The d -Methyl Group in β-Lactamase Evolution: Evidence from the Y221G and GC1 Mutants of the Class C β-Lactamase of Enterobacter cloacae P99 †

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The D-Methyl Group in β-Lactamase Evolution: Evidence from the Y221G and GC1 Mutants of the Class C β-Lactamase of *Enterobacter cloacae* P99†

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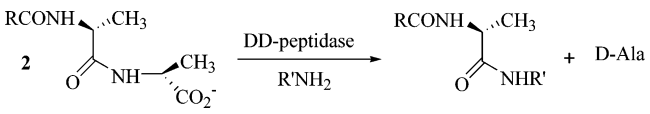
ABSTRACT: The β-lactam antibiotics act through their inhibition of D-alanyl-D-alanine transpeptidases (DD-peptidases) that catalyze the last step of bacterial cell wall synthesis. Bacteria resist β-lactams by a number of mechanisms, one of the more important of which is the production of β-lactamases, enzymes that catalyze the hydrolysis of these antibiotics. The serine β-lactamases are evolutionary descendants of DD-peptidases and retain much of their structure, particularly at the active site. Functionally, β-lactamases differ from DD-peptidases in being able to catalyze hydrolysis of acyl-enzyme intermediates derived from β-lactams and being unable to efficiently catalyze acyl transfer reactions of D-alanyl-D-alanine terminating peptides. The class C β-lactamase of *Enterobacter cloacae* P99 is closely similar in structure to the DD-peptidase of *Streptomyces* R61. Previous studies have demonstrated that the evolution of the β-lactamase, presumably from an ancestral DD-peptidase similar to the R61 enzyme, included structural changes leading to rejection of the D-methyl substituent of the penultimate D-alanine residue of the DD-peptidase substrate. This seems to have been achieved by suitable placement of the side chain of Tyr 221 in the β-lactamase. We show in this paper that mutation of this residue to Gly 221 produces an enzyme that more readily hydrolyzes and aminolyzes acyclic D-alanyl substrates than glycyl analogues, in contrast to the wild-type β-lactamase; the mutant is therefore a more efficient DD-peptidase. Molecular modeling showed that the D-alanyl methyl group fits snugly into the space originally occupied by the Tyr 221 side chain and, in doing so, allows the bound substrate to assume a conformation similar to that on the R61 DD-peptidase, which has a hydrophobic pocket for this substituent. Another mutant of the P99 β-lactamase, the extended spectrum GC1 enzyme, also has space available for a D-alanyl methyl group because of an extended Ω loop. In this case, however, no enhancement of activity against D-alanyl substrates with respect to glycyl was observed. Accommodation of the penultimate D-alanyl methyl group is therefore necessary for efficient DD-peptidase activity, but not sufficient.

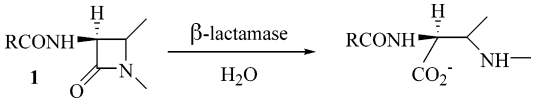
The biosynthesis of bacterial cell walls is completed by a transpeptidation reaction linking adjacent peptidoglycan strands and catalyzed by a class of enzymes known variously as D-alanyl-D-alanine transpeptidases, DD-peptidases, or penicillin-binding proteins (Scheme 1) (*1*). The latter name reflects the important point that inhibition of the enzymes by penicillins, and β-lactams in general, **1**, leads to the antibiotic properties of the β-lactams. Bacterial resistance to β-lactams derives from a number of sources (*2*) but the clinically most important is probably from the ability of bacteria to produce enzymes, the β-lactamases, that catalyze hydrolysis of β-lactams (Scheme 2).

On the basis of the structural resemblance between the substrates of the DD-peptidases, *viz.*, D-alanyl-D-alanine terminating peptides, **2**, and those of the β-lactamases, Tipper and Strominger suggested that the latter enzymes might have

Scheme 1



Scheme 2



evolved from the former (*3*). Functional (*4*, *5*) and structural (*6*, *7*) studies have now shown that this proposition is probably correct. Crystal structures show that the DD-peptidases and most β-lactamases (excluding the metallo-β-lactamases) possess the same general backbone fold and a generally similar collection of active site residues for catalysis. Both groups of enzymes react, to some degree at least, with appropriately designed peptides, analogous depsipeptides, and β-lactams, catalyzing the hydrolysis and, in some cases, aminolysis of these molecules by a double displacement mechanism involving a now well-characterized acyl-enzyme intermediate (Scheme 3). The enzymes differ

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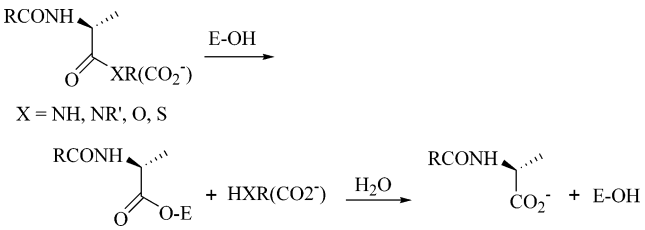
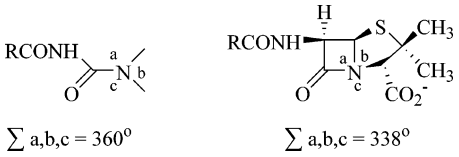
Scheme 3



Scheme 4



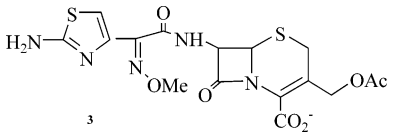
in the inability of the DD-peptidases to efficiently catalyze hydrolysis of acyl-enzymes derived from β-lactams and the inability of β-lactamases to form acyl-enzymes from peptides.

These deficiencies, neither of which is well understood with respect to molecular detail, are important: the first, of course, is the source of the antibiotic activity of β-lactams while the latter is presumably important to bacteria in preventing interference of β-lactamases in peptidoglycan biosynthesis.

The evolution of β-lactamases from DD-peptidases, which occurred millions of years ago (*8*), must therefore have resulted from selection of enzymes that were unable to attack peptides but that could hydrolyze acyl-enzymes derived from β-lactams. The former of these goals may have been met by a subtle change in the shape of the active site such that planar (at nitrogen) peptides could not be productively bound although "bent" (Scheme 4) structures such as bicyclic β-lactams could (*9*). The latter goal appears to have been met by the repositioning of a general acid/base catalytic group or incorporation of a new amino acid side chain to play this role in β-lactam deacylation. These issues have been reviewed recently in detail (*10*).

