Evidence for Structural Elasticity of Class A β-Lactamases in the Course of Catalytic Turnover of the Novel Cephalosporin Cefepime

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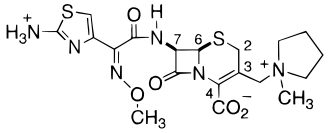
Received August 28, 1995[⊗]

Abstract: The mechanism of hydrolysis of cefepime, a novel cephalosporin, by the class A TEM_{pUC19} β-lactamase has been investigated. Models for the active-site binding of this antibiotic indicate severe steric interactions between the active site of the enzyme and the $C_{7\beta}$ function of cefepime. Specific interactions with the side-chain functions of Pro-167 and Asn-170, amino acids present in the Ω-loop spanning residues 164−179, have been singled out as important in the interactions with the antibiotic. These interactions displace the hydrolytic water (Wat-712) from its preferred position for the deacylation step. These observations are consistent with experimental evidence that deacylation is the rate-limiting step in the turnover of cefepime by this β-lactamase. Furthermore, it has been shown in circular-dichroic measurements that hydrolysis of cefepime by this β-lactamase is accompanied by an unprecedented relaxation of the structure of the enzyme in order to accommodate the bulky $C_{7\beta}$ side chain of the antibiotic in the active site. These findings are in good agreement with dynamics simulations of the structure of the acyl-enzyme intermediate, which support the possibility for the structural relaxation of the protein once this intermediate forms. The class C β-lactamase Q908R, lacking the Ω-loop structural motif, turns over cefepime, and the kinetic parameters for this process were evaluated. In contrast to the class A β-lactamase which we studied, the kinetics were sufficiently fast that circular-dichroic experiments with the Q908R enzyme during hydrolysis of cefepime could not be carried out. Two mutant variants of the class A TEM_{pUC19} β-lactamase, Asp-179-Gly and Arg-164-Asn, were prepared to explore whether an enlargement of the active site would facilitate turnover of cefepime. Both mutant enzymes showed improved interactions with cefepime, consistent with our expectations. Kinetic analyses for turnover of cefepime by the parental enzyme and both of its mutant derivatives are presented.

Over the past 10−15 years a variety of new broad-spectrum β-lactam antibacterials have been introduced into clinical use, including extended-spectrum penicillins (*e.g.*, azlocillin and piperacillin), third-generation cephalosporins (*e.g.*, cefotaxime, ceftriaxone, cefoperazone, and ceftazidime), a carbapenem (*e.g.*, imipenem/cilastatin combination, *i.e.*, Primaxin), a monobactam (aztreonam), and mechanism-based inactivators of β-lactamases in combination with older penicillins (clavulanate with amoxicillin or ticarcillin, and sulbactam with ampicillin). The utility of third-generation cephalosporins has been compromised by mutational derepression of class C β-lactamases in important nosocomial pathogens, such as *Enterobacter* spp., *Serratia marcescens*, and *Pseudomonas aeruginosa*. In order to reduce this problem, new cephalosporins, such as cefepime (**1**), were developed, which have useful activity against strains with derepressed class C enzymes that confer resistance to third-generation cephalosporins.[1] In general, all these newer β-lactams are resistant to the action of most β-lactamases and their activity against a broad spectrum of Gram-negative bacteria is due in part to this property. The underlying structural and mechanistic bases for resistance to the action of β-lactamases for various extended-spectrum β-lactams are distinct. We have recently addressed the details of interactions of mechanism-based inactivators[2−4] and imipenem[5,6] with class A β-lactamases. We will disclose in this paper new insights on the unique features of the turnover chemistry of the new cephalosporin cefepime (**1**). We will present evidence that turnover of this molecule by the TEM_{pUC19} β-lactamase, a prototypic class A enzyme, involves conformational changes by the protein in the course of enzymic hydrolysis of the lactam bond, which lead to the unraveling of the structure of the enzyme in a process which is not entirely reversible. Furthermore, we will present a kinetic analysis for turnover of this novel β-lactam molecule.



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[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1996.

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S0002-7863(95)02975-1 CCC: $12.00

Experimental Section

Amino acid substitution at position 179 of the TEM_{pUC19} β-lactamase[7] was performed as reported elsewhere.[8] The parental TEM_{pUC19} β-lactamase and its Arg-164-Asn and Asp-179-Gly mutant derivatives were purified according to the method of Zafaralla *et al*.,[9] and the Q908R β-lactamase was purified as described by Ross.[10] The enzymes were purified to apparent homogeneity, as judged by SDS polyacrylamide gel electrophoresis. Cefepime was obtained from Bristol-Myers Squibb. Terrific Broth was purchased from Gibco. Kinetic measurements were carried out with a Hewlett-Packard 452 diode array instrument. Circular-dichroic (CD) spectra were recorded on a Jasco Model J-600 spectropolarimeter. Crystal coordinates for the TEM-1[11] β-lactamase were used in the three-dimensional modeling and energy minimization. Computations were performed on a Silicon Graphics R4000 Indigo computer.

Mutagenesis. *Escherichia coli* JM83[12] was the recipient strain for plasmids. Plasmid pUC19::kan was constructed by cloning the kanamycin-resistance gene, *aph*A1, from Tn903[13] into the *Bam*HI site in the polylinker of pUC19.[12] This plasmid encodes the TEM-1 β-lactamase[14] containing two mutations, Val-84-Ile and Ala-184-Val, which are removed from the active site and are considered to be enzymologically inconsequential.[15] The Arg-164-Asn mutation was obtained as one of a series of mutants with replacement of Arg-164 by site-saturation mutagenesis, using the transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA) and a mutagenic oligonucleotide mixture with all four bases at each of the three positions corresponding to the codon for residue 164. The mutated plasmid DNA was introduced into the recipient *E. coli* strain JM83 by electroporation, and transformants were selected by growth on LB agar medium containing kanamycin (30 μg/mL). The DNA for nucleotide sequencing was isolated by the alkaline method.[16] The entire β-lactamase structural gene and its promoter region in each putative mutant plasmid were sequenced by the method of Sanger *et al*.,[17] using a Sequenase Version 2.0 DNA sequencing Kit (US Biochemicals, Cleveland, OH) and [α-^{35}S]-dATP (Dupont NEN, Boston, MA). The Asn-164 mutation was identified, and the remainder of the sequences of the entire structural gene for the β-lactamase and its promoter region were verified as identical to the parental gene.

