Kinetics of Turnover of Cefotaxime by the *Enterobacter cloacae* P99 and GCl β-Lactamases: Two Free Enzyme Forms of the P99 β-Lactamase Detected by a Combination of Pre- and Post-Steady State Kinetics†

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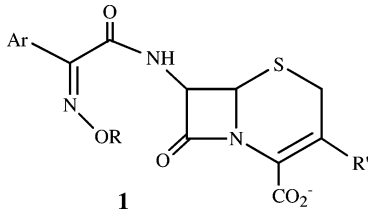
ABSTRACT: Third-generation cephalosporins bearing oximino side chains are resistant to hydrolysis by class C β-lactamases such as that from *Enterobacter cloacae* P99. For example, steady state parameters for hydrolysis of cefotaxime by this enzyme are as follows: $k_{cat} = 0.41\ s^{-1}$, $K_m = 17.2\ \mu M$, and $k_{cat}/K_m = 2.3 \times 10^4\ s^{-1}\ M^{-1}$. On the other hand, however, the K_i value for cefotaxime as an inhibitor of cephalothin hydrolysis is 27 nM. The discrepancy between K_m and K_i indicated that a real steady state had not been achieved in at least one of these experiments. Analysis indicated that only two to three cefotaxime turnovers occurred during the K_i determination. This suggested that the first few turnovers of cefotaxime by the P99 β-lactamase may be different from those in the subsequent steady state. A direct pre-steady state experiment confirmed this hypothesis. The simplest reaction scheme that fitted the data involved replacement of the initial enzyme form, E, which bound cefotaxime tightly, with a second more weakly binding form, E′, by partitioning of the acyl−enzyme intermediate during the first few turnovers. Steady state turnover of cefotaxime then largely involved E′ as the free enzyme form. E′ slowly reverted to E in the post-steady state regime. Further evidence for this scheme included quantitative analysis of the post-steady state and observation of a difference in the catalytic activity of E and E′ in 2 M ammonium sulfate. The kinetics of P99 β-lactamase-catalyzed hydrolysis of an acyclic depsipeptide substrate bearing a third-generation cephalosporin side chain showed that the side chain is necessary but not sufficient for production of resistance to β-lactamase; a combination of the side chain and the dihydrothiazine ring of a cephalosporin is required. The β-lactamase of *E. cloacae* GC1, an extended spectrum mutant of the P99 enzyme, rapidly hydrolyzes third-generation cephalosporins, without the structural transition described above. The flexibility of the extended Ω loop of the GC1 enzyme probably leads to this situation. Conformational restriction of the loop in the P99 enzyme is probably responsible for the long-lived acyl−enzyme intermediate and the transition to E′ induced by cefotaxime.

The class C β-lactamases have evolved in Gram-negative bacteria as important resistance factors against cephalosporin antibiotics (*1*, *2*). Third-generation cephalosporins bearing (oximino)acetamido side chains at the 7-position of the cephalosporin nucleus, **1**, have been successfully used as β-lactam antibiotics for more than a decade because of their stability to both class A and class C β-lactamases (*3*).



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Recently, however, mutant extended spectrum β-lactamases (ESBLs)[1] that are capable of hydrolyzing most third-generation cephalosporins have evolved (*4*). Most of the known ESBLs are class A enzymes, but in 1995, Nukaga et al. reported the isolation of a chromosomal class C β-lactamase from a virulent clinical strain of *Enterobacter cloacae* (named GCl) that exhibited resistance to third-generation cephalosporins (*5*). This enzyme displayed extended spectrum activity against β-lactams.

Structural analysis of the new enzyme showed that it differed from the common *E. cloacae* P99 β-lactamase by duplication of three amino acids (Ala208, Val209, and Arg210) in the Ω loop. This loop abuts the active site in the side chain binding region. A crystal structure of the GCl β-lactamase showed localized disorder of the loop at residues 213−215 (*6*); this region includes one amino acid of the tripeptide insertion (residues 211−213) and is likely to be the section of the loop most closely associated with a substrate side chain (note that the sequence numbers in this

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[1] Abbreviations: BMHB, benzyl *m*-hydroxybenzoate; DCC, dicyclohexylcarbodiimide; DME, dimethoxyethane; DMF, dimethylformamide; DMAP, 4-(dimethylamino)pyridine; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide; ESBL, extended spectrum β-lactamase; ESI^-, negative ion electrospray; MOPS, 3-morpholinopropanesulfonic acid; MS, mass spectrum; NMR, nuclear magnetic resonance.

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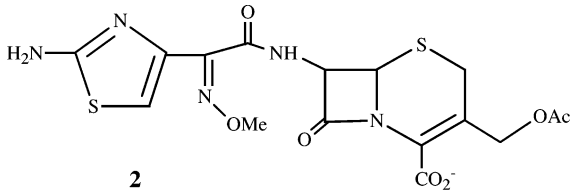
sentence refer to GC1). Crichlow et al. (*6*) suggested that the conformational flexibility of the expanded Ω loop may facilitate the hydrolysis of third-generation cephalosporins by permitting greater mobility of the acyl moiety and thus its more optimal access to water and to the catalytic apparatus. More recently, the same group has described the crystal structure of the GCl enzyme covalently bound to a mechanism-based cephem sulfone inhibitor (*7*). In this structure, the Ω loop is immobilized by interaction with the inhibitor. They suggest that the mobility of the Ω loop is the primary basis of the extended substrate specificity of the GCl β-lactamase. At this time, also, the crystal structure of a third-generation cephalosporin bound to a wild-type narrow spectrum class C β-lactamase (amp C from *Escherichia coli*) was described by Powers et al. (*8*). The kinetic stability of this complex was interpreted in terms of restricted access of the deacylation water to the acyl−enzyme intermediate and the concomitant steric crowding of the deacylation transition state.

Although, as described above, a considerable amount of structural information is now available that is relevant to the resistance of third-generation cephalosporins to class C β-lactamases, detailed kinetics studies are rather less common. In this paper, we demonstrate that the turnover of cefotaxime (**2**) by the P99 β-lactamase is a complicated process involving a branched pathway after acyl−enzyme formation and an accumulating second free enzyme form where the steady state rate constants are significantly different from those of the original form.