Beyond the difference in geometry of the scissile bond, described above, another striking difference between **1** and **2** is the presence of the D-alanyl methyl group in **2**. Selection against this methyl group could also be part of the evolutionary process leading to a β-lactamase. Indeed, research in this laboratory has shown that, in the case of one closely related DD-peptidase/β-lactamase pair (the DD-peptidase of *Streptomyces* R61 and a typical class C β-lactamase, that of *Enterobacter cloacae* P99), the β-lactamase preferred to hydrolyze glycyl derivatives rather than D-alanyl while the DD-peptidase had the reverse preference (*11*). Similar observations have been made by others (*12*, *13*). Inspection of the relevant crystal structures, aided by molecular modeling of complexes of glycyl and D-alanyl substrates, showed that the DD-peptidase had a nice hydrophobic pocket for the penultimate D-methyl group. During evolution of the β-lactamase, the distinctive Ω loop that lies under the active site became twisted at the neck, reversing the direction of the loop (*14*). The effect of this was to position Tyr 221 beneath the active site such that its β-methylene group effectively removes the D-alanyl methyl group pocket (*11*). If this analysis were correct, then replacement of Tyr 221 with glycine would be predicted to promote the reactivity of the P99 β-lactamase with D-alanyl substrates. We describe in this paper the catalytic properties of the Y221G mutant of the P99 β-lactamase. The predicted change in substrate specificity was in fact observed. Molecular models were constructed to support these observations.

β-Lactamases have continued to evolve. In particular, over the last 50 years, mutants capable of efficiently catalyzing the hydrolysis of β-lactamase-resistant penicillins and third generation cephalosporins have arisen, been selected for, and spread through bacterial populations (*15*). These clinically important β-lactams share a structural feature, a bulky side chain where the bulk is concentrated directly adjacent to the β-lactam carbonyl group. One strategy employed by these mutants, therefore, is to expand the space available to the substrate directly adjacent to the carbonyl group, i.e., in just the area where a DD-peptidase requires more space for the penultimate D-alanyl methyl group of its peptide substrate. One particular example of an extended spectrum β-lactamase of this type, capable of efficiently hydrolyzing third generation cephalosporins, is a variant of the P99 β-lactamase isolated from *E. cloacae* GCl (*16*). This enzyme has more space available for β-lactam side chain binding by virtue of a three amino acid (AlaValArg) insertion into the Ω loop (*17*). This extended loop can then adopt a conformation where Tyr 221, which, in the wild-type enzyme, closely abuts the side chain of a bound third generation cephalosporin such as cefotaxime (**3**), can move away and allow the side chain



3

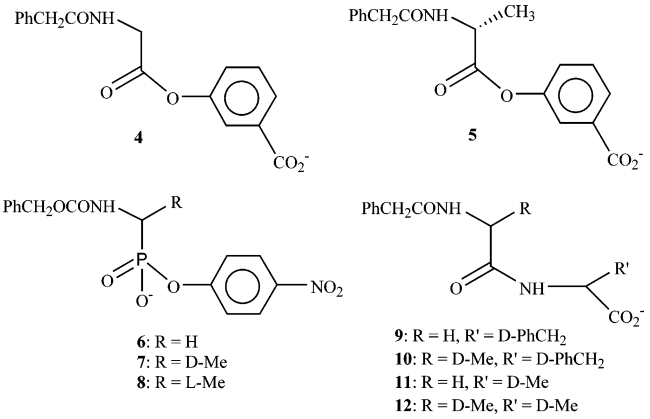
to bind more closely and productively (*18*). The effect of the GCl mutation on the affinity of a class C β-lactamase for acyclic D-alanyl substrates is also described below.

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 Y221G mutant of this enzyme and the extended spectrum β-lactamase from *E. cloacae* GCl were prepared and purified as previously reported (*16*, *19*). Cephalothin was a gift from Eli Lilly and Co., and cefotaxime and cefoxitin were from Merck. CENTA[1] was prepared in this laboratory in the manner reported previously (*20*). 3-{[*N*-(Phenylacetyl)glycyl]oxy}benzoic acid (**4**) (*11*), 3-{[*N*-(phenylacetyl)-D-alanyl]oxy}benzoic acid (**5**) (*11*), *p*-nitrophenyl (benzyloxycarbonylamido)methylphosphonate (**6**) (*21*), *p*-nitrophenyl D- and L-1-(*N*-benzyloxycarbonylamido)-ethylphosphonate (**7** and **8**, respectively) (*22*), *N*-(phenylacetyl)glycyl-D-phenylalanine (**9**) (*11*), and *N*-(phenylacetyl)-D-alanyl-D-phenylalanine (**10**) (*11*), were available from previous research in this laboratory. *N*-(Phenylacetyl)glycyl-D-alanine (**11**) and *N*-(phenylacetyl)-D-alanyl-D-alanine (**12**) were prepared by means of classical peptide chemistry

[1] Abbreviations: CENTA, 7β-[(thien-2-yl)acetamido]-3-[(4-nitro-3-carboxyphenylthio)methyl]-3-cephem-4-carboxylic acid; DMSO, dimethyl sulfoxide; MOPS, 3-morpholinopropanesulfonic acid; NMR, nuclear magnetic resonance.

procedures. *N*,*N*′-Diacetyl-L-lysyl-D-alanyl-D-alanine was purchased from Sigma Chemical Co.



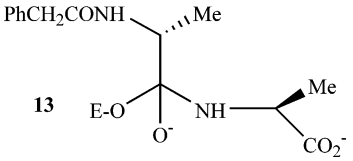
Kinetics Methods. All kinetics measurements were carried out in 20 mM MOPS buffer, pH 7.5, at 25 °C, unless otherwise stated. Absorption spectra and spectrophotometric reaction rates were measured with a Hewlett-Packard 8452A spectrophotometer. Steady-state enzyme-catalyzed reactions of **4** and **5** were monitored at 290 nm ($\Delta\epsilon$ =1760 cm^{-1} M^{-1}) or 300 nm ($\Delta\epsilon$ = 940 cm^{-1} M^{-1}), of cefotaxime at 264 nm ($\Delta\epsilon$ = 6830 cm^{-1} M^{-1}) or 280 nm ($\Delta\epsilon$ = 1830 cm^{-1} M^{-1}), of cefoxitin at 260 nm ($\Delta\epsilon$ = 6470 cm^{-1} M^{-1}), of cephalothin at 278 nm ($\Delta\epsilon$ = 3200 cm^{-1} M^{-1}), and of CENTA at 410 nm ($\Delta\epsilon$ = 6230 cm^{-1} M^{-1}). Steady-state kinetics parameters were then obtained from initial rates by nonlinear least squares fitting of data to the Michaelis−Menten equation.

