On the Importance of a Methyl Group in β-Lactamase Evolution: Free Energy Profiles and Molecular Modeling†

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ABSTRACT: β-Lactam antibiotics are generally thought to inhibit their target enzymes, the bacterial cell wall-synthesizing DD-peptidases, because of their resemblance to D-alanyl-D-alanine peptides. Although a favorable conformation of the latter does structurally resemble the β-lactams with respect to backbone conformation, a significant difference is the presence of a D-methyl substituent on the penultimate alanine residue of the cell wall peptide. A classical β-lactam antibiotic has a hydrogen in the corresponding position. In the process of evolution of a β-lactamase from a DD-peptidase, it seems likely that this D-methyl group would be selected against, to ensure that the former enzyme would hydrolyze β-lactams rather than peptides. In this paper, the effect of the penultimate D-alanine residue (as opposed to a glycine residue) has been examined in peptide substrates of a present-day DD-peptidase and a β-lactamase. The peptides *N*-(phenylacetyl)-D-alanyl-D-phenylalanine and *N*-(phenylacetyl)glycyl-D-phenylalanine were used as a test pair against the DD-peptidase of *Streptomyces* R61 and the structurally very similar class C β-lactamase of *Enterobacter cloacae* P99. The kinetics of turnover of both of these substrates were determined for both enzymes. To quantify the partitioning of the acyl−enzyme intermediate, the aminolysis by D-phenylalanine of a cognate pair of depsipeptides was also studied. Thus, free energy−reaction coordinate diagrams were constructed for turnover of both peptides by both enzymes. Comparison of these profiles showed that the D-methyl group is preferred over hydrogen by the DD-peptidase at all stages of catalysis (acyl−enzyme and acylation and deacylation transition states), whereas the β-lactamase selects against the D-methyl group only at the peptide acylation transition state. A process of evolution by uniform dissociation of the methyl group by the β-lactamase has apparently occurred. These results were explored structurally by computational models of the acylation tetrahedral intermediates. A methyl group pocket on the DD-peptidase, less favorable on the β-lactamase, was identified. The interaction of the leaving group, the terminal D-alanine residue, with the two enzymes was interesting, since it seemed that different positively charged active site residues were directly associated with the carboxylate, Lys 315 in the β-lactamase and Arg 285 (rather than His 298) in the case of the DD-peptidase. The problems posed by larger substituents on the penultimate residue of the peptide, and in particular by the heterocyclic substituent present in a bicyclic β-lactam, were analyzed. Qualitative and quantitative analysis of the models support the proposed importance of the penultimate D-alanine in β-lactamase evolution.

The effectiveness of β-lactam antibiotics has been eroded by a variety of resistance mechanisms that have been selected for and then proliferated among bacteria (*1*). The most widespread and clinically important of these mechanisms is that of β-lactamase production (*2*). The β-lactamase enzymes are exported by bacteria to catalyze the hydrolysis of β-lactams before the latter are able to reach their targets, the transpeptidases or DD-peptidases that catalyze the final step of peptidoglycan biosynthesis. For some fifty years now, therefore, a goal of pharmaceutical companies has been to develop β-lactams that would avoid the β-lactamase active site but acylate and inhibit the DD-peptidase site. This task has proved difficult however for a very good reason; the active sites of these two classes of enzyme are very similar. This, in turn, can be readily understood. β-Lactams must obviously bind to both sites, and both sites catalyze acyl transfer reactions. Not surprisingly perhaps, as first suggested by Tipper and Strominger (*3*), β-lactamases are evolutionary descendents of DD-peptidases.

A considerable amount of evidence, both functional (*4*) and structural, in support of this proposition has now accumulated. Crystallographic studies of these enzymes clearly demonstrate their similarity in tertiary structure (*5*−*7*). The active site functionality is also very similar such that it is difficult to be sure at present whether an enzyme is a DD-peptidase or β-lactamase, only on the basis of inspection of the catalytic residues of the active site. Discussions about the nature of the active site structural changes involved in the evolution of a β-lactamase from a DD-peptidase have included suggestions concerning the overall size and shape of the active site (*5*, *8*) and about the positioning of hydrolytic water molecules in it (*9*, *10*), the latter presumably influenced by the former.

Another aspect of the question of the evolutionary process arises from further consideration of the forces that must have driven it. An early goal, selected for in β-lactamase evolution, would presumably be the loss of the enzyme's ability to catalyze hydrolysis (Scheme 1) of cell wall D-alanyl-D-

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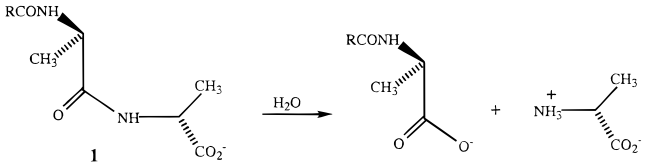
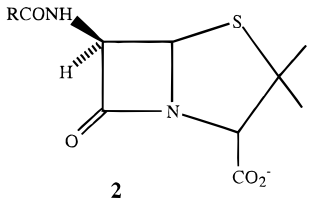
Scheme 1



alanine peptides (**1**). It would not be profitable for a bacterium to produce large amounts of β-lactamases for β-lactam resistance which also destroy the cell wall precursors.