Bacterial Growth. A 5-mL seeded culture was incubated at 37 °C for 6 h in the LB medium. One-milliliter portions of this culture were transferred into four Erlenmeyer flasks containing 1 L of the Terrific Broth medium (47 g/L). The culture was incubated at 37 °C for 18 h. After centrifugation, 45−50 g of wet cell paste was obtained, which was used for enzyme purification.

Steady-State Rate Measurements. Hydrolysis of cefepime was monitored by the decrease in absorbance at 260 nm ($\Delta\epsilon_{260} = 750$ M^{-1} cm^{-1}) in 50 mM sodium phosphate, 100 mM NaCl, pH 7.0. The values of k_{cat} and K_m were determined from the Lineweaver−Burk plots of the initial steady-state velocities at various cefepime concentrations (30−200 μM) with the TEM_{pUC19} enzyme, its Asn-164 and Gly-179 mutant derivatives, and the Q908R enzyme. Initial rates were generally determined from the first 5−10% (over 30 min) of the reaction with five substrate concentrations flanking the K_m values. A delay of over 200 s was observed for hydrolysis of cefepime by the wild-type TEM_{pUC19} enzyme before the onset of the steady-state kinetics. Rate measurements were made during the steady-state phase of hydrolysis in these cases.

We determined the activity of the TEM_{pUC19} and Q908R β-lactamases after their hydrolysis of cefepime. The measurements were made in three different determinations. Typically, the enzyme (3.5 μM) was incubated with 200 μM cefepime overnight (20 h) at room temperature. Then, a 5-μL aliquot of the solution was added to 995 μL of penicillin G (2 mM), and hydrolysis of penicillin G was monitored ($\Delta\epsilon_{240} = 570$ M^{-1} cm^{-1}). A control experiment was also carried out for which everything was identical to the above experiment, except no cefepime was added to the mixture.

Determination of the Dissociation Constants. The dissociation constants (K_s) for cefepime with the TEM_{pUC19} enzyme, its Asn-164 and Gly-179 mutant derivatives, and the Q908R enzyme were determined according to the procedure of Zafaralla *et al*.[9] Two concentrations (300 and 500 μM) of substrate ([2-[[*p*-(dimethylamino)-phenyl]azo]pyridino]cephalosporin, PADAC; $\Delta\epsilon_{610} = 1240$ M^{-1} cm^{-1}) in 100 mM sodium phosphate buffer, pH 7.0, were used in each case. A series of mixtures containing both PADAC and various concentrations of cefepime (0−33 mM for the wild-type TEM_{pUC19}, 0−13 mM for the Asn-164 mutant enzyme, 0−2 mM for the Gly-179 mutant enzyme, and 0−2 mM for the Q908R enzyme) were prepared in 100 mM phosphate buffer, pH 7.0, in a total volume of 0.3 mL. A portion of the enzyme was added to start the inhibition assays (final concentrations of 170, 88, 300, and 200 nM for the TEM_{pUC19}, Asn-164 and Gly-179 mutant derivatives, and the Q908R β-lactamase, respectively). We determined that in the course of the initial-rate measurements for hydrolysis of PADAC over typically 2 min much less than 1% hydrolysis of cefepime took place. Invariably, cefepime served as a competitive inhibitor for hydrolysis of PADAC by all β-lactamases that were studied. Hence, the measurements would evaluate the influence of the noncovalent interactions of cefepime in the enzyme active site on hydrolysis of PADAC.

Calculations of the Microscopic Rate Constants. In light of the fact that turnover of cefepime by our enzymes takes place exceedingly slowly, the determinations of the dissociation constants (K_s) were made very accurately and conveniently. Turnover of these molecules follows the kinetic scheme shown below:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E{-}S \xrightarrow{k_3} E + P \qquad (1)$$

For this scheme the following kinetic parameters are derived: $k_{cat} = (k_2k_3)/(k_2 + k_3)$, $K_m = (k_3K_s)/(k_2 + k_3)$, and $k_{cat}/K_m = k_2/K_s$, assuming that $k_{-1} > k_2$. This assumption is valid for a poor substrate such as cefepime. The values of k_2 and k_3 were conveniently calculated using the equations given above and experimentally determined values of the kinetic parameters k_{cat}, K_m, and K_s.

Circular-Dichroic Measurements. A protein concentration of 3.5 μM for the various β-lactamases in 10 mM sodium phosphate, pH 7.0, was used in these experiments. The parental TEM_{pUC19} β-lactamase (3.5 μM) and 240 μM of cefepime were incubated to investigate the time course of the hydrolytic reaction. The concentration of cefepime used was that of the corresponding K_m for the TEM_{pUC19} β-lactamase. A CD cell with a path length of 1 mm was used in the measurements. Neither cefepime nor the hydrolytic product had any CD spectrum.

Computational Models. We fitted the structures of the acyl-enzyme intermediates for cefepime (**2** and **3**) in the crystal coordinates for the active site of the TEM-1 β-lactamase, and then only the energy of the substrate portion of the complex was minimized within the confines of the active site. The energy minimization was carried out with the MOPAC method,[18] using AM1 parametrization. In our hands, AM1 provides a better root-meat-square deviation for β-lactam molecules than the other semiempirical methods such as MNDO and PM3, when the results are compared to the crystallographic data. Subsequently, charges were calculated for the atoms of cefepime using MOPAC MNDO ESP charges,[18] which approach the values for the GAUSSIAN charges closely. Hydrogen atoms were added in the calculated positions in the crystal structure of the TEM-1 β-lactamase, and the energy was minimized by the geometry optimization algorithm MAXMIN2, using the Tripos force-field parameters implemented in Sybyl-6.22. Kollman[19] charges were used for the protein. Finally, the complexes **2**

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Table 1. Kinetic Parameters for Turnover of Cefepime by the Class A TEM_{pUC19} β-Lactamase, Its Structural Variants, and the Class C Q908R β-Lactamase[a]

