Lobkovsky et al., 1993, 1994; Oefner et al., 1990), which for this reason, and because its tertiary structure is closer to this DD-peptidase than to the class A β-lactamase, has been labeled a more primitive β-lactamase (Lobkovsky et al., 1993).

The Pivotal Role of Thr301. As the active site of DD-peptidase is rather open and free of flexible loops, it is not unexpected that acylation with cephalosporins produces in the native structure only localized conformational changes. One such change, a side chain rotation, occurs in residue Thr301, but the rotation occurs only in the cefotaxime complex. All β-lactamoyl and phosphonyl intermediates previously studied by crystallography (Chen & Herzberg, 1992; Chen et al., 1993; Kelly et al., 1989; Lobkovsky et al., 1994; Oefner et al., 1990; Strynadka et al., 1992) are bound to the *backbone* CO of this residue (237/318 in class A/C β-lactamases). In the case of cefotaxime, however, we have found that steric crowding of its rigid oxime substituent has necessitated its hydrogen bonding to the *side chain* OH of Thr301 after a 110° rotation from the native conformation.

Site-directed mutation of Thr301 to isoleucine produced little change in the binding and kinetic parameters of a group of seven β-lactams, unfortunately not including cefotaxime



(Wilkin et al., 1994). As six of these contained flexible C7 substituents presumably not dependent on the side chain OH of Thr301, this null result is expected. However, one of the β-lactams in the group was cefuroxime, which contains a rigid substituent similar to that in cefotaxime and which might be expected to utilize the side chain OH on the wild-type enzyme. It would be useful to measure the kinetic parameters of cefotaxime with the Thr301Ile mutant of DD-peptidase.



Thr301 and a Natural Mutation in PBPs and β-Lactamases. Another instance in which the side chain of residue 301 is uniquely implicated in cefotaxime binding is found in the microbiology literature. Altered PBPs with low affinity for cefotaxime have arisen spontaneously in *Streptococcus pneumoniae* grown in the presence of cefotaxime (Laible & Hakenbeck, 1991). On the basis of a sequence alignment of *S. pneumoniae* PBPs and the title DD-peptidase (Ghuysen, 1994), it appears that the cefotaxime-resistant PBP-2x of *S. pneumoniae* contains a Thr-to-Ala change at a position corresponding to 301 in DD-peptidase (Table 7). The weaker affinity of this mutant for cefotaxime is therefore entirely consistent with our findings and proposed role for the hydrogen-binding side chain of threonine in cefotaxime binding.

We note also the spontaneous mutability of this residue in TEM-type β-lactamases, two natural variants of which have been found to contain an alteration of the equivalent residue (237) from alanine to threonine (Jacoby, 1994; Jacoby & Medeiros, 1991). These so-called extended-spectrum class A β-lactamases have the troublesome ability to hydrolyze third-generation cephalosporins such as cefotaxime. Hydrogen binding by cefotaxime to the new Thr237 side chain in these mutant β-lactamases may therefore facilitate forma-

Table 7: Mutations in PBP-2x and TEM β-Lactamase Corresponding to Position 301 in DD-Peptidase

enzyme	equivalent position
DD-peptidase (*S.* R61)	T301
PBP-2x mutant (*S. pneumoniae*)[a]	T550A
TEM-5 and −24 β-lactamases[b]	A237T

[a] Does not bind cefotaxime (Laible & Hakenbeck, 1991). [b] Hydrolyzes cefotaxime (Jacoby, 1994).

tion of the Michaelis complex and/or may help align the acyl intermediate for attack by the hydrolytic water molecule.

These crystallographic binding studies, with the observation of a conformational change in the side chain at 301 and its pattern of hydrogen bonding to several cephalosporin intermediates, have provided a significant clue to understanding a possible mechanism used by emerging β-lactam-resistant PBPs and extended-spectrum β-lactamase mutants arising in response to third-generation β-lactam antibiotics.

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