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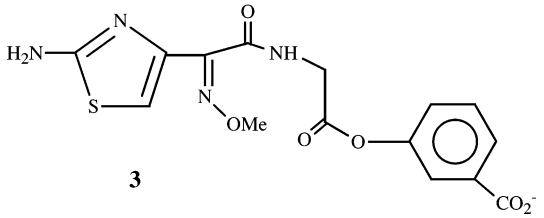

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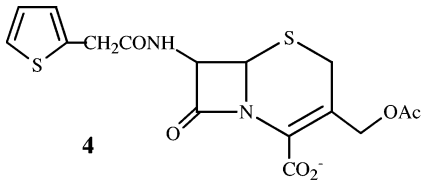
The "post-steady state" kinetics phase was found to be useful in detecting reaction schemes of this sort. This complexity in the kinetics is not observed with the GCl enzyme. To determine the effect of a third-generation side chain alone, we have synthesized the depsipeptide **3**, and also describe the kinetics of the interaction of this molecule with the normal and mutant enzymes.

MATERIALS AND METHODS

The *E. cloacae* P99 β-lactamase was purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.). The extended spectrum β-lactamase from *E. cloacae* GCl was prepared as reported previously (*5*). Cephalothin (**4**) and cefotaxime (**2**) were gifts from Eli Lilly and Co. and Merck, respectively.

Cefuroxime (**5**) was purchased from Sigma-Aldrich. The chromogenic substrate CENTA (**6**) was prepared in this



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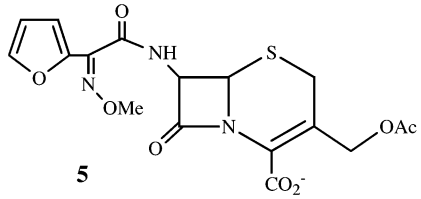

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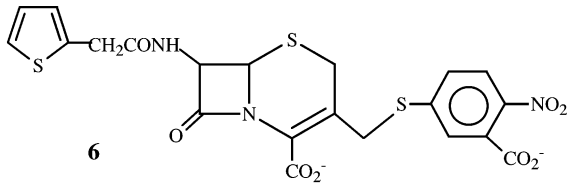

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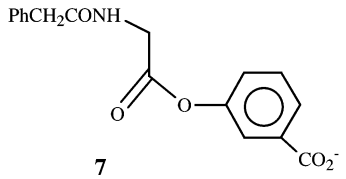

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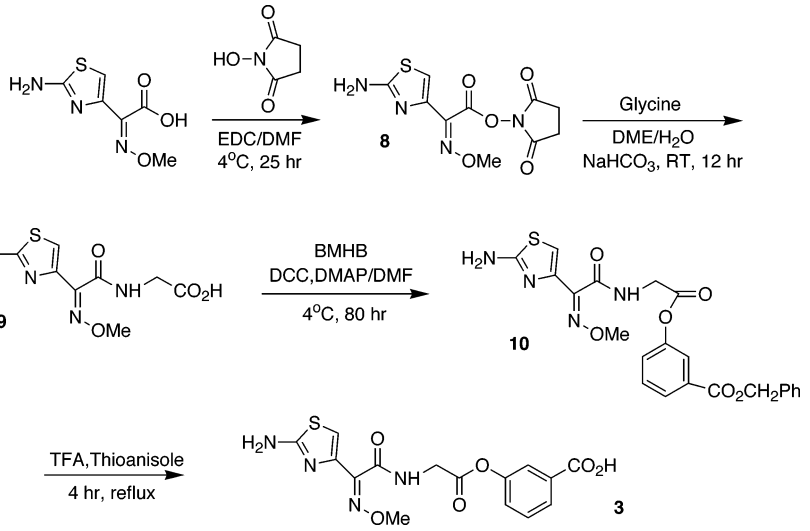
laboratory by R. Nagarajan as described in a published protocol (*9*). 3-{[*N*-(Phenylacetyl)glycyl]oxy}benzoic acid (**7**) was available from previous studies in this laboratory (*10*).

*Synthesis of 3-[2-(2-Aminothiazol-4-yl)-2-(Z)-methoxyiminoacetylglycyl]oxybenzoic Acid (**3**).* This compound was prepared by the reaction sequence outlined in Scheme 1. First, the *N*-hydroxysuccinimide ester of 2-(2-aminothiazol-4-yl)-2-(*Z*)-methoxyiminoacetic acid (**8**) was prepared. Thus, to an ice-cooled solution of 2-(2-aminothiazol-4-yl)-2-(*Z*)-methoxyiminoacetic acid (21.0 g, 105 mmol; Acros Organics) and *N*-hydroxysuccinimide (12.0 g, 105 mmol) in dry dimethylformamide (400 mL) was added 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (20.0 g, 104 mmol), and the reaction mixture was stirred in the cold room for 24 h. After the solvent was removed by rotary evaporation, the residual yellow oil was partitioned between ethyl acetate (500 mL) and water (1200 mL). The precipitated solid, which was the required product, was removed by filtration, washed with more water, and dried: yield 20.3 g (65%); ^{1}H NMR ([$^2H_6$]DMSO) δ 2.88 (s, 4H, $2CH_2$), 3.98 (s, 3H, OCH_3), 7.12 (s, 1H, thiazole-5H), 7.44 (br s, 2H, NH_2).

Condensation of **8** with glycine then afforded 2-(2-aminothiazol-4-yl)-2-(*Z*)-methoxyiminoacetylglycine (**9**). A solution of glycine (2.5 g, 33.3 mmol) and sodium bicarbonate (5.6 g, 66.6 mmol) in water (100 mL) was added to a stirred solution of **8** (10.0 g, 33.5 mmol) in 1,2-dimethoxyethane (600 mL) at room temperature. After 12 h, the volume of solvent was reduced to 100 mL by rotary evaporation. The product, a pale yellow solid, which precipitated on acidification of the ice-cooled solution to pH 2.1 with 1 M HCl, was collected by filtration and dried *in vacuo*: yield 7.1 g (82%); ^{1}H NMR (^2H_2O, Na^2HCO_3) δ 3.90 (s, 2H, CH_2), 3.96 (s, 3H, OCH_3), 7.20 (s, 1H, thiazole-5H).