The absence of a D-methyl substituent at the penultimate amino acid residue (i.e., at C-6) is characteristic of classical β-lactams such as the penicillins **2**.



Although β-lactams are able to achieve a backbone conformation resembling that of D-Ala-D-Ala peptides (*11*), they very strikingly lack this methyl group. Selection for the absence of this methyl group would logically therefore be an important aspect of β-lactamase evolution. This absence of the D-methyl substituent in β-lactams was noted by Tipper and Strominger, who suggested that 6-α-methylpenicillins should therefore be effective antibiotics (*3*). This did not turn out to be true (*12*), although 6-α-methoxypenicillins and 7-α-methoxycephalosporins (the cephamycins, e.g., cefoxitin) were subsequently found to have considerable antibiotic activity (*13*). Other small 6-α-substituents have also proved to be very effective in other β-lactam systems, e.g., in carbapenems such as imipenem (*14*).

It seems likely, then, that the selection process leading to β-lactamases with poor DD-peptidase activity, as present-day β-lactamases indeed have, was correlated with selection against the D-methyl substituent (*15*). The preference for the D-methyl substituent in acyclic peptide substrates of DD-peptidases has been noted (*15*, *16*). Further, it seems likely that the inability of β-lactamases to efficiently catalyze peptide hydrolysis is probably expressed in the acylation step of turnover (*5*, *15*). There is however at present no clear quantitative data about the effects of D-methyl substitution on the complete free energy pathway of turnover of peptides by a DD-peptidase in comparison with that by a β-lactamase, and there has been no attempt yet at the interpretation of the effects of this substitution in terms of enzyme structure.

To directly address these issues, we have examined the effect of the D-methyl substituent (vs hydrogen) on the free energy of the acylation and deacylation transition states and of the acyl−enzyme intermediate for turnover of a peptide substrate by a DD-peptidase and by a structurally similar β-lactamase (Scheme 2, where EOH represents the enzyme with its serine hydroxyl nucleophile at the active site). The enzymes that were chosen were the DD-peptidase of *Streptomyces* R61 and the class C β-lactamase of *Enterobacter cloacae* P99, because crystal structures are available for both (*17*, *18*), and they have very similar active site structure (*5*, *7*). The peptides chosen for the kinetics studies were **3** and **4**. To relate the free energy of the acyl−enzyme intermediate to that of the acylation transition state, the reverse reaction of the enzyme acylation step of Scheme 2, i.e., the aminolysis of the acyl−enzyme **5** by D-phenylalanine, was studied. For the latter experiments, the acyl−enzyme **5** was generated by reaction of the enzyme with the depsipeptides **6** and **7** (Scheme 3). The chosen DD-peptidase and β-lactamase are known to catalyze the hydrolysis and aminolysis by D-phenylalanine of the depsipeptide **6** (*19*−*22*).

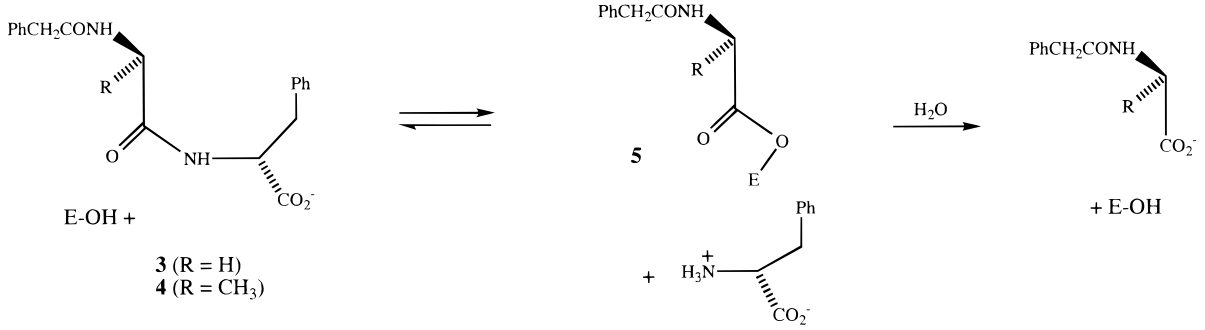
The data from these experiments were used to construct free energy−reaction coordinate diagrams for hydrolysis of **3** and **4** by the DD-peptidase and β-lactamase in assessing the comparative effect of the D-methyl substituent on these enzymes at all stages of catalysis. Molecular modeling methods were then employed to help us understand the structural basis of these energy profiles. The results provide convincing evidence for the important role of this methyl group in β-lactamase evolution.