β-lactamases	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	K_s (mM)	k_2 (s^{-1})	k_3 (s^{-1})
TEM_{pUC19}	0.6 ± 0.1	240 ± 50	$(2.5 \pm 0.6) \times 10^3$	10.3 ± 0.8	26 ± 7	0.6 ± 0.2
TEM_{pUC19} D179G	0.024 ± 0.001	15 ± 2	$(1.6 \pm 0.2) \times 10^3$	1.7 ± 0.2	2.7 ± 0.5	0.024 ± 0.006
TEM_{pUC19} R164N	290 ± 30	320 ± 17	$(9.1 \pm 1.1) \times 10^5$	0.7 ± 0.1	635 ± 117	535 ± 130
Q908R[b]	121 ± 12	422 ± 44	$(2.9 \pm 0.4) \times 10^5$	0.8 ± 0.1	232 ± 44	253 ± 63

[a] Experiments were carried out in 50 mM sodium phosphate, 100 mM NaCl, pH 7.0. [b] The class C Q908R β-lactamase is a variant of the β-lactamase from *Enterobacter cloacae* P99, which is different only in four amino acids at sites remote from the active site. The two enzymes are believed to be virtually identical in both structure and mechanism.

and **3** in the active site of the TEM-1 β-lactamase were energy-minimized by the MAXMIN2 method within a radius of 15 Å from the active site until the energy-gradient change was less than 0.01 kcal/(mol·Å) between two successive iterations.

We carried out 10 ps of dynamics simulation on the energy-minimized structures for **2** at 0, 50, and 300 K. The complexes were heated over 0.5 ps to 50 K and over 1.5 ps (plus 0.5 ps of equilibration) to 300 K, before the 10 ps of simulation was carried out. For each dynamics run, the time step was 1 fs, nonbonded lists were updated every 25 fs, and nonbonded cutoff was 12 Å. The conformations were sampled each 100 steps. The Tripos force-field parameters were used.[20] Kollman charges for protein and water molecules[19] and MOPAC MNDO ESP charges[18] for small molecules were applied for the simulation.

Product of Cefepime Hydrolysis. A solution of 20 mg of cefepime in 10 mM phosphate buffer, pH 7.0 (5 mL), was incubated with the Q908R enzyme (1×10^{-6} M final concentration) at room temperature for 24 h. The progress of reaction was followed by monitoring the disappearance of cefepime by reverse-phase C_{18} thin-layer chromatography (R_f 0.30 for cefepime in 1/1 MeOH/H_2O). After a duration of 24 h, the mixture was subsequently filtered through an Amicon YM-10 membrane to remove the enzyme. The hydrolytic product in the filtrate was purified by HPLC: $t_R = 11.7$ min (Vydac, C_{18} column, 25 × 1.0 cm, 5-μm particle size, linear gradient 2−98% acetonitrile in water over 50 min, 1.0 mL/min, 260 nm); mp 190 °C dec.; IR (KBr) 1628, 1640 (enol), 1646 (amide carbonyl), 1656 (oxime) cm^{-1}. ^1H-NMR (D_2O): while this analysis was not very informative, it was quite apparent that the product lacked the six-membered ring of the cephalosporin and the 3′ substituent. MS FAB^+: 286 (M + H, 4), 200 (1), 156 (1), 131 (11).

Attempt at Detection of a Covalent Enzyme−Cefepime Species during the Lag Time and Other Intermediates in the Course of Catalysis. A solution of TEM_{pUC19} β-lactamase (final concentration 9.2×10^{-4} M) was incubated with cefepime (final concentration 9 mM). At 30-, 60-, 90-, and 180-s intervals, a 20-μL portion of the mixture was added to 280 μL of 1% SDS in 100 mM phosphate buffer, pH 7.0. The UV spectra for the denatured protein were then recorded. A similar experiment was carried out in the absence of cefepime. The denatured protein samples were passed through a Centricon-10 filter to remove small molecules, and the protein portions were each reconstituted in 300 μL of the same buffer to measure their spectra. No change in the UV spectra for the samples from the reaction mixture and the native denatured enzyme was seen.

To gain insight into the spectroscopic character of potential intermediates after attack of the nucleophile at the β-lactam carbonyl and the attendant β-lactam ring opening, we carried out a model study. A solution of cefepime (1.6 mg) in 160 μL of 4 M ammonia was stirred at room temperature. For monitoring of the progress of the reaction and study of the nature of the chromophores for the reaction mixture, the reaction was monitored initially every few minutes, and subsequently every few hours. Aliquots (3 μL each) were removed at various time intervals and diluted into 997 μL of 100 mM sodium phosphate buffer, pH 7.0 (to give a final concentration of 60 μM), and the UV−vis spectra were recorded.

A similar experiment was carried out for monitoring by circular dichroism. A 20 mM solution of cefepime in 4 M ammonia was prepared. A 24 μM aliquot of this mixture was added to 1.976 mL of 100 mM sodium phosphate buffer, pH 7.0, and the circular-dichroic spectrum was recorded.

Results and Discussion

Cefepime was developed as a β-lactam antibiotic resistant to the hydrolytic activity of β-lactamase. The kinetic results summarized in Table 1 support the expectation of this property for cefepime. The value for k_{cat}/K_m is low, and K_s is in the high millimolar range, as measured for interactions of cefepime with the TEM_{pUC19} β-lactamase. The high value for K_s argues for low affinity for cefepime by the β-lactamase. The extended-spectrum cephalosporins, including cefepime, have been assumed to be poor substrates for β-lactamases because of the bulk of their $C_{7\beta}$ side-chain functionalities. Our modeling indicates that this is indeed correct for cefepime. Some steric interactions of cefepime with the active site of the TEM-1 enzyme were so unfavorable that energy minimizations of the structures failed several times, and adjustment of the structure was necessary to permit the energy-minimization procedure to reach its conclusion. For modeling of the starting position for energy minimization of the acyl-enzyme intermediate, we have had access to two crystallographic structures for DD-peptidase/transpeptidase with its active site modified with either cefotaxime or cephalothin, two cephalosporins (generously communicated by Professors Judith Kelly and James Knox).[21] DD-Peptidase/transpeptidase is a bifunctional penicillin-binding protein (PBP), whose active-site topology closely resembles those of β-lactamases; indeed, these two types of enzymes are assumed to be related to each other from an evolutionary point of view.[22] Two starting conformations for the immediate acyl-enzyme intermediate of cefepime appeared equally plausible. These two structures are essentially identical to each other with the sole exception of the orientation of the side-chain substituent at $C_{7\beta}$. A 140° rotation about the bond between the side chain amide carbonyl and the oxime carbon is the sole distinction between the two conformations. One conformation fits closely the orientation of the side-chain of cefotaxime in the active site of DD-peptidase/transpeptidase (Figure 1A). While the other orientation for the side chain does not approach that of cefotaxime, it seems equally plausible because of the constraints imposed by the active site (Figure 1B).