Scheme 1



The acid **9** was then condensed with benzyl 3-hydroxybenzoate to afford the protected ester **10**. Thus, 1,3-dicyclohexylcarbodiimide (5.4 g, 26.2 mmol) and the phenol (3.96 g, 17.4 mmol) were added simultaneously to a stirred solution of **9** (4.5 g, 17.4 mmol) in *N*,*N*-dimethylformamide (180 mL) at 0 °C. The catalytic base 4-(dimethylamino)-pyridine (0.18 g, 1.47 mmol) was then added and the reaction mixture stirred in the cold room for 80 h. After filtration of the reaction mixture, the filtrate was evaporated to dryness under vacuum. The resulting crude product was taken up into ethyl acetate (600 mL) and the resulting solution washed with water and a sodium bicarbonate solution. The solid product was then obtained from the solution (dried over $MgSO_4$) by rotary evaporation. The product was purified by recrystallization from benzene in 55% yield (4.5 g): ^{1}H NMR ([$^2H_6$]DMSO) δ 3.81 (s, 3H, OCH_3), 4.25 (d, $J = 4.5$ Hz, 2H, NCH_2), 5.36 (s, 2H, OCH_2), 6.86 (s, 1H, thiazole-5H), 7.23 (br s, 2H, NH_2), 7.3−7.9 (m, 9H, ArH), 9.2 (t, $J = 4.5$ Hz, 1H, NH).

The final required product could then, in principle, be obtained from **10** by removal of the benzyl protecting group. This could not be achieved by catalytic hydrogenation, presumably because of the sulfur present, and thus, the method of Weygand and Steglich (*11*) was employed. For this procedure, a solution of **10** (2.0 g, 4.3 mmol) was stirred in a solution of trifluoroacetic acid (12 mL) and thioanisole (1.0 g, 8.2 mmol) at room temperature for 11 h. After removal of the trifluoroacetic acid under vacuum, the brown residue was partitioned between ethyl acetate (75 mL) and water (55 mL) containing $NaHCO_3$. The aqueous layer was extracted with ethyl acetate (3 × 100 mL) and freeze-dried. Final purification of the product from the freeze-dried solid was achieved by Sephadex G10 column chromatography carried out in the cold room with water as the eluent: yield (as the sodium salt) 0.16 g (10%); ^{1}H NMR (^2H_2O) δ 3.98 (s, 3H, OCH_3), 4.51 (s, 2H, CH_2), 7.10 (s, 1H, thiazole-5H), 7.3−7.9 (m, 4H, ArH); MS (ESI^-) 377.3. This salt was rather hygroscopic and unsuitable for combustion analysis. Fluorimetric analysis for *m*-hydroxybenzoate after alkaline hydrolysis, however, indicated a purity of 96%.

Kinetics Methods. Steady state and pre-steady state kinetics measurements were obtained from a Hewlett-Packard 8452A spectrophotometer and a Durrum D-110 stopped-flow spectrophotometer, respectively. All kinetics studies were performed in 20 mM MOPS buffer (pH 7.5) at 25 °C. Enzyme stock solution concentrations were determined spectrophotometrically. Intermediate dilutions of enzymes at concentrations of <1 μM contained bovine serum albumin (1 mg/mL).

Steady state kinetics parameters were routinely obtained from spectrophotometric initial rate measurements at a series of appropriate substrate concentrations. These data were fitted to the Michaelis−Menten equation by a nonlinear least-squares program. Extinction coefficient changes for the substrates that were employed were 6510 cm^{-1} M^{-1} (260 nm) or 1830 cm^{-1} M^{-1} (280 nm) for cefotaxime, 5860 cm^{-1} M^{-1} (260 nm) for cefuroxime, 3200 cm^{-1} M^{-1} (278 nm) for cephalothin, 1240 cm^{-1} M^{-1} (290 nm) for **3**, and 5570 cm^{-1} M^{-1} (410 nm) for CENTA.

Values of K_m for the third-generation cephalosporins were also obtained as inhibition constants (K_i) for inhibition of turnover of a good substrate. To determine the K_i value for cefotaxime and cefuroxime with the P99 β-lactamase, cephalothin (50 μM; $K_m = 15$ μM) was used as the substrate. Concentrations of the enzyme, cefotaxime, and cefuroxime were 1 nM, 0−1 μM, and 0−0.6 μM, respectively. An experiment to obtain the K_i value for cefotaxime with the GCl β-lactamase employed CENTA (10 μM; $K_m = 2.6$ μM) as the substrate; concentrations of the enzyme and cefotaxime were 2.5 nM and 0−100 μM, respectively. Values of K_i were then obtained by fitting the initial rates, v, of substrate (S) turnover at a series of inhibitor (I) concentrations to eq 1 by means of a nonlinear least-squares program.

$$v = V_{max}[S]/[K_m(1 + [I]/K_i) + [S]] \quad (1)$$

In the limited-turnover stopped-flow experiments, concentrations of the P99 β-lactamase were 10, 10, and 5 μM and of cefotaxime 5.51, 2.76, and 5.51 μM, respectively. Multiple-turnover total progress curves for turnover of cefotaxime by the P99 β-lactamase were generated by employing an enzyme concentration of 50 nM and substrate concentrations of 10, 20, and 30 μM. The total progress curves that were generated were fitted to potential reaction

Table 1: Steady State Kinetics Parameters for Turnover of Substrates by the P99 β-Lactamase

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
cefotaxime	0.41 ± 0.02	17.2 ± 2.2	2.3×10^4
cefuroxime	0.28 ± 0.01	5.6 ± 0.7	5.0×10^4
cephalothin	690 ± 30	15.4 ± 1.9	4.5×10^7
depsipeptide **3**	19 ± 2	59 ± 15	3.3×10^5
depsipeptide **7**[a]	125	230	5.4×10^5

[a] Data from ref *20*.

schemes by means of the Dynafit program (*12*). This program was also used for all simulations of kinetic data. Methanolysis experiments to demonstrate the identity of the rate-determining step for substrate turnover and to determine the relative nucleophilicity of methanol and water toward acyl−enzyme intermediates were conducted and analyzed as previously described (*10*). Methanol concentrations were 0−2.5 M with both enzymes.