Attempts were made to directly monitor spectrophotometrically at 236 nm the reactivity of the β-lactamase with the peptide substrates **9**, **10**, and *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine. This method, however, was not sufficiently sensitive so the required rates were determined by means of a colorimetric assay for D-alanine (*23*). Typically, the peptide substrate, **11** or **12** (10 mM), was incubated with enzyme (10 μM) at 37 °C. At appropriate intervals over several days, aliquots were removed and assayed for D-alanine. This method yielded intial rates that could be directly converted into k_{cat}/K_m values since measurements of the inhibition of turnover of **4** by **11** and **12** showed that $K_m \gg 10$ mM for all three enzymes and both peptides.

Methanolysis Kinetics. The effects of methanol on initial rates of solvolysis of both depsipeptides **4** and **5** in the presence of the Y221G P99 β-lactamase were determined spectrophotometrically in aqueous methanolic buffer as previously described (*24*). Methanol concentrations of up to 2.5 M were employed. The concentration of **4** was 5 mM, and that of **5** was 4 mM. The variations of the initial rates of reaction of both **4** and **5** (monitored at 300 nm) were linear, and the partition ratios, $k_M/k_{cat'}$, of methanolysis to hydrolysis were determined from least squares fits to the data (*24*). A similar procedure was used for the GC1 β-lactamase.

Aminolysis Kinetics. Spectrophotometric measurements, as described above, were also used to obtain initial rates of reactions of **4** in the presence of D-phenylalanine and the Y221G β-lactamase. Two experimental protocols were employed, one with fixed substrate (5 mM) and varying D-phenylalanine concentration (0−40 mM) and the other with fixed D-phenylalanine concentration (15 mM) and varying substrate concentration (0−20 mM). For these experiments, stock solutions of **4** were prepared in DMSO, and the kinetics measurements were carried out in 100 mM MOPS buffer, pH 7.5, in order to keep better control of the pH. The DMSO concentration in the reaction mixtures was 5% (v/v). These data were fitted by a nonlinear least squares method to the appropriate equation (see below). In other cases, an abbreviated procedure was employed. The substrate (**4** or **5**), at a concentration above K_m, was reacted with the appropriate enzyme in the presence of D-alanine or D-phenylalanine at varied concentrations. These experiments were performed in 20 mM MOPS, pH 7.5. Initial rates were obtained at sufficiently low amino acid concentrations as to yield linear plots of initial velocity vs amino acid concentration. These data were fitted by a linear least squares procedure and interpreted as described in the text below.

Molecular Modeling. Models of tetrahedral intermediates **13** formed during acylation of the Y221G and GCl enzymes by **11** and **12** were constructed as described previously (*11*).



The complex of the Y221G mutant was constructed from the crystal structure of a phosphonate (transition state analogue) complex of the P99 enzyme (PDB file 1BLS; *23*) by deletion of the Y221 side chain and its replacement by hydrogen. Two conformations of the bound ligand were examined. One ("methyl out") was obtained by "mutation" of the structure shown in Figure 5A of ref *11*, and the second ("methyl in") was from Figure 6 of that paper. These structures (entire system, protein plus water molecules) were subjected to short molecular dynamics runs (20 ps) to relax local structure, and typical snapshots were energy minimized as previously described. Interaction energies between ligand and active site, E_{int}, were then calculated (*11*, *26*). These E_{int} values include interactions between the ligand and active site residues, interactions between the active site residues themselves, and internal energies of the ligand and active site residues. Residues included in these calculations were Ser 64, Lys 67 (side chain cationic), Tyr 150 (side chain neutral), Asn 152, Lys 315 (side chain cationic), Thr 316, Gly 317, and Ser 318.

Analogous tetrahedral structures were constructed at the active site of the GCl β-lactamase. The source in this case was the crystal structure of a phosphonate inhibitor complex (tetrahedral intermediate analogue) of this enzyme (PDB file 1RAZ; *18*). Energy-minimized structures were again obtained and E_{int} values calculated.

Models of the tetrahedral intermediates formed on reaction of **11** and **12** with the P99 β-lactamase and the R61 DD-peptidase were available from previous studies (*11*). Molecular dynamics simulations (200 ps) were carried out on these to judge their mobility. To obtain a measure of this, 2 ps snapshots from 50 to 200 ps were averaged and the averaged structures compared with the initial, energy-minimized structures in each case. The rms differences in atom positions between these structures were calculated by means of the ProFit program (*27*).

Models of noncovalent complexes of **11** and **12** with the P99 β-lactamase and the R61 DD-peptidase, respectively,

Table 1: Steady-State Kinetic Parameters for Hydrolyses of **4** and **5** Catalyzed by the P99, Y221G, and GC1 β-Lactamases

	P99		Y221G		GC1	
parameter	**4**[a]	**5**[b]	**4**	**5**	**4**	**5**
k_{cat} (s^{-1})	125	69	5.5 ± 0.2	21.4 ± 0.7	125 ± 6	7.8 ± 0.6
K_m (mM)	0.23	2.5	0.7 ± 0.1	1.2 ± 0.1	0.94 ± 0.05	1.5 ± 0.3
K_D (mM)	3.0[c]	*d*	60 ± 10	*d*	1.0 ± 0.1	*d*
k_{cat}/K_m ($s^{-1}\,M^{-1}$)	5.4×10^5	2.8×10^4	7.9×10^3	1.7×10^4	1.3×10^5	5.2×10^3

[a] Data from ref *44*, except for K_D. [b] Data from ref *11*. [c] Data from ref *24*. [d] No evidence for ED_1D_2 (Scheme 5) at the concentrations employed.