The hydrocarbon portions of the C_3 functionality fit in the hydrophobic pocket created by the side chains of amino acid residues Tyr-105 and Val-216 in both conformations; this binding interaction was first described by us for binding of a hydrophobic moiety in *N*-sulfonyloxy monobactam mechanism-based inactivators in the active site of the TEM-1 enzyme.[23] The structures shown in Figure 1 are those for the immediate product of acylation. The release of a leaving group such as

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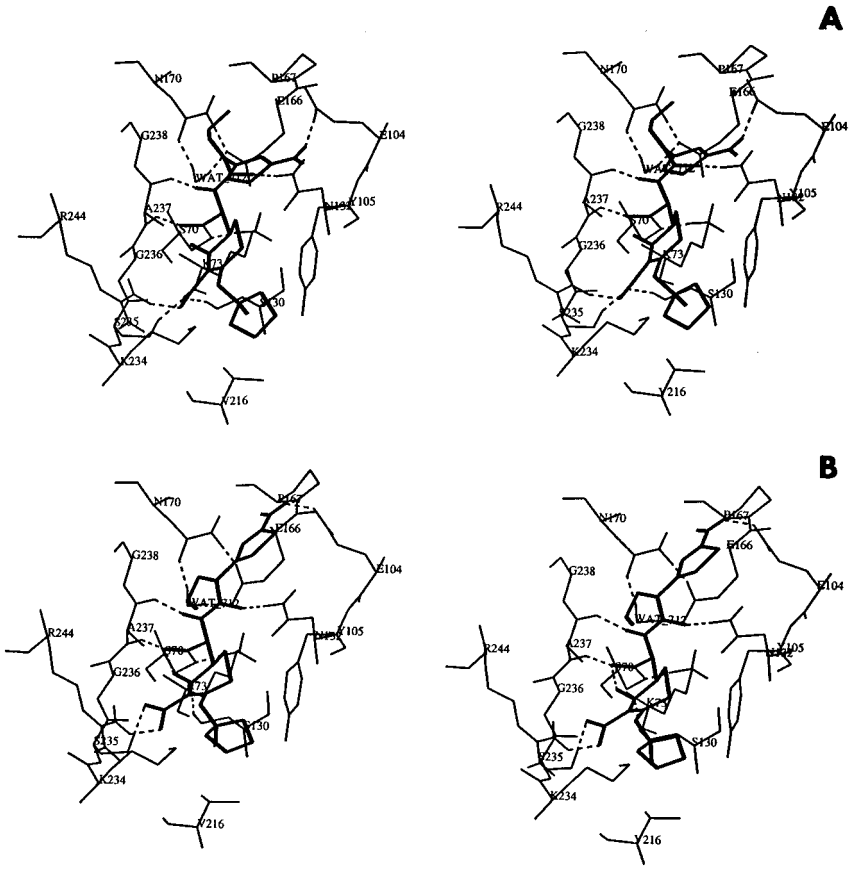

Figure 1. Energy-minimized structures for the acyl-enzyme intermediate for cefepime in the active site of the TEM-1 β-lactamase. The antibiotic $C_{7\beta}$ side-chain function is positioned (A) according to the orientation of the side chain of cefotaxime in the active site of DD-peptidase/transpeptidase, and (B) in the alternative plausible orientation.

the *N*-methylpyrrolidine moiety from the cefepime nucleus would take place subsequently (*vide infra*), as shown for other cephalosporins.[24] In these conformers, the C_7 side-chain function of cefepime interacts unfavorably in a steric sense with the side-chain functions of amino acid residues Pro-167 and Asn-170 in the "top" of the Ω-loop function (spanning residues 164−179; detailed discussion will follow). The consequence of these unfavorable interactions is the observation that the structurally conserved hydrolytic water molecule, Wat-712,[25] is displaced by 0.5 Å from its ideal position for the deacylation step. As will be discussed below, this displacement would make deacylation rate-limiting for turnover of cefepime by the TEM_{pUC19} β-lactamase. Another interesting observation about the side-chain function at $C_{7\beta}$ of cefepime is the fact that the π orbitals of the amide carbonyl, the oxime double bond, and the 2-amino-4-thiazolyl group may not be in conjugation with one another because of the steric constraints of the active site. The extent of the out-of-plane bend for each of these moieties varies between the two conformers somewhat, but that for the oxime double bond and the 2-amino-4-thiazolyl group shown in Figure 1B approaches orthogonality. This lack of conjugation would be expected to increase the energy of the two complexes by as much as 1−3 kcal/mol.

We have investigated the kinetics of turnover of cefepime by the class A TEM_{pUC19} β-lactamase and the class C Q908R enzyme (Table 1). As discussed above, the bulky side-chain function for cefepime would make unfavorable contacts in the active site of the TEM_{pUC19} enzyme. This is manifested by a large dissociation constant for cefepime (10.3 ± 0.8 mM) measured for the TEM_{pUC19} β-lactamase. The turnover constant (k_{cat}) is small, giving a k_{cat}/K_m value of 2500 M^{-1} s^{-1}. Because of the steric encumbrance of the substrate within the active site, both acylation and deacylation are slow processes. The difference between k_2 (rate constant for acylation) and k_3 (rate constant for deacylation) indicates that the rate-limiting step for hydrolysis of cefepime is the deacylation of the wild-type enzyme. Furthermore, the value for k_3 equals that for k_{cat}. These findings are consistent with our discussion that acylation of the active site of the TEM_{pUC19} β-lactamase by cefepime would entail a displacement of the hydrolytic water from its preferred position for deacylation of typical substrates. The same type of displacement of Wat-712 has been suggested to make deacylation rate-limiting for turnover of carbapenems.[6,26]

During kinetic measurements with cefepime, we noticed a lapse time of 2−3 min before the onset of steady-state kinetics. This lag period might possibly result from a two-step reaction, wherein the first step, the enzyme acylation, with the attendant β-lactam ring opening associated with a small change in the extinction coefficient, is followed by another, for example, the release of *N*-methylpyrrolidine from the 3′ site. We ruled out this possibility on the basis of two reasons. First, the change in the extinction coefficient for the β-lactam opening, while small, is always measurable. In the case of cefepime, the lag phase appeared like a period of "inaction" in catalysis by the enzyme. Nonetheless, we explored this possibility further by denaturing the β-lactamase after 30, 60, 90, and 180 s of exposure to cefepime to investigate if any chromophore from

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(25) The water molecules in the crystal coordinates for the TEM-1 β-lactamase which were made available to us were not numbered. Hence, the numbering system for the water molecules is that of the related *Bacillus licheniformis* enzyme, as proposed by Knox and Moews (Knox, J. R.; Moews, P. C. *J. Mol. Biol.* **1991**, 220, 435).