EXPERIMENTAL PROCEDURES

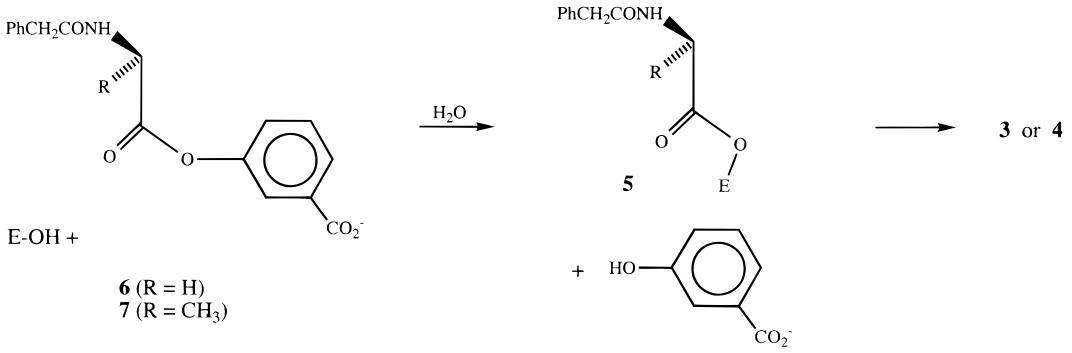
Materials. The β-lactamase of *E. cloacae* P99 was obtained from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as supplied. The *Streptomyces* R61 DD-peptidase was generously supplied by J.-M. Frére of the University of Liège (Liège, Belgium). *m*-[[(Phenylacetyl)glycyl]oxy]benzoic acid (**6**) and (phenylacetyl)glycyl-D-phenylalanine (**3**) were available from previous studies in this laboratory (*21*).

m-[[(Phenylacetyl)-D-alanyl]oxy]benzoic Acid (7). This compound was prepared in a manner completely analogous to that used to obtain **6** (*19*). Thus, D-alanine was acylated with phenylacetyl chloride and the resulting (phenylacetyl)-D-alanine condensed with *m*-[(benzyloxy)carbonyl]phenol in the presence of carbonyldiimidazole to give the benzyl ester of **7**. The benzyl protecting group was removed by catalytic

Scheme 2



Scheme 3



hydrogenation to give the required product. This was recrystallized from 1/1 (v/v) aqueous ethanol, after which its melting point was 158−161 °C and its ^{1}H NMR spectrum was as anticipated [(CD_3CN) δ 1.49 (3H, d, J = 8 Hz, CH_3), 3.63 (2H, s, CH_2Ph), 4.52 (1H, quint, J = 8 Hz, CH), 7.2−7.9 (9H, m, ArH)]. Anal. Calcd for $C_{18}H_{17}NO_5$: C, 66.04; H, 5.23; N, 4.28. Found: C, 66.35; H, 5.37; N, 4.14.

(Phenylacetyl)-D-*alanyl*-D-*phenylalanine (**4**)*. This material was prepared from (phenylacetyl)-D-alanine, via its hydroxysuccinimide ester, and D-phenylalanine according to the method of Anderson et al. (*23*). After recrystallization from 2/1 ethanol/water, **4** had a melting point of 189−191 °C and a ^{1}H NMR spectrum in accord with expectation [(DMSO-d_6) δ 1.15 (3H, d, J = 8.0 Hz, CH_3), 2.88, 3.02 (2H, ABX, J = 13.8, 8.5, 5.4 Hz, CH_2CH), 3.41 (2H, AB, J = 14.8 Hz, CH_2Ph), 4.07 (1H, quint, J = 8 Hz, $CH$$CH_3$), 4.37 (1H, dt, J = 8.5, 5.4 Hz, $CH$$CH_2$), 7.1−7.3 (10H, m, ArH), 8.10 (1H, d, J = 8 Hz, NH), 8.14 (1H, d, J = 8 Hz, NH)]. Anal. Calcd for $C_{20}H_{22}N_2O_4$: C, 67.78; H, 6.25; N, 7.90. Found: C, 67.72; H, 6.28; N, 7.91.

Analytical Methods. Absorption spectra and spectrophotometric reaction rates were measured with Perkin-Elmer Lambda 4B and Hewlett-Packard 8453 spectrophotometers. The concentrations of stock enzyme solutions were obtained using published extinction coefficients (*24*, *25*).

Steady-State Depsipeptide Hydrolysis Kinetics. All kinetics runs were carried out in 1 mL samples at 25 °C in a 20 mM MOPS buffer (pH 7.5). Enzyme concentrations in the reaction mixtures were approximately 50 nM. The P99 β-lactamase was diluted into buffer containing 1% BSA prior to final dilution into the reaction cuvette. Steady-state kinetic parameters were determined by the method of Wilkinson (*26*) from spectrophotometric initial velocity measurements. The wavelength employed for hydrolysis of **7** was 300 nm ($\Delta\epsilon$ = 940 M^{-1} cm^{-1}).

Depsipeptide Methanolysis Kinetics