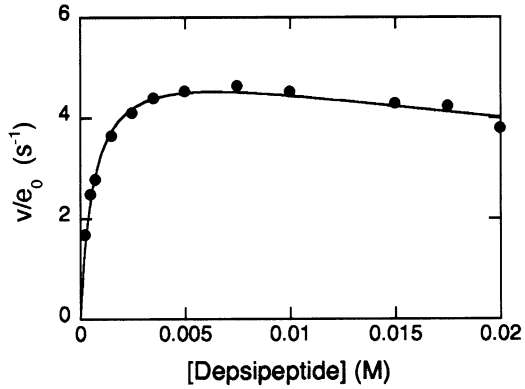
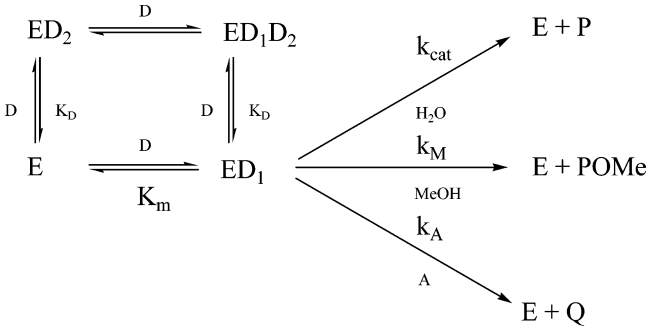


FIGURE 1: Initial rates of hydrolysis of the depsipeptide **4**, catalyzed by the Y221G mutant of the P99 β-lactamase (0.32 μM). The points are experimental, and the line is calculated from the fit of the data to Scheme 5.

Scheme 5



were obtained as described previously (*28*). In these structures, the side chains of Lys 67 and Tyr 150 (β-lactamase) or Lys 65 and Tyr 159 (DD-peptidase) were cationic and anionic, respectively.

RESULTS AND DISCUSSION

The Y221G and GC1 mutants of the classical *E. cloacae* P99 class C β-lactamase catalyze the hydrolysis of the depsipeptides **4** and **5**, as does the wild-type enzyme (*24*, *29*). In the case of **4**, substrate inhibition was observed with both mutant enzymes; Figure 1 shows the data for the Y221 mutant. Although such inhibition had not been directly observed in earlier experiments with **4** and the wild-type P99 β-lactamase, there was ample evidence from studies of the aminolysis of **4** by D-amino acids for a second depsipeptide binding site on this enzyme (*30*); this result has been confirmed by direct binding studies (*31*). In accord with precedent, therefore, the steady-state data from the mutant enzymes were fitted to Scheme 5. In this scheme, on the present occasion, the tertiary complex ED_1D_2, where D represents the depsipeptide substrate and P the hydrolysis products, is represented as inert to hydrolysis. This may not be absolutely true [the ED_1D_2 complex of **4** with the wild-type enzyme is as reactive as ED_1 (*24*, *29*)] but the assumption yields the simplest scheme to fit the data at the achievable substrate concentrations. Scheme 5 also includes steps for methanolysis and aminolysis by D-amino acids (A) of the binary complex (see below). The methanolysis and aminolysis products are represented as POMe and Q, respectively.

Steady-state parameters derived from the fit of the hydrolysis data to Scheme 5 are presented in Table 1. In Scheme 5, k_M and k_A are second-order rate constants, whereas k_{cat}, by custom, is a pseudo-first-order rate constant, incorporating the effective water concentration; the second-order rate constant corresponding to k_{cat}, and given by $k_{cat}/[H_2O]$, where $[H_2O]$ is taken to be 55.5 M, will henceforward in this paper be designated $k_{cat'}$. The partition ratios $k_M/k_{cat'}$ and $k_A/k_{cat'}$ will therefore be dimensionless. All precedent (*11*, *24*) would suggest that ED_1, the accumulating intermediate in the steady state, represents the acyl-enzyme intermediate, and therefore that k_{cat} represents the deacylation rate constant. Indeed, an experiment to investigate the effect of the alternative nucleophile methanol on the rates of reaction of the Y221G mutant with **4** and **5** showed that methanol accelerated the reaction in both cases. Analysis of the data (*24*) gave values of $k_M/k_{cat'}$ of 54 ± 6 and 64 ± 1 for **4** and **5**, respectively. These values are larger than those for the wild-type enzyme, 28 and 26 for **4** and **5** respectively (*11*), which suggests that methanol has, relative to water, greater access to the acyl-enzyme in the mutant than in the wild-type enzyme. This result may indicate a general loosening of the active site on elimination of the Y221 side chain (see below). A similar result was obtained for the GC1 mutant: $k_M/k_{cat'}$ values of 47 ± 3 and 41 ± 2 for **4** and **5**, respectively. The latter numbers also suggest relatively greater access of methanol to the acyl-enzyme.

The reactions of **4** and **5** in the presence of both mutant enzymes were also accelerated by D-amino acids, which further supports the conclusion that deacylation is rate-determining under saturating substrate conditions. ^{1}H NMR spectra showed that the rate increase correlated with formation of the corresponding peptide product. Figure 2 shows kinetics data for the effects of D-phenylalanine on the rate of reaction of **4** in the presence of the Y221G mutant enzyme. Panel A shows the effect of varying depsipeptide concentration at constant D-phenylalanine concentration (15 mM) and panel B the effect of varying phenylalanine concentration at constant depsipeptide concentration (5 mM). The fit of these data to Scheme 5 (*11*) yielded a value of $(5.3 \pm 0.3) \times 10^4$ for $k_A/k_{cat'}$, the partition ratio of ED_1 for aminolysis with respect to hydrolysis. Values of this ratio were obtained as described in Materials and Methods for the other combina-

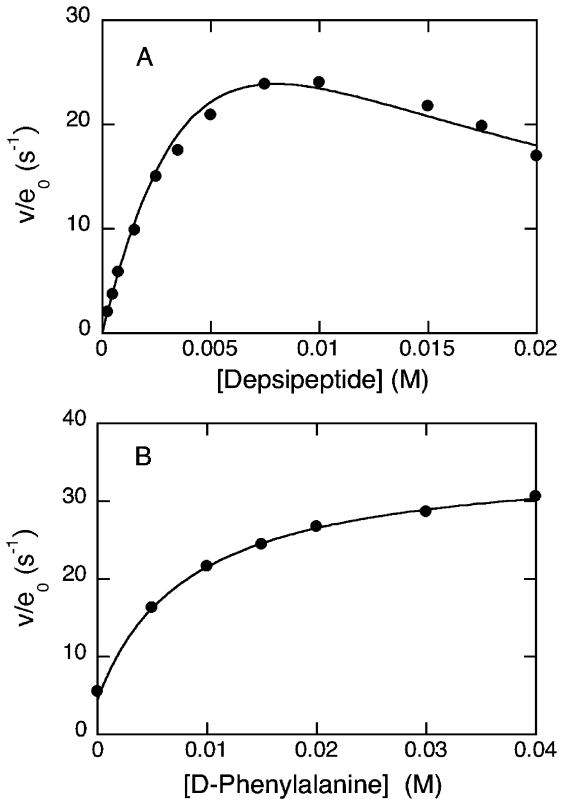


FIGURE 2: (A) Initial rates of total reaction (hydrolysis and aminolysis) of the depsipeptide **4** in the presence of D-phenylalanine (15 mM) and the Y221G mutant of the P99 β-lactamase (0.32 μM). (B) Initial rates of total reaction (hydrolysis and aminolysis) of the depsipeptide **4** (5 mM) in the presence of D-phenylalanine and the Y221G mutant of the P99 β-lactamase (0.32 μM). In both (A) and (B), the points are experimental, and the lines are calculated from the fit of the data to Scheme 5.