The perturbation experiment with ammonium sulfate involved, first, incubation of the enzyme (1.0 μM) with cefotaxime (10 μM) in MOPS buffer until the cefotaxime was completely hydrolyzed and the free enzyme restored, as monitored by the absorption at 284 nm and by a Dynafit (*12*) simulation from Scheme 4. An aliquot of the reaction mixture (1.0 μL) was then added to a cuvette containing the substrate cephalothin (1.0 mL, 200 μM) in MOPS buffer containing 2 M ammonium sulfate, and the hydrolysis of the latter monitored spectrophotometrically at 278 nm. Controls involved incubation of the enzyme in the absence of cefotaxime and also quenching the reaction mixture in the absence of ammonium sulfate. The return of activity rate constants after the ammonium sulfate quench (Scheme 5) were obtained by fitting the data (Figure 4B) to eq 2 by means of a nonlinear least-squares program. In this equation,

$$A = A_o - v_\infty t + \left(\frac{v_\infty - v_o}{k_8}\right)(1 - e^{-k_8 t}) \qquad (2)$$

A represents the substrate (cephalothin) absorbance at any time, A_o represents the absorbance at time zero, v_o and v_∞ represent the time zero and final velocities, respectively, and k_8 (Scheme 5) represents the return of activity rate constant.

RESULTS AND DISCUSSION

Cefotaxime, a third-generation cephalosporin, is a relatively poor substrate of the *E. cloacae* P99 β-lactamase. Steady state kinetics parameters for turnover of this substrate are reported in Table 1. The data yielding these parameters are shown in Figure 1A. On the other hand, Matagne et al. (*13*) reported a value of K_m that was some 500 times lower, viz., 0.033 μM. The latter value, however, was derived from measurement of the K_i value of cefotaxime as an inhibitor of substrate (nitrocefin) hydrolysis. In general, under steady state conditions, the K_i value derived in this fashion should be the same as the K_m value derived from the cefotaxime turnover experiment (*14*). To check the reported K_i value, we determined the K_i of cefotaxime as an inhibitor of cephalothin hydrolysis with the results shown in Figure 1B; the calculated K_i from these data was 0.029 ± 0.003 μM, in good agreement with the value reported by Matagne et al. (*13*). The discrepancy between the steady state turnover K_m value and this K_i value suggested that the turnover of

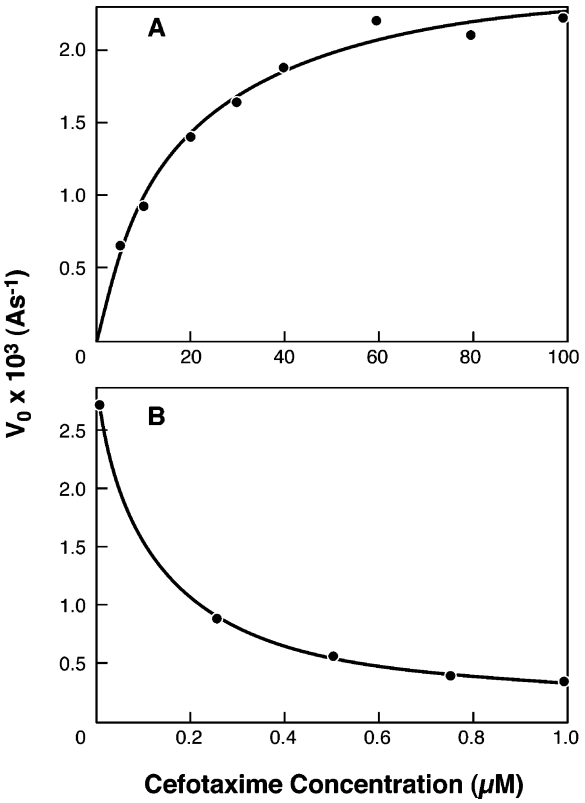


FIGURE 1: (A) Initial velocity (absorbance change at 260 nm/s) of hydrolysis of cefotaxime by the P99 β-lactamase (1.0 μM) as a function of cefotaxime concentration. The solid line represents a fit of the data to Scheme 2 (a Michaelis−Menten scheme), and the points are experimental. (B) Inhibition of cephalothin (50 μM) hydrolysis by cefotaxime. Shown is the initial velocity of cephalothin turnover (absorbance change at 278 nm/s) by the P99 β-lactamase (1.0 nM). The solid line represents a fit of the data to eq 2 for competitive inhibition; the points shown are experimental.

cefotaxime by this enzyme may, under some circumstances, involve kinetics more complicated than Michaelis−Menten kinetics.

The same pair of experiments were performed with cefuroxime **5**, another oximinocephalosporin, with similar results. Directly measured steady state parameters are also presented in Table 1. The K_i value of cefuroxime as an inhibitor of cephalothin hydrolysis was 0.013 ± 0.001 μM. Thus, the sharp difference between K_m and K_i observed for cefotaxime was also found with cefuroxime. The important common feature in the structures of cefotaxime and cefuroxime, which causes them to be relatively poor substrates and perhaps leads to the anomalous effects described above, is the third-generation oximino side chain. Cephalothin **4**, lacking the oximino moiety in the side chain, is a good substrate, whose steady state parameters for turnover by the P99 β-lactamase are also presented, for reference, in Table 1.

To determine the degree to which the oximino side chain itself was responsible for the kinetics properties of the third-generation cephalosporins, the acyclic depsipeptide analogue **3**, bearing the cefotaxime side chain, was prepared. The steady state kinetics parameters for hydrolysis of this compound, catalyzed by the P99 β-lactamase, are presented in Table 1, along with those of the reference depsipeptide **7** which possesses the side chain of a good β-lactam substrate. It is clear from these data that the oximino side chain of **3** does not negatively influence turnover to the extent that it

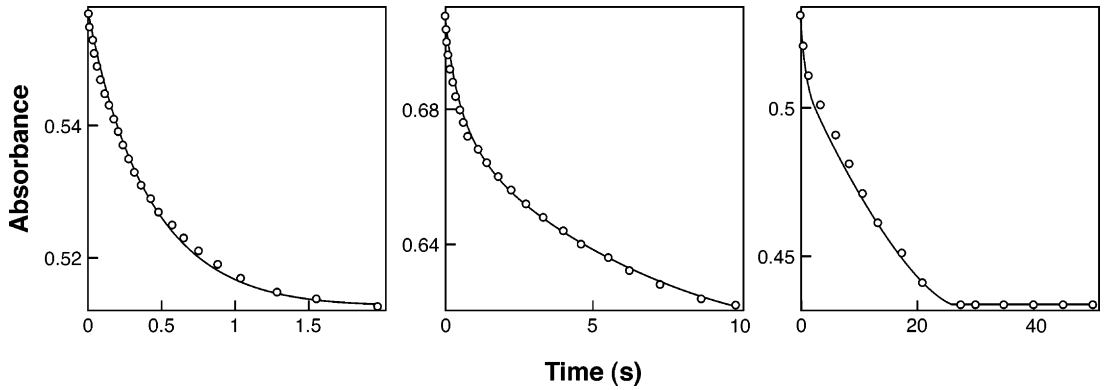


FIGURE 2: Stopped-flow absorbance changes at 260 nm as a function of time after mixing the P99 β-lactamase (5.51, 5.51, and 2.76 μM from left to right, respectively) with cefotaxime (5.0, 10.0, and 10.0 μM, respectively). The points are experimental, and the solid lines are derived from Scheme 4 as described in the text.