(26) Matagne, A.; Lamotte-Brasseur, J; Frère, J. M. *Eur. J. Biochem.* **1993**, *217*, 61. Monk, J.; Waley, S. G. *Biochem. J.* **1988**, *253*, 323.

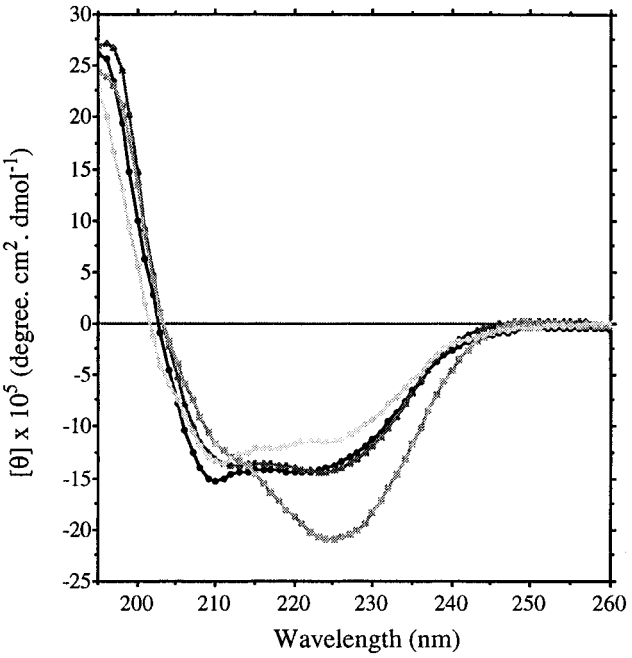


Figure 2. CD spectra of 3.5 μM each of the parental TEM_{pUC19} β-lactamase (●), TEM_{pUC19} β-lactamase after hydrolysis of cefepime (▲) TEM_{pUC19} β-lactamase 30 min into incubation with cefepime (■), and the Arg-164-Asn mutant TEM_{pUC19} β-lactamase (◆); gray-scale coding of the spectra is from black (for the parental) to the lightest shade of gray (for the Arg-164-Asn mutant β-lactamase), respectively. The spectrum of the TEM_{pUC19} β-lactamase 30 min into incubation with cefepime was recorded when less than 4% of cefepime was hydrolyzed.

cefepime would become incorporated into the spectrum of protein. Under conditions whereby such chromophore incorporation would have been detected readily, none was observed. Therefore, the lag period does not involve covalent interaction between the enzyme and cefepime.

Consequently, we attributed this lapse time to the unfavorable noncovalent interactions between the enzyme and cefepime, which may require a conformational change by the enzyme prior to hydrolysis of cefepime. Since turnover of cefepime is slow, we looked at the circular-dichroic (CD) spectrum of the enzyme as a function of time during catalysis. As shown in Figure 2, catalysis is accompanied by changes in the CD spectrum of the enzyme. Specifically, we saw a decrease in helicity of the protein in the course of cefepime turnover, as judged by an increase in the ellipticity (*i.e.*, a smaller negative value) of the minimum at 208 nm (Figure 2; the helical minimum at 222 nm was masked by the minimum centered at 225 nm; discussed below). This conformational change would be initiated by movement of stretches of amino acids flanking the binding site for the $C_{7\beta}$ side chain of the antibiotic upon active-site binding, such as residues 167−170 (a 3_{10} helix; a portion of the Ω-loop), residues 103−106 (a surface bend), and residues 237−239 (β-strand B3). Experimental quantification of these changes in the CD spectrum is difficult, so we resorted to dynamics simulations of our energy-minimized models to gain insight into this novel structural elasticity that we detected in the course of turnover of cefepime.

A limitation of dynamics simulation is that catalysis takes place over a longer time frame than the duration for dynamics simulation that can reasonably be carried out (in our case, 10 ps); the long computing time required for these procedures is a major hindrance to this type of analysis. Nonetheless, we are aware that the time scale for internal motions of flexible hinged domains in proteins approaches the 10-ps time frame.[27] Molecular dynamics simulations of our complexes showed no appreciable change in the protein structure at 0 and 50 K from the starting position. However, simulation at 300 K showed movement for the Ω-loop (containing a 3_{10} helix at residues 168−170) and the bend formed by residues 103−106 and its adjacent residues. A striking observation was that the 3_{10} helix adjacent to this bend (residues 99−101) unraveled within the first 3 ps of simulation. Also, small but significant movement was seen near Val-216.[28] There are two additional helices, α-helix H3 (one turn) and α-helix H5, which may remain as potential sites for structure relaxation only by indirect means, but no effect was seen at these sites during our simulation. α-Helix H3 is adjacent to the bend formed by residues 103−106, which is in contact with the C_7 side chain of antibiotic. On the other hand, the side-chain function of Asn-132 (on α-helix H5) makes a hydrogen bond to the amide carbon of the C_7 side chain of the antibiotic. We believe that these are less likely sites for manifestation of the observed CD measurements.

Hydrolysis of cefepime was correlated with the course of the CD experiments by thin-layer chromatography of the reaction mixture (cefepime R_f 0.30, reverse-phase C_{18} TLC; 1/1 MeOH/H_2O). At the end of the CD experiment, at which time the spectrum had reverted mostly back to that for the native protein, no trace of cefepime remained, but a new compound had appeared (R_f 0.76). Nonetheless, our structural analysis of the purified product, which relied heavily on mass spectral analysis, indicated **4** as the structure for the isolated product. On the basis of the precedent for the nonenzymic fragmentation of cephalosporins subsequent to hydrolysis of the lactam bond,[24] one can propose the sequence of events depicted in Scheme 1 to account for the formation of **4**.