Table 2: Aminolysis/Hydrolysis Partition Ratios for Reaction of **4** and **5** in the Presence of D-Amino Acids, Catalyzed by the P99, Y221G, and GC1 β-Lactamases

enzyme	substrate	amine	partition ratio (k_A/k_{cat})	k_A ($s^{-1}\,M^{-1}$)
P99	**4**[a]	D-Phe	8×10^4	1.8×10^5
	5[a]	D-Phe	1.4×10^3	1.74×10^3
	4	D-Ala	90 ± 40	200
	5	D-Ala	50 ± 35	60
Y221G	**4**	D-Phe	$(5.3 \pm 0.3) \times 10^4$	5.3×10^3
	5	D-Phe	$(3.1 \pm 0.3) \times 10^4$	1.2×10^4
	4	D-Ala	300 ± 40	30
	5	D-Ala	1000 ± 60	385
GC1	**4**	D-Phe	$(8.9 \pm 1.3) \times 10^4$	2×10^5
	5	D-Phe	270 ± 80	38
	4	D-Ala	530 ± 30	1200
	5	D-Ala	50 ± 35	7

[a] Data from ref *11*.

tions of enzyme and substrate and also for the acceptor D-alanine. The results are collected in Table 2.

The DD-peptidase activity of these enzymes was also tested. The P99 β-lactamase, like all other known β-lactamases, has only very weak peptidase activity (*32*). Preliminary experiments with peptides **9**, **10**, and *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine showed that the mutants were also poor peptidases, if at all. Closer investigation of **11** and **12** revealed the weak activities reported in Table 3. The values obtained for **11** and **12** with the P99 enzyme are similar to but larger than those reported by Rhazi et al. (*32*); the latter authors, however, used phosphate buffer which is known to be inhibitory to this enzyme (*24*).

Table 3: DD-Peptidase Activites of the P99, Y221G, and GC1 β-Lactamases

	$k_{cat}/K_m \times 10^2$ ($s^{-1}\,M^{-1}$)	
enzyme	**11**	**12**
P99	10.5 ± 1.7	4.4 ± 0.5
Y221G	1.8 ± 0.3	13.0 ± 3.4
GC1	10.9 ± 1.8	3.3 ± 1.0

Several interesting conclusions can be made on the basis of the data of Tables 1−3. First, from Table 1, it is apparent that the catalytic effectiveness of the P99 β-lactamase toward **4** and **5** has decreased on mutation of Tyr 221 to Gly 221. This change is apparent in both acylation (k_{cat}/K_m) and deacylation (k_{cat}). The most easily interpretable result is probably the decrease in activity against **4** where the bulk of the methyl group of the penultimate D-alanine is not an issue. The decrease in this case may reflect the greater mobility of the active site and substrate when the Tyr 221 side chain is removed; greater mobility unrelated to catalysis would, of course, produce an entropic decrease in the observed rate constants. More interesting, however, is the change in the relative effectiveness of **4** and **5** as substrates produced by the Y221G mutation. The D-alanyl substrate **5** is more effective than its glycyl analogue **4** in the mutant whereas the reverse is true in the wild-type P99 enzyme. This strongly suggests that removal of the Tyr 221 side chain opens up space for more productive binding of a D-alanyl substrate such as **5**.

Although the GC1 mutation also opens up space in the active site (*17*, *18*), it is clear from Table 1 that the relative reactivity of **4** and **5** with the enzyme remains unchanged. It seems, therefore, that space alone, although it may be necessary to promote reaction of D-alanyl substrates, is not sufficient. If binding of the D-alanyl methyl group leads to a more conformationally productive complex of enzyme and substrate, it appears that a closer approximation to an optimal conformation is afforded by the Y221G mutation than by the GC1.

The hydrolysis data of Table 1 is reinforced by the aminolysis results of Table 2. The greater relative preference of the Y221G mutant for a D-alanyl substrate such as **5** is seen in the aminolysis/ hydrolysis partition ratio and, more particularly, in the aminolysis rate constants themselves. With both D-alanine and D-phenylalanine as acyl acceptors, the reactivity of **5** is greater than that of **4** with the mutant although not with the wild-type enzyme. The GC1 mutant retains the relative reactivities of the native enzyme, in agreement with the hydrolysis results described in the previous paragraph.

The enhanced reactivity of **5** with D-amino acid acceptors in the presence of the Y221G mutant raised the question of whether this enzyme would have significant DD-peptidase activity. Although preliminary experiments showed that the mutant was not a significant peptidase, the quantitative data of Table 2 show that the trend observed with the depsipeptides, described above, is also present in peptide hydrolysis. Thus, although the wild-type P99 enzyme is more effective against the glycyl peptide **11**, the reverse is true for the Y221G mutant. Again, the removal of the Tyr 221 side chain

Table 4: Second-Order Rate Constants for the Inhibition of β-Lactamases by the Phosphonates **6**−**8**

	k_i ($s^{-1} M^{-1}$)	
inhibitor	P99	Y221G
6	3.3×10^4 [a]	15.2 ± 0.5
7	3.4[b]	480 ± 30
8	610[b]	720 ± 60

[a] Data from ref *21*. [b] Data from ref *22*.

has promoted reactivity of the D-alanyl substrate. In further agreement with the results described above for the GC1 β-lactamase, this enzyme exhibits comparable DD-peptidase activity against **11** to the wild-type enzyme and also the smaller activity against **12**.