Scheme 2

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

does in cefotaxime. Methanolysis experiments showed that turnover of **3** is limited by the deacylation rate as it is for **7** (*10*). The slower turnover of **2** and **5** therefore must arise through a combination of the presence of the side chain and of the cephalosporin nucleus. This conclusion has been previously reached by Shoichet and co-workers on structural grounds (*8*). They proposed that unfavorable interaction of the ceftazidime side chain with the enzyme enforced a conformation of the pendant leaving group (the dihydrothiazine ring) that sterically inhibited deacylation (*8*, *15*). In the absence of the leaving group, as in the acyl−enzyme intermediate derived from **3**, deacylation is apparently not greatly affected (cf. that of **7**) (Table 1).

The kinetics anomaly described in the first paragraph of Results and Discussion can now be addressed. One difference between the two kinds of experiments described therein was the number of turnovers per enzyme molecule in each. The initial velocity measurements of the steady state turnover experiment were obtained from data where the initial substrate to enzyme concentration ratio was varied between 10 and 100. On the other hand, the K_i values were obtained from experiments where only two or three molecules of cefotaxime were hydrolyzed per molecule of enzyme [calculated by a Dynafit (*12*) simulation]. Consequently, a possibility to be examined was one where the first or first few turnovers by the enzyme are kinetically different from subsequent steady state turnovers. This idea was tested by limited turnover experiments carried out by means of a stopped-flow spectrophotometer. The results from these experiments are shown in Figure 2. The initial substrate/enzyme ratio in the three experiments whose results are shown were 0.9, 1.87, and 3.6. It is evident from the latter two experiments that the progress curves are biphasic, with the indication of a faster phase covering between one and two turnovers.

Scheme 2 is the simplest reaction sequence that might fit the biphasic curves of Figure 2. In Scheme 2, ES represents the noncovalent Michaelis complex and E−S the covalent acyl−enzyme intermediate. The fast phase of the reaction would thus correspond to rapid formation of E−S. This would lead to a spectral change similar to that which occurs on formation of P since the β-lactam ring is opened and the good acetoxy leaving group lost in both cases (*16*). That

Scheme 3

$$E + S \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} E\text{-}S \xrightarrow{k_3} E + P$$
$$E\text{-}S \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E\text{-}S' \xrightarrow{k_5} E + P$$

deacylation of E−S (k_3) is slower than acylation (k_2) was shown by the increase in the rate with methanol (data not shown) under substrate saturation (500 μM) conditions (*10*). [This conclusion agrees with that reached by Monnaie et al. (*17*) on the basis of pre-steady state kinetics experiments.] Attempts to fit (Dynafit) the data of Figure 2 to Scheme 2, where $k_3 = k_{cat} = 0.41\ s^{-1}$, k_1 (arbitrarily) $= 2 \times 10^8\ s^{-1}\ M^{-1}$, $k_2 \geq 10k_3$, and $K_m = k_{-1}k_3/k_1k_2 = 17.2\ \mu$M, were unsuccessful. The problems in fitting Scheme 2 were evident on inspection. The calculated rate of the fast (burst) phase was much slower than the data required, and the observed amplitude of the burst appeared to be somewhat greater than one enzyme concentration equivalent; Scheme 2 requires that the burst amplitude be stoichiometric or substoichiometric with the enzyme.

To proceed further, we noted that the acyl−enzyme intermediates formed on reaction of β-lactamases with poor substrates often partition into different forms. Usually, these are more inert to hydrolysis than the original, and thus, many such substrates are inhibitory. Scheme 3 is usually employed to describe these phenomena (*18*). This scheme, however, is also unable to fit the data of Figures 1 and 2, taken together. Sets of parameters that are able to generate the tight curves of Figure 2 at low substrate concentrations are unable to fit the substrate dependence at the high concentrations seen in Figure 1.

The curious kinetics of the cefotaxime reaction is also seen in the total progress curve of Figure 3A. Simple reaction schemes such as Schemes 2 and 3 have the problem of reconciling the curvature early in the reaction with the sharp break at the end of the reaction (the "post-steady state"). Figure 3A also shows (dashed line) the calculated progress curve of a reaction following Scheme 2 with the steady state parameters of Table 1. The initial rates match, but the latter line has much less curvature than the experimental line in the post-steady state region. Product inhibition, were it present, would of course produce even shallower curvature.

The simplest reaction scheme that is able to fit the data appeared to be that shown in Scheme 4. This combines the motifs of a branching acyl−enzyme intermediate with the

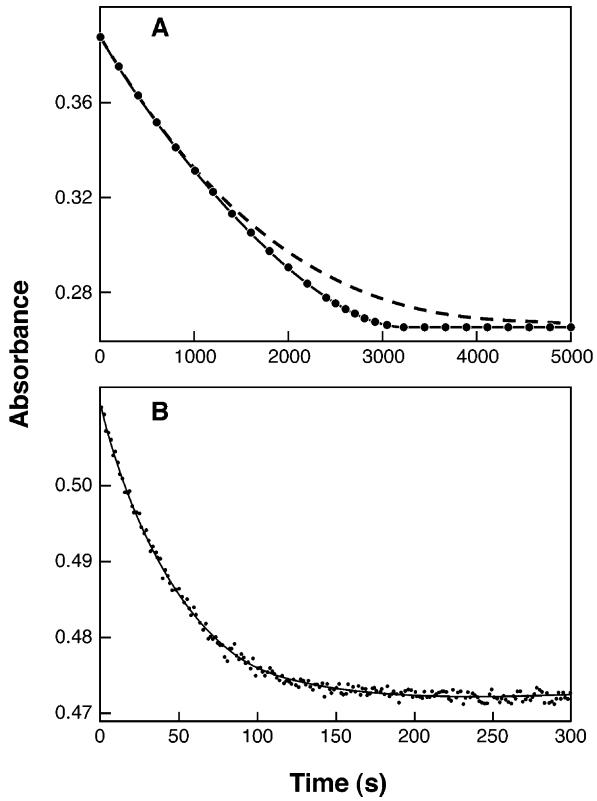
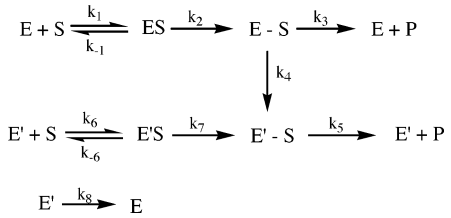


FIGURE 3: (A) Total progress curve for hydrolysis of cefotaxime (20 μM, monitored at 260 nm) by the P99 β-lactamase (50 nM). The points are experimental, and the solid line is derived from Scheme 4 as described in the text. The dashed line represents a calculated progress curve for the hydrolysis of cefotaxime (20 μM) by the P99 β-lactamase (50 nM) in accord with Scheme 1 and the steady state parameters of Table 1. (B) Total progress curve for the hydrolysis of cefotaxime (20 μM, monitored at 260 nm) by the GC1 β-lactamase (10 nM). The points are experimental, and the solid line is derived from Scheme 1.

Scheme 4



generation of a different free enzyme form, E′, on breakdown of the rearranged acyl−enzyme intermediate, E′−S. This free enzyme form either reacts with substrate leading to further turnover, although with kinetics constants different from those valid for E, or spontaneously reverts to the thermodynamically more stable free enzyme form E. To fit this scheme to the data depicted in Figure 2, k_1 and k_6 were set to 2×10^8 s^{-1} M^{-1}, k_5 was set to 0.41 s^{-1}, the steady state k_{cat} value (Table 1), k_7 was set to 10 s^{-1}, arbitrarily, but at least 10-fold higher than k_5 (since deacylation is rate-determining in the steady state), and k_{-6} was set to 8.85×10^4 s^{-1} so that $k_5k_{-6}/k_6(k_5 + k_7) = K_m$ (Table 1), the steady state value. Optimization of k_{-1}, k_2, k_3, k_4, and k_8 then yielded values for these parameters of 68, 1.5, 0.15, 0.14, and 0.04 s^{-1}, respectively, and the solid lines in Figure 2. The same set of parameters fitted the progress curve of Figure 3A (solid line) and those obtained at two other substrate concentrations (10 and 30 μM, not shown). Even the tight post-steady state curvature is fitted well (Figure 3A). Linear schemes generating a different free enzyme form were unable to fit all of the data.

The conversion of E−S to E′−S may be reversible to some degree, but this extension was not required to fit the data. The rate constant for reversion of E to E′ in solution must be small with respect to k_8, or significant amounts of E′ would exist at equilibrium; if the latter were so, another transient should have been observed in the experiments of Figure 2.

The kinetics parameters obtained for Scheme 4 were then used to simulate progress curves from which initial rates could be estimated. Such initial rates generated under the concentration conditions of Figure 1, when fitted to the Michaelis−Menten equation, yielded a k_{cat} of 0.30 ± 0.01 s^{-1} and a K_m of 14.3 ± 1.2 μM, in good agreement with those directly measured (Table 1).

Finally, simulated progress curves for turnover of cephalothin in the presence of cefotaxime were calculated, under the conditions of the experiment in Figure 1B, and using Scheme 4 and the kinetic constants for it that were obtained as described above. From initial rates measured from the simulated curves, a K_i value for cefotaxime of 0.029 μM was calculated. This result lends further support to Scheme 4.

A number of attempts were made to independently provide evidence for Scheme 4 by a demonstration that after all substrate and covalent enzyme−substrate complexes disappeared from the solution, the free enzyme remaining, E′ according to Scheme 4, differed in some fashion from the original enzyme E, but subsequently reverted to E. Dynafit (*12*) simulations of a reaction between 1 μM β-lactamase and 10 μM cefotaxime, and using the rate constants fitting Scheme 4 given above, showed that after 100 s the residual concentrations of cefotaxime and enzyme−cefotaxime complexes should be essentially zero. After this time, the only reaction happening in solution should be conversion of ca. 0.7 μM E′ to E, a process with a rate constant, from the fitting described above, of 0.04 s^{-1}, and thus essentially complete in an additional 100 s.

In one experiment, the protein fluorescence of the reaction mixture was monitored over a 200 s time period after the enzyme and cefotaxime had been mixed. A fast fluorescence (λ_{ex} = 285 nm, λ_{em} = 332 nm) decrease was observed on acylation (*19*) followed by a slow increase over 90 s as deacylation proceeded, but there was no further change in fluorescence intensity over the next 100 s. In another experiment, an aliquot of an *m*-(dansylamido)benzeneboronic acid solution was added after 90 s (final concentration of the boronate was 1 μM), but no change in dansyl fluorescence (emission at 540 nm) was observed over the next 100 s. This aryl boronate is an inhibitor of the P99 β-lactamase [$K_i \sim 2$ μM (*20*)], where inhibition is accompanied by a fluorescence emission enhancement (*20*); apparently, E and E′ have comparable affinity for this ligand and comparable effects on its fluorescence.

The reactivity of the post-cefotaxime reaction mixture with a second substrate was also assessed as described in Materials and Methods. An aliquot of such a reaction mixture after 90 s (when, according to both observation and simulation based on Scheme 4, essentially all of the enzyme should be free and most in the form of E′) was added to a solution of cephalothin and hydrolysis of the latter followed at 278 nm.

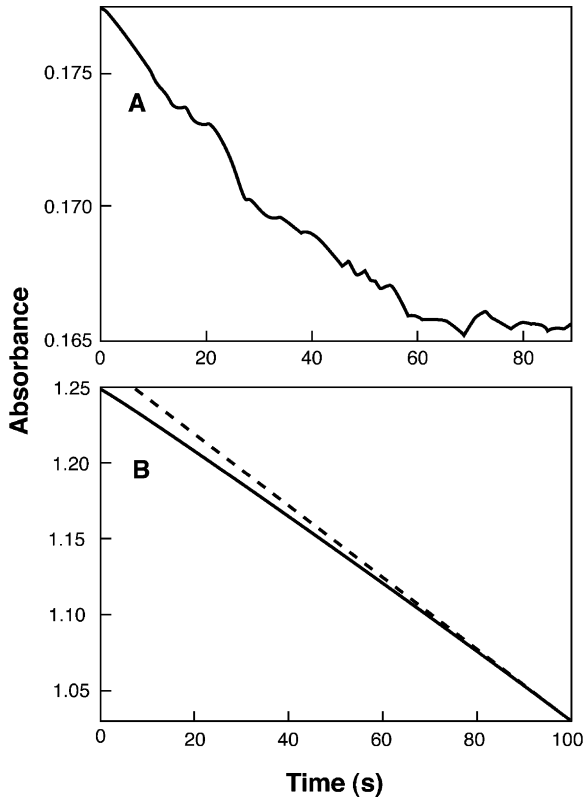
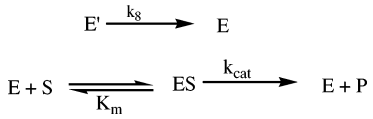


FIGURE 4: (A) Absorbance changes at 284 nm as a function of time after mixing the P99 β-lactamase (1.0 μM) with cefotaxime (10 μM) and reflecting the hydrolysis of cefotaxime. (B) Ninety seconds after cefotaxime and the β-lactamase had been mixed as described for panel A, an aliquot of the reaction mixture was withdrawn and added to a cephalothin (200 μM) solution containing 2 M ammonium sulfate. This panel shows the subsequent absorbance changes at 278 nm, reflecting the hydrolysis of cephalothin. The dashed line is a visual aid representing the finally achieved activity of the enzyme.

Scheme 5



No difference in rate or change in rate with time, with respect to a control in the absence of cefotaxime, was observed. Then, the same experiment was repeated, but where a perturbation to the assay (cephalothin) medium was included in the form of 2 M ammonium sulfate. In this case, but not in a control experiment where the enzyme was incubated in the absence of cefotaxime, a time-dependent increase in reactivity against cephalothin was observed. The results of such an experiment are shown in Figure 4. Panel A shows the disappearance of cefotaxime, complete after 90 s, and panel B the absorption of cephalothin as a function of time after addition of an aliquot of the reaction mixture to a second cuvette. As seen in the second panel, an increase in enzyme activity with time was observed under these conditions. If an additional 100 s elapsed before transfer of an aliquot to the cuvette containing cephalothin, no time-dependent increase in enzyme activity was observed. Application of Scheme 5 to the time-dependent data led to a rate constant (k_8) of conversion of E′ to E of 0.068 ± 0.018 s^{-1}. The interpretation of these data in terms of Scheme 4 would be that E′ is less reactive in ammonium sulfate with cephalothin than E but is converted to E with the above rate constant; the latter is apparently not greatly different from that in the absence of ammonium sulfate, viz., 0.04 s^{-1}. We have previously noticed that transition states of the P99 β-lactamase can be distinguished by the effect of ammonium sulfate (*21*).

Table 2: Steady State Kinetics Parameters for Turnover of Substrates by the GCl β-Lactamase

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
cefotaxime	62 ± 3	8.1 ± 1.5	7.6×10^6
cephalothin[a]	74.8	31	2.4×10^6
depsipeptide **3**	11.0 ± 0.1	46 ± 7	2.4×10^5
depsipeptide **7**	25 ± 1	130 ± 20	1.9×10^5

[a] Data from ref *5*.

Although an independent demonstration of the existence of E′ was not easy, it seems that it has been achieved by the final experiment described above. Scheme 4 therefore may be a good representation of the interaction of the P99 β-lactamase with cefotaxime.

Experiments with the E. cloacae GCl β-Lactamase. Steady state kinetics parameters for turnover of cefotaxime, cephalothin, and depsipeptides **3** and **7** are presented in Table 2. Comparison with the data given in Table 1 shows that the GCl β-lactamase is capable of catalyzing hydrolysis of cefotaxime essentially as effectively as it does the first-generation cephalosporin cephalothin. Hence, the ability of this enzyme to produce bacterial resistance to third-generation cephalosporins. As would be expected from the data given in Table 1, the GCl β-lactamase is also capable of catalyzing hydrolysis of depsipeptides **3** and **7** comparably well.

Measurement of substrate turnover rates of cefotaxime, benzylpencillin, and depsipeptide **3** ([S] ≥ $10K_m$) in the presence of methanol showed that, as with the P99 β-lactamase, the initial rate increased with methanol concentration. This indicates that, under conditions of substrate saturation, deacylation of the GCl β-lactamase is rate-determining with these substrates. This correlation is firmly established for the P99 β-lactamase where deacylation is usually rate-determining (*10*, *22*). Treatment of the methanolysis data by methods previously described (*10*) yielded values for the partition ratio of rate constants for methanolysis versus hydrolysis of the acyl−enzyme intermediate. These values were 12, 36, and 34 for cefotaxime, benzylpenicillin, and **3**, respectively. The interesting result here is the lower value for cefotaxime. This suggests a hindrance, possibly steric, of the approach of the larger nucleophile methanol to the acyl−enzyme intermediate derived from cefotaxime which is not present in the other two substrates. This result is also in accord with those described above which demonstrate the striking effects generated by a combination of a third-generation cephalosporin side chain and the β-lactam nucleus.

An experiment of particular relevance to this paper involved determination of the K_m of cefotaxime for the GCl β-lactamase from its K_i as an inhibitor of turnover of the first-generation cephalosporin CENTA (*9*). CENTA was used rather than cephalothin because determination of the K_i value in this case required cefotaxime concentrations that made direct observation of cephalothin turnover impossible. The K_i value of cefotaxime obtained by this method was 7.8 ± 0.6 μM. This value is the same, within experimental uncertainty, as the value of K_m determined directly (Table

2). It appears therefore that the complications observed in the turnover of cefotaxime by the P99 β-lactamase are not present with the GCl enzyme. This is indicated visually by inspection of a total progress curve for the latter enzyme shown in Figure 3B. The distinctive, sharp post-steady state cutoff observed with the P99 β-lactamase (Figure 3A) is not observed for the latter enzyme.

General Discussion. The difficulty that the P99 β-lactamase, a typical wild-type class C β-lactamase, experiences with the third-generation cephalosporins such as cefotaxime and cefuroxime is readily appreciated from the data in Table 1. A 10^3-fold decrease in the acylation rate (k_{cat}/K_m) with respect to that of cephalothin, a typical first-generation cephalosporin, is accompanied by a 10^3-fold decrease in the deacylation rate (k_{cat}); K_m, therefore, remains essentially unaffected. As elucidated above, however, these steady state (multiple-turnover) parameters disguise a more complex situation. Limited turnover experiments, both direct (pre-steady state) and indirect (inhibition kinetics), and post-steady state observations show that initial interactions between the P99 β-lactamase and cefotaxime are much stronger than the steady state parameters indicate. All of these results have been successfully rationalized, both qualitatively and quantitatively, by Scheme 4, where tight interaction of substrate S with free enzyme E yields acyl−enzyme intermediate E−S which partitions between turnover and rearrangement to a second acyl−enzyme form E′−S which yields product P and altered free enzyme form E′. The latter may either take up S, although more slowly than E, and catalyze hydrolysis or revert to E. Reversion to E is the preferred path at low S concentrations, so E will predominate in solution under these conditions. This situation would occur, for example, under post-steady state conditions (Figure 3A). Simulations of Scheme 4 (Dynafit) show that E comprises more than 50% of the free enzyme when the cefotaxime concentration falls below ~2 μM.

Numerical fitting of the cefotaxime data to Scheme 4 produced several interesting points. The acylation rate constant for the first turnover by E (k_1k_2/k_{-1}) has a value of 4.4×10^6 s^{-1} M^{-1}, only 10-fold lower than that of cephalothin. Deacylation is, however, much slower ($k_3 = 0.15$ s^{-1}). Conversion to E′ in the steady state leads to a 100-fold decrease in the acylation rate without a significant effect on the rate of deacylation (k_5). It therefore decreases the effectiveness of cefotaxime as a steady state competitive inhibitor of this enzyme by some 500-fold; K_i (K_m) for E $[k_{-1}k_3/k_1(k_2 + k_3)]$ would be 0.031 μM, while that for E′ $[k_{-6}k_5/k_6(k_5 + k_7)]$ would be 17.2 μM.

Scheme 4 represents an example of enzyme hysteresis (*23*) or of a mnemonic enzyme (*24*). Both of these phenomena involve slow equilibration of various enzyme forms. There have been previous suggestions that more than one free enzyme form of class C β-lactamases exist in solution under certain conditions, for example, by Page and co-workers (*25*, *26*), although the quantitative fitting of data to a specific reaction scheme has not previously been described. The partitioning of acyl−enzyme intermediates of β-lactamases into catalytically less active forms is well-established and thought, in many cases, to arise from conformational transitions (*18*). In other cases, a covalent contribution is also present. For example, the expulsion of the 3′-leaving group from a cephalosporin at the acyl−enzyme stage of turnover generally leads to a more stable acyl−enzyme intermediate (*16*, *27*). In the instance presented here, however, k_4 is much smaller than the expected rate constant for acetate elimination (*27*), and thus, the partitioning to E′ (Scheme 4) probably involves other processes after the elimination; the reversion of E′ to E, of course, most likely involves protein conformational events, related to movement of the Ω loop, for example.

The P99 β-lactamase catalyzes hydrolysis of depsipeptide **3**, which incorporates the cefotaxime side chain, as well as it does that of **7** which has a classical first-generation cephalosporin side chain (Table 1). Apparently, the deacylation problem found with cefotaxime is not induced by the side chain alone. Powers et al. (*8*) have suggested that the deacylation problem with third-generation cephalosporins arises from a combination of the steric effects of the dihydrothiazine leaving group, the side chain, and the Ω loop of the enzyme which abuts the active site. The results with **3** presented here appear to support this proposal.

Cefotaxime, therefore, is only slowly destroyed by classical class C β-lactamases, and thus, third-generation cephalosporins, in general, have been very important in the battle against β-lactam resistance in bacteria. The response of the P99 β-lactamase to cefotaxime is interesting in this regard, however, since the transition to E′ in the presence of micromolar concentrations of cefotaxime actually leads to more free enzyme. Our results suggest that E′ is as effective against the first-generation cephalosporin, cephalothin, as is E. Thus, even in the presence of substantial concentrations of cefotaxime (2−100 μM), free enzyme is available that could be used to tackle other β-lactams to which a bacterial cell might be exposed. It may be that the ability to undergo the E to E′ transition is an evolutionary strategy for maintaining some degree of β-lactam resistance even under specific β-lactam assault (although, presumably, not by cefotaxime which is not, to our knowledge, a natural product).

A more generally effective evolutionary strategy for maintaining resistance is derived from the selection of β-lactamase mutants that more effectively hydrolyze third-generation cephalosporins. One such extended spectrum class C β-lactamase is the GC1 enzyme of *E. cloacae* described in the introductory section. As is clear from Table 2 and elsewhere (*5*), the GC1 β-lactamase catalyzes the hydrolysis of cefotaxime as well as it does cephalothin. It also catalyzes the hydrolysis of depsipeptides **3** and **7** equally well. It has been suggested (*6*, *8*) that the greater flexibility of the extended Ω loop allows a conformational adaptation of the enzyme and ligand, leading to a conformation where facile deacylation is possible.

It seems possible from these results therefore that an Ω loop rearrangement may be responsible for the complicated response of the P99 β-lactamase to the third-generation cephalosporins such as cefotaxime. Although acylation is facile, the resulting acyl−enzyme intermediate may be conformationally restricted by the Ω loop, especially by the position of the Tyr221 side chain (*6*, *8*), such that rapid hydrolysis cannot occur. Although a conformational relaxation, perhaps of the Ω loop, can then compete with deacylation, this does not, in the case of the P99 β-lactamase, in contrast to that of the GC1 enzyme, lead to faster deacylation. It does, however, after deacylation, result in a conformationally altered free enzyme form (E′) which is slower to react with third-generation cephalosporins and slow

to revert to the thermodynamically stable form of the free enzyme (E). It is also possible that the E to E′ transition could, partly at least, be derived from movement of the loop containing helix 10a that hovers above the active site. Electron density maps of the P99 and ampC β-lactamases show fuzziness in this area, suggesting mobility (J. R. Knox, personal communication; *28*), and mutations in this area lead to significant catalytic changes (*29*−*31*).

Powers et al. have described the crystal structure of an acyl−enzyme intermediate formed by reaction of the class C ampC β-lactamase with the third-generation cephalosporin ceftazidime (*8*). Since the conformations of the Ω loop and of the helix 10a loop in this complex are essentially identical to those of the free enzyme (*32*), it is likely that the enzyme form in the crystal is E. The conformational change required to form E′ may not be readily achievable in the crystalline state. As demonstrated in this paper, the consequences of these structural problems for the activity of the enzyme in solution may be readily detected and studied by a combination of pre- and post-steady state kinetics experiments.

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