We have also carried out ammonolysis experiments with cefepime. A significant finding from these experiments was that within 1 min from mixing of the reagents, the *N*-methylpyrrolidine attached at the 3′ position was released. These results are consistent with the prior literature on cephalosporin fragmentation.[24] The resultant intermediate in the nonenzymic reaction showed a chromophore at 230 nm, consistent with the assignment of structure **3** to this species.[24] Neither cefepime nor the final product of fragmentation of cefepime hydrolysis (**4**) showed a CD spectrum, but intermediate **3** for the ammonolysis reaction possessed one at 230 nm. These results indicate that the minimal kinetic scheme of eq 1 is valid, since the release of the leaving group from the 3′ position of the acyl-enzyme intermediate is fast (*i.e.*, within 1 min from acylation of the active site), and that deacylation of the acyl-enzyme intermediate(s) takes place over several hours. In eq 1, E−S is the acyl-enzyme intermediate after the departure of the leaving group (species **3**). Correspondingly, we interpret the minimum at 225 nm for the CD spectrum in the course of hydrolysis of cefepime (Figure 2) by the TEM_{pUC19} β-lactamase to be contributed by species **3** and by an increase in the β-sheet content, since the side chain of cefepime makes hydrogen bonds to the protein reminiscent of the β-sheet motif.

As discussed above, the sterically hindered C_7 side-chain function of the antibiotic makes contacts with amino acid residues Pro-167 and Asn-170 for both **2** and **3** (the position of the side-chain function for the energy-minimized structure **3** is not any different compared to **2**; data not shown), and this interaction in turn affects the position of Wat-712. These two

(27) Brooks, C. L.; Karplus, M.; Pettitt, B. M. In *Proteins: A Theoretical Perspective of Dynamics, Structure, and Thermodynamics*; Advances in Chemical Physics, Volume LXXI; John Wiley & Sons: New York, 1988.

(28) We suspect that the Tripos force-field parameters may underestimate some interactions (*e.g.*, hydrogen bonding) and overestimate others, with the effects becoming more pronounced with elevated temperature. However, these opposing effects have a tendency to even each other out. Although we are aware of this caveat, the results of the simulations are generally reliable, and in this case, they are supported by experimental observations.

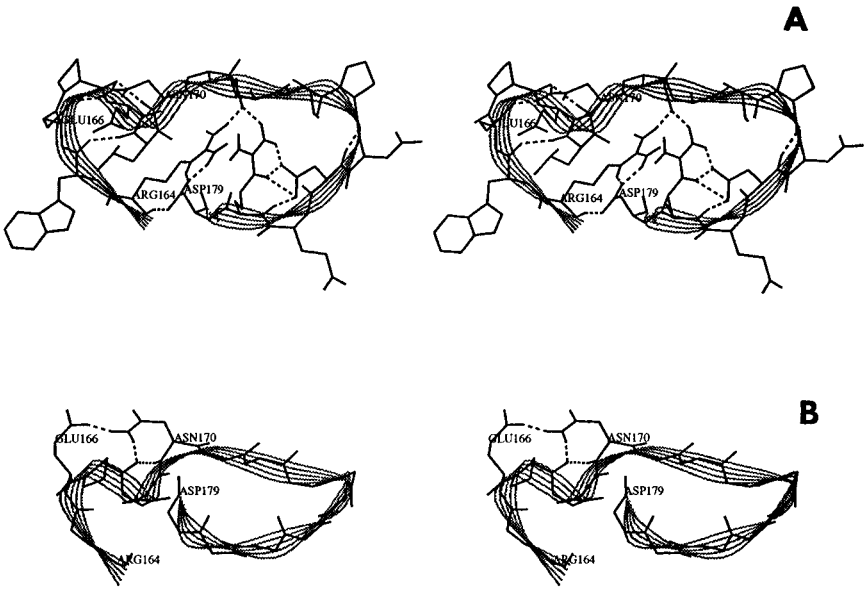
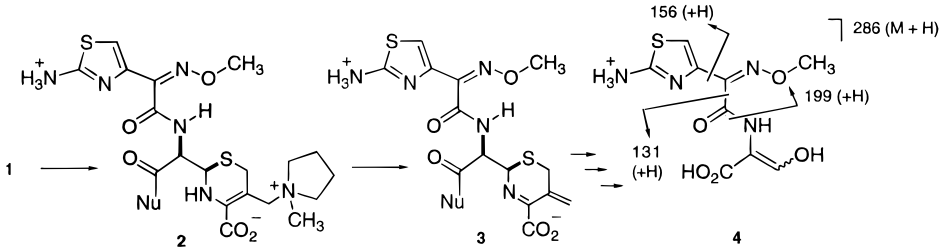


Figure 3. The Ω-loop spanning residues 164−179. Hydrogen atoms are shown only when a hydrogen bond (dashed lines) exists, and the orientation of the backbone is indicated by the overlapping gray ribbon presentation. (A) All the side chains of amino acids within the Ω-loop are shown. (B) A different orientation of the Ω-loop highlighting the 3_{10} helix at the active site; only the side chains of Glu-166, Pro-167, and Asn-170 are shown.

Scheme 1



amino acids are within the Ω-loop spanning residues 164−179 (Figure 3). It has been observed in some clinical isolates that a serine substitution at position 164 enhances activity toward some third-generation cephalosporins.[15,29−31] We believe that the effect of this substitution on the enzyme is to create a cavity in the middle of the Ω-loop, since the side chain of serine is substantially shorter than that of arginine. Since the interactions of the side chains of residues Arg-164 and Asp-179 would also be abolished by such mutation, the "top" of the loop would collapse to fill the void created in the middle of the loop. Such a movement of the loop would create more room at the active site to accommodate the large C_7 side-chain function of the antibiotic. To gain experimental insight into this possibility, we prepared two mutant derivatives of the TEM_{pUC19} β-lactamase, the Asp-179-Gly and Arg-164-Asn enzymes.[32] The Asp-179-Gly enzyme is not expected to be able to maintain the two critical hydrogen bonds to the Arg-164 side chain; as a consequence, the arginine side chain would be expected to be forced out of the middle of the loop for steric reasons. This mutant enzyme has not been detected in clinical isolates. We reasoned that this mutant enzyme may have properties similar to those of the Arg-164-Asn mutant enzyme.[32]

The expected immediate consequence of these mutations at positions 164 and 179 was that binding of cefepime to the enzymes improved, as judged by a lowering of the values for the dissociation constants (K_s) for cefepime with both mutant enzymes (Table 1). The K_s values of approximately 1 mM are not atypical for the first-generation cephalosporins, which are in general reasonably good substrates for class A β-lactamases.[9] Because catalysis by β-lactamase involves acylation and deacylation steps, alterations in the active-site topology would potentially affect both steps. Movement of the "top" of the Ω-loop as a consequence of mutations at positions 164 and 179 would create more room for the antibiotic to fit in the active site potentially favoring acylation, but also would affect deacylation. This is especially so because the general base responsible for activation of Wat-712 for the deacylation step has been suggested to be Glu-166, which is an amino acid residue positioned in the Ω-loop as well.[33] Therefore, these mutations must modulate activity such that acylation should be favored, while not abolishing deacylation. In general, a discussion of macroscopic rate constants between mutants and wild-type enzymes should be tempered in light of the analysis presented by Waley.[34] Nonetheless, certain general observations can be made consistent with the discussion given above. An increase in k_{cat}/K_m of 364-fold for hydrolysis of cefepime was seen for the Arg-164-Asn enzyme. Remarkably, both acylation and deacylation rates were enhanced, and the two rate constants are similar to each other in magnitude (Table 1). Hence, it is

(29) Collatz, E.; Tran Van Nhieu, G.; Billot-Klein, D.; Willianson, R.; Gutmann, L. *Gene* **1989**, *78*, 349.

(30) Palzkill, T.; Le, Q. Q.; Venkatachalam, K. V.; LaRocco, M.; Ocera, H. *Mol. Microbiol*. **1994**, *12*, 217.

(31) Raquet, X.; Lamotte-Brasseur, J.; Fonzé, E.; Goussard, S.; Courvalin, P.; Frère, J. M. *J. Mol. Biol*. **1994**, *244*, 625.

(32) We have observed that Asn-164 mutant TEM_{pUC19} β-lactamase is in general even more active than the Ser-164 enzyme (unpublished results). However, Asn-164 has not yet been observed clinically.

(33) Adachi, H.; Ohta, T.; Matsuzawa, H. *J. Biol. Chem*. **1991**, *266*, 3186. Escobar, W. A.; Tan, A. K.; Fink, A. L. *Biochemistry* **1991**, *30*, 10783. Miyashita, K.; Massova, I.; Taibi, P.; Mobashery, S. *J. Am. Chem. Soc*. **1995**, *117*, 11055. Maveyraud, L.; Massova, I.; Birck, C.; Miyashita, K.; Samama, J.-P.; Mobashery, S. *J. Am. Chem. Soc.* **1996**, *118*, 7435.

(34) Waley, S. G. In *The Chemistry of β-Lactams*; Page, M. I., Ed.; Blackie Academic: London, 1992; pp 223−224.

misleading to talk of a unique rate-limiting step for hydrolysis of cefepime by this enzyme. This observation is also true for turnover of cefepime by the class C Q908R β-lactamase; for this case, the k_2 value is essentially equal to that for k_3. Perhaps these are examples of β-lactamases which seem capable of hydrolyzing this nonclassical β-lactam for which neither of the catalytic steps is rate-limiting.[35] We add here that the CD experiment in the course of hydrolysis of cefepime was not carried out with the Asp-179-Gly mutant of the TEM_{pUC19} since we could not prepare enough of this enzyme (*vide infra*). Furthermore, the rates of cefepime hydrolysis were sufficiently fast for the Arg-164-Asn mutant and the class C Q908R β-lactamases that such an experiment could not be performed for these enzymes. As stated earlier, an interesting observation was that the CD spectrum of the parental class A enzyme experienced a decrease in its helical content (Figure 2).[36] Neither the helical content of the spectrum (Figure 2) nor the enzyme activity was entirely regained after complete hydrolysis of cefepime, indicative of a partial irreversible inactivation of the enzyme by its incubation with cefepime. Consistent with this observation, at the end of the hydrolysis, the TEM_{pUC19} enzyme regained only 92% (average of three determinations) of its original activity. Under the identical condition, in the absence of cefepime, β-lactamase did not lose any activity. Interestingly, the CD spectrum of the Arg-164-Asn enzyme (Figure 2) seems more similar—in terms of the molar ellipticity minimum at 208 nm—to that for the cefepime-parental β-lactamase mixture than to that for the parental enzyme itself; this suggests that this mutant enzyme may have a lower helical content than the parental enzyme.[37]

The crystal structure for the *Staphylococcus aureus* P54 mutant class A β-lactamase, which has a substitution of asparagine in place of Asp-179, has been determined.[38] Although the Ω-loop of the *S. aureus* β-lactamase is somewhat different from that of the TEM enzymes, it appears that in the *S. aureus* P54 enzyme the structure of the Ω-loop was disordered. The mutant enzyme was shown to possess weak activity in turnover of penicillin G, and the deacylation step was the rate-limiting step for turnover of this penicillin. As stated above, deacylation is the rate-limiting step for turnover of cefepime by our Asp-179-Gly enzyme as well (Table 1).

Typically, stopped-flow experiments are performed to obtain the individual microscopic rate constants. This is, of course, a powerful technique that has been applied in a few cases to turnover of both penicillins[35,38,39] and cephalosporins[40−43] by class A β-lactamases. Inherent difficulties in these experiments are the need for large quantities of enzyme, the need for special instruments, and often a large spectroscopic change during catalysis (*e.g.*, $\Delta\epsilon$).[44] A further complicating factor for the study of turnover of extended-spectrum β-lactams by β-lactamases is the profoundly slow rates. Indeed, typically k_{cat}/K_m is estimated for turnover of these substrates under approximating conditions of $K_m > [S]_0$, which may not be valid for turnover of most nonclassical β-lactams. The simple technique reported here for calculation of k_2 and k_3 actually takes advantage of these slow rates. Since the rates are exceedingly slow, evaluation of K_s values for various substrates can be performed with accuracy, permitting the facile calculation of the microscopic rate constants from the expressions for k_{cat} and K_m. This technique should be applicable for such evaluations of other extended-spectrum β-lactams as well.

It is well recognized that the structures of proteins are in a state of constant motion at ambient temperature.[27] This fact does not diminish the importance of protein crystal structure, which shows the average atomic positions; rather, it indicates that movement about the average positions can exist and would play a significant role in protein function. β-Lactamase conformational flexibility in catalysis has been assumed for many years in the literature.[45] However, the direct evidence for such molecular motion has been lacking. Recently, Vijayakumar *et al*. reported on molecular dynamics simulation of the structure of the class A β-lactamase from *S. aureus*.[46] The dynamics computations revealed that, whereas the secondary structural elements in the enzyme were in general rather rigid, significant deviation from the crystal structure was noted in the positions of loops and turns. Specifically, the N-terminal portion of the Ω-loop (residues 164−173) was shown to move in the direction of the active site, and the C-terminal portion (residues 174−179) moved outward. We reported on our experimental results recently which indicated that turnover of imipenem (**5**), a clinically used carbapenem, is accompanied by a conformational change, as judged by changes in the CD spectra of the TEM_{pUC19} β-lactamase in the course of turnover. In this case, we noted that the enzyme enhanced its helicity during catalysis.[6] It would appear that the turnover process requires that the enzyme acquire more structure to hydrolyze imipenem, which itself has a relatively small chemical structure compared to other β-lactams. We see the opposite in the turnover of cefepime. For this substrate, the bulk of the $C_{7\beta}$ side chain of the antibiotic requires accommodation by a structure relaxation of the enzyme, manifested by changes in the CD spectra characterized primarily by a decrease in the helical content. It is presently difficult to infer from these observations structural factors useful in explaining the chemistry of catalysis. However, this catalytic ability to accommodate substrates of diverse structures is a clear indication of the versatility of β-lactamases to adapt their structures to carry out a vital function for the survival of bacteria in the face of the challenge by novel β-lactams.

(35) Christensen, H.; Martin, M. T.; Waley, S. G. *Biochem. J.* **1990**, *266*, 853.

(36) For an analysis of CD spectra of proteins, consult: Jirgenson, B. In *Optical Activity of Proteins and Other Macromolecules*; Springer Verlag: New York, NY, 1973; pp 66−74.

(37) For reasons not readily obvious to us, we have not been able to purify the Asp-179-Gly mutant β-lactamase to homogeneity in the large quantities needed for the CD measurements.

(38) Herzberg, O.; Kapadia, G.; Blanco, B.; Smith, T. S.; Coulson, A. *Biochemistry* **1991**, *30*, 9503.

(39) Martin, M. T.; Waley, S. G. *Biochem. J.* **1988**, *254*, 923.

(40) Bicknell, R.; Waley, S. G. *Biochem. J.* **1985**, *231*, 83.

(41) Anderson, E. G.; Pratt, R. F. *J. Biol. Chem.* **1981**, *256*, 11401.

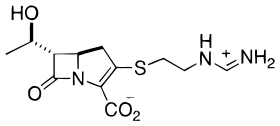
(42) Anderson, E. G.; Pratt, R. F. *J. Biol. Chem.* **1983**, *258*, 13120.

(43) Fraci, W. S.; Pratt, R. F. *Biochemistry* **1985**, *24*, 903.

(44) In derivations of the integrated forms for the equations that describe the pre-steady-state treatment of enzyme kinetics, some assumptions are made which may not be valid at all times. These assumptions would introduce errors in the measured values for microscopic constants from stopped-flow experiments which are often not appreciated.

(45) Samuni, A.; Citri, N. *Biochem. Biophys. Res. Commun.* **1975**, *62*, 7. Citri, N.; Samuni, A.; Zyk, N. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1048. Virden, R.; Bristow, A. F.; Pain, R. H. *Biochem. Biophys. Res. Commun.* **1978**, *82*, 951. Klemes, Y.; Citri, N. *Biochem. J.* **1980**, *187*, 529. Citri, N.; Kalkstein, A.; Samuni, A.; Zyk, N. *Eur. J. Biochem.* **1984**, *144*, 333. Persaud, K. C.; Pain, R. H.; Virden, R. *Biochem. J.* **1986**, *237*, 723. Page, M. G. P. *Biochem. J.* **1993**, *295*, 295. Dubus, A.; Normark, S.; Kanai, M.; Page, M. G. P. *Biochemistry* **1995**, *34*, 7757. The above references correlate the observation of a decrease in the activity of β-lactamases in the course of turnover of certain substrates as evidence for conformational changes for the enzyme during catalysis, but no direct evidence besides the attenuation of activity is presented in these reports. We wish to point out that Charnas and Knowles (*Biochemistry* **1981**, *20*, 2732) also noted such attenuation in the rate of turnover of an olivanic acid substrate by the TEM β-lactamase, but they dismissed the argument for a conformational change on kinetic grounds in that case. However, it may be likely that in some cases discussed in the above references conformational changes may be playing a role in catalysis. More recently Jamin *et al*. have observed changes in the NMR spectrum of the *B. licheniformis* β-lactamase, which they attribute to conformational changes in the course of catalysis (Jamin, M.; Damblon, C.; Bauduin-Misselyn, A. M.; Durant, F.; Roberts, G. C. K.; Charlier, P.; Llabres, G.; Frère, J. M. *Biochem. J.* **1994**, *301*, 199).

(46) Vijayakumar, S.; Ravishanker, G.; Pratt, R. F.; Beveridge, D. L. *J. Am. Chem. Soc.* **1995**, *117*, 1722.



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Acknowledgment. This work was supported in part by grants from the National Institutes of Health, Bristol-Myers Squibb, and a predoctoral fellowship from the Philippe Foundation (to P.T.). I.M. is a recipient of the Rumble and Heller Fellowships. We thank Professor Michael James for providing us with the crystal coordinates of the TEM-1 β-lactamase. We are indebted to Professors James Knox and Judith Kelly for making their coordinates for DD-peptidase/transpeptidase available to us. Cefepime was a generous gift from Dr. Robert Kessler of Bristol-Myers Squibb.

JA9529753