The effect of the Y221G mutation was also observed in the reaction of the β-lactamase with phosphonate inhibitors such as **6**−**8**. Table 4 presents the second-order rate constants for inactivation of the P99 β-lactamase and its Y221G mutant by these phosphonates. These data show that although the reactivity of **8**, the L-alanyl phosphate, is only slightly affected by the Y221G mutation, that of the D-alanyl compound **7** is considerably enhanced. The latter compound is also significantly more reactive with the mutant enzyme than is the glycyl analogue. It seems then that the additional space afforded by removal of the Tyr 221 side chain permits formation of a more reactive complex with these phosphonate inhibitors as well as with substrates. Very noticeable also, however, is the fact that the original glycyl compound **6** is a much better inhibitor of the wild-type enzyme than of the mutant. It may be that the conformation of the inhibitor at the active site after loss of the Tyr 221 side chain strongly reduces the reactivity of all phosphonates of this type. This is interesting because the *Streptomyces* R61 DD-peptidase, a DD-peptidase that has tertiary structure and active site structure very similar to those of the P99 β-lactamase (*7*), is virtually unaffected by **6** and **7** (*22*). It seems, therefore, that, in this regard at least, the Y221G mutation has achieved a distinct DD-peptidase attribute. As has been discussed elsewhere (*10*, *21*, *33*), the reactivity of phosphonates, where a pentacoordinated intermediate/ transition state is involved, need not closely parallel that of analogous substrates with their tetrahedral intermediates/transition states.

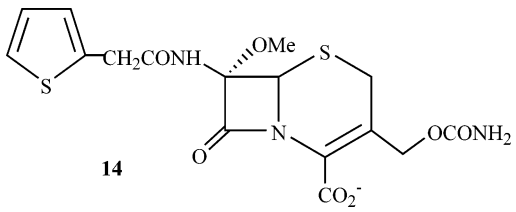
Despite the lack of similarity between the Y221G and GC1 mutants with respect to their response to a D-alanyl substrate, they both show an extended specificity toward β-lactam substrates with bulky side chains. This class of substrate includes third generation cephalosporins (*16*, *19*) such as cefotaxime (**3**). A collection of data demonstrating this phenomenon is presented in Table 5. The advantage of the mutant enzymes in catalyzing cefotaxime hydrolysis is present at both low (k_{cat}/K_m) and high (k_{cat}) substrate concentrations. It is also interesting that neither the Y221G nor the GC1 mutant displays the transition after a few turnovers from tight binding to weak binding of cefotaxime that is characteristic of the wild-type enzyme. This phenomenon is observable in the difference between the K_m value determined from inhibition of reporter substrates and that from total progress curves (*34*). The wild-type enzyme is thought to undergo a conformational transition because of the close and unfavorable interaction between the substrate side chain and the Ω loop that contains Tyr 221 (*18*). It seems likely that the additional space available in both the Y221G and GC1 mutations makes the transition unnecessary, reduced in scope, or more facile.

Table 5: Steady-State Kinetics Parameters for the Hydrolyses of Cefotaxime and Cefoxitin by β-Lactamases

		enzyme		
substrate	parameter[a]	P99	Y221G	GC1
cefotaxime	k_{cat} (s^{-1})	0.41[b]	93 ± 4 124 ± 32[c]	62[b]
	K_m (μM)	17[b] 0.029[b,d]	15.5 ± 2.4 17.5 ± 1.8[e] 25 ± 8[c]	8.1[b]
	k_{cat}/K_m ($s^{-1} M^{-1}$)	2.3×10^4 [b] 1.41×10^7 [b,d]	6.0×10^6	7.6×10^6 [b]
cefoxitin	k_{cat} (s^{-1})	0.50 ± 0.01	$(3.5 \pm 0.3) \times 10^{-2}$	$(4.4 \pm 0.8) \times 10^{-2}$
	K_m (μM)	0.25 ± 0.02[d]	$(4.4 \pm 0.2) \times 10^{-3}$ [d]	$(25 \pm 2) \times 10^{-3}$ [d]
	k_{cat}/K_m ($s^{-1} M^{-1}$)	2.0×10^6	8.0×10^6	1.8×10^6

[a] Determined from initial rate measurements unless noted otherwise. [b] Data from ref *34*. [c] Determined from total progress curve analysis. [d] Determined from competition experiments with cephalothin as the reporter substrate. [e] Determined from competition experiments with CENTA as the reporter substrate.

The cephamycin cefoxitin, **14**, has a methoxy group in a position apparently analogous to the D-alanyl methyl of **5**.



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It was therefore of interest to examine the effect of the Y221G and GC1 mutations on turnover of this substrate. Its interaction with the wild-type enzyme is characterized by rapid acylation (k_{cat}/K_m) but slow deacylation (k_{cat}) (Table 4). The acylation rate is somewhat less than that observed with a good substrate, such as the first generation cephalosporin cephalothin, but the deacylation is much slower. The 7α-methoxy substituent must obstruct water attack at the acyl-enzyme carbonyl group, either directly or, more probably, since water attack on the acyl-enzyme of a class C β-lactamase is probably from the re (outside) face (*25*, *35*), indirectly, by reducing the ability of the acyl-enzyme to conformationally rearrange into a hydrolytically reactive conformation after acylation. The experimental results (Table 4) show that this situation is not improved by the Y221G and GC1 mutations: acylation rates are maintained but the deacylation rates decrease further by at least an order of magnitude. The additional space and, presumably, flexibility afforded to the active site by the mutations do not lead to more facile hydrolysis of cefoxitin. Indeed, it may be that occupation of the additional space by the methoxy group leads to a more rigid and inert intermediate. It seems likely, on the basis of these results, that the methyl group of acyclic D-alanyl substrates does not directly overlap with α-substituents on a β-lactam ring when both are bound to a class C β-lactamase; this difference has, in fact, been observed with the R61 DD-peptidase (*36*). Model building of a 7α-methoxy group onto the crystal structure of the covalent complex

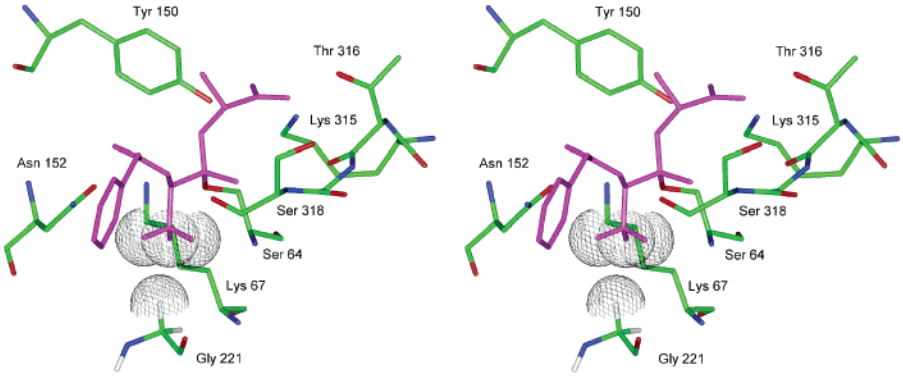
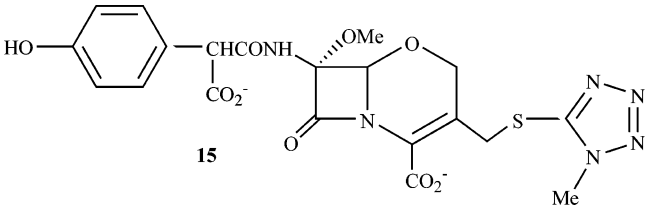


FIGURE 3: Stereoview of an energy-minimized tetrahedral intermediate structure formed on reaction of the Y221G mutant P99 β-lactamase with the peptide **12**. Only heavy atoms are shown except for the hydrogens of the D-alanyl methyl group and Gly 221. van der Waals spheres are shown for the D-alanyl methyl group and the α-hydrogens of Gly 221.

between cephalothin and the ampC β-lactamase (essentially identical in structure to the P99 enzyme; PDB file 1KVM; *37*) shows that it would interact unfavorably with the enzyme. The result of this interaction may be seen in the crystal structure of the complex of the ampC β-lactamase with moxalactam, **15** (*38*), where the dihydrothiazine ring, along with the methoxy group, is once again in a position to impede nucleophilic attack on the acyl-enzyme carbonyl group (*10*).



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Molecular Modeling. A model of the Y221G mutant was constructed, as described in Materials and Methods, and the tetrahedral intermediate **13**, derived from reaction of the enzyme with the peptide **12**, built into the active site in two conformations, A, where the D-alanyl methyl group is directed into the enzyme, and B, where the methyl group is directed more outward into the solvent (*11*). In the P99 β-lactamase, the B conformation was more stable (*11*), but in the Y221G mutant, the A conformation with the D-alanyl methyl group occupying the space vacated by the Y221 side chain was more stable (a larger negative E_{int} value by 15.5 kcal/mol). In the wild-type enzyme, the D-methyl group unfavorably interacts with the side chain of Tyr 221 (11). An energy-minimized A conformation of the Y221G mutant is shown in Figure 3. It was noticeable that in the A conformation but not in the B (see Figure 5A of ref *11*), the usual hydrogen bonding of the side chain amido group to the side chain of Asn 152 and to the backbone carbonyl of Ser 321 (Ser 318 in the P99 sequence) was readily achieved. This loss of contact with the active site in the B conformation may generally mobilize and entropically reduce the reactivity of the B conformation. Similar models of the GC1 β-lactamase (not shown) also indicated that the A conformation was more stable than the B (E_{int} more negative by 17.5 kcal/mol), although, as demonstrated by the kinetics results described above, the reactivity of **12**, presumably in this conformation, was not greater than with the wild-type enzyme.

Molecular dynamics simulations were also performed on the tetrahedral adduct complexes, **13**, of the P99 β-lactamase with **11** and of the R61 DD-peptidase with **12**. Analogous simulations on another series of substrates have recently suggested an inverse correlation between the general mobility of the active site in such complexes and the substrate activity (k_{cat}/K_m values) (*28*). In the present case, the active site mobility of the R61/**12** complex was considerably less than that of the P99/**11** complex, indicating that the former may be more productive in enzyme acylation. Estimates of mobility, obtained as described in Materials and Methods, were derived from rms distance differences between the average structure of the active site plus ligand present between 50 and 200 ps of the molecular dynamics simulation and the initial energy-minimized structure (*28*). These values were 1.15 and 1.70 Å for the R61/**12** and P99/**11** complexes, respectively.

Noncovalent complexes of the peptides **11** and **12** with the P99 β-lactamase and the R61 DD-peptidase, respectively, were also constructed from the tetrahedral intermediates, as previously described (*28*). Energy minimization of the latter of these yielded a stable complex, shown in Figure 4. This structure seems to be a good representation of a reactive complex: the peptide carbonyl oxygen atom is in the oxyanion hole, the terminal carboxylate interacts with Arg 285 and Thr 299, Ser 62 O_γ, the active site nucleophile, is closely attended (hydrogen bond distances) by the auxiliary functional groups of Lys 65 and Tyr 159, and the side chain phenyl group appears in hydrophobic contact with the side chains of Phe 120 and Trp 233. These features are in good accord with relevant crystal structures (*36*, *39*). In contrast, the P99/**11** complex was not stable during energy minimization: the peptide group broke free from the oxyanion hole and moved away from the active site. This result also suggests a weaker or less productive complex of a peptide with the β-lactamase than with the DD-peptidase.

An interesting view of the structural differences between the β-lactamase and DD-peptidase active sites in these structures is shown in Figure 5. This shows equivalently, but not completely, energy-minimized structures of the peptide complexes (the peptide has not yet pulled out of the β-lactamase active site). Of particular interest are the positions of the positive charges that anchor the terminal carboxylate in these complexes, Lys 315 in the β-lactamase and Arg 285 in the DD-peptidase. These are clearly differently placed with respect to the oxyanion hole and the side chain binding sites. This difference may powerfully influence how strongly and productively a peptide substrate may be bound

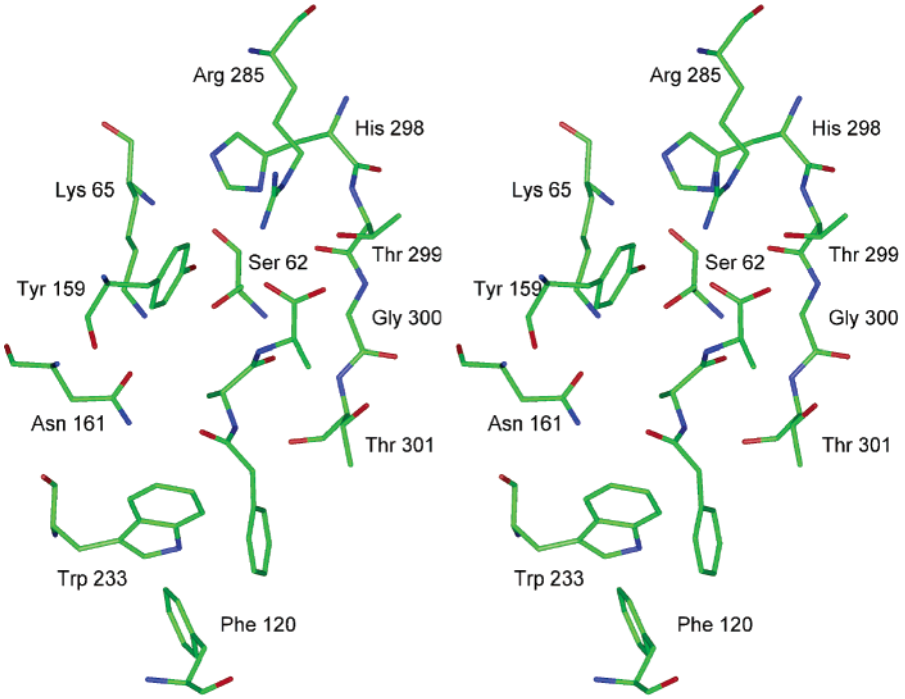

FIGURE 4: Stereoview of an energy-minimized noncovalent complex between the R61 DD-peptidase and the peptide **12**. Only heavy atoms are shown.

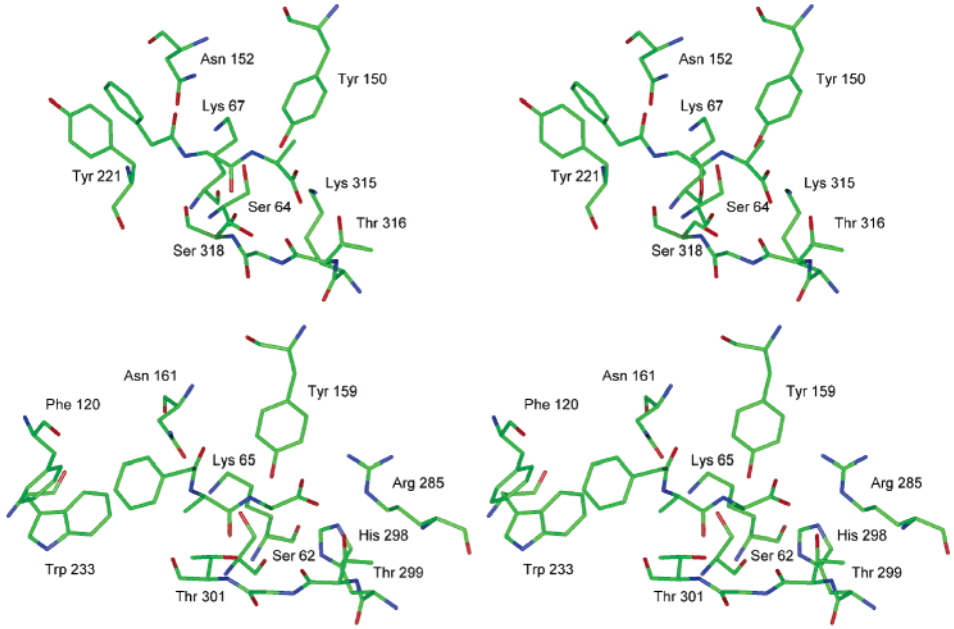

FIGURE 5: Stereoviews of noncovalent complexes of the peptide **11** with the P99 β-lactamase (upper pair) and of the peptide **12** with the R61 DD-peptidase (lower pair). Only heavy atoms are shown.

to the respective active sites and thus the acylation rates of the P99 β-lactamase and R61 DD-peptidase by peptides. On relaxation of the peptide structure by conversion of the peptide carbonyl carbon atom into the tetrahedral carbon of the intermediate, both active sites can accommodate peptide-derived ligands with binding of the terminal carboxylate to the respective positively charged residue and oxyanion in the hole, although, as described above, not as stably in the case of the β-lactamase. It is possible that replacement of the positive charge of Lys 315 in the β-lactamase with one placed in a position analogous to that of Arg 285 in the R61 DD-peptidase may further enhance DD-peptidase activity in the β-lactamase.

Recent QM/MM calculations have suggested to Gherman et al. (*40*) that the greater ability of the P99 β-lactamase than the R61 DD-peptidase to catalyze hydrolysis of acyl-enzymes derived from β-lactams may derive from the immediate electrostatic environment of Tyr 150 in the former enzyme as opposed to that of the homologous Tyr 159 in the latter. This environment was found to affect the electron density on the side chain oxygen atom of the tyrosine and thus, presumably, its ability as a general acid/base catalyst. This electronic difference may exist, but, if so, it might be expected to exert its effect on acyclic substrates as well; experiment shows that this is not the case. It thus seems likely to the present authors that other structural factors, such as

those discussed above and elsewhere (*7*, *10*, *41*), must also contribute to the substrate specificity difference between the P99 β-lactamase and the R61 DD-peptidase. These issues, of course, are germane to all discussions of β-lactamase evolution.

General Conclusions. The results of the experiments described above demonstrate that, as predicted (*11*), deletion of the Tyr 221 side chain from the class C P99 β-lactamase does lead to reversal of substrate preference from glycyl to D-alanyl at the penultimate C-terminal position of an acyclic substrate. Both esterase and DD-peptidase preferences were enhanced by this mutation. Molecular modeling shows that the D-methyl group can be comfortably accommodated in the space formerly occupied by the tyrosine side chain. This mutation alone, however, does not, in itself, produce an efficient DD-peptidase. This must mean that, in the real evolutionary process, where β-lactamases derived from ancestral DD-peptidases, selection against the methyl group was not the dominant change. Nonetheless, such selection may well have been one of the initial steps in the process so that evolving β-lactamases did not continue to hydrolyze peptidoglycan biosynthetic intermediates. Enzyme molecules in the early stages of β-lactamase evolution, before the appearance of a mechanism of facile hydrolysis of the acyl-enzyme, may well have been simple stoichiometric β-lactam traps with preference for glycyl rather than D-alanyl peptide substrates. No primitive protein of this type seems to have yet been isolated from present day organisms.

Most indications to date suggest that subtle factors controlling the shape of the active site, enabling the site to distinguish between a planar peptide and a "bent" bicyclic β-lactam, were crucial in the evolution of β-lactamases (*7*, *9*, *11*, *29*). There is also evidence that β-lactamases have lost the ability to productively bind extended peptidoglycan-like substrates (*41*−*43*). The specific details of the former changes have not yet been well-defined, but the modeling described above suggests that the position of the positive charge employed by the enzyme to anchor the terminal carboxylate of the peptide substrate may be crucial. This hypothesis will be tested.

The results with the GC1 β-lactamase show that sufficient space for the binding of the D-methyl group may be necessary for DD-peptidase activity but it is certainly not sufficient. There must be coordinated specific interaction between the side chain binding site and the reaction center to achieve high DD-peptidase activity (*36*, *39*). The GC1 active site may be too mobile to allow optimal interaction; the crystal structure of the GC1 enzyme exhibits a gap in electron density in the Ω loop, presumably due to mobility or to the presence of multiple conformations (*17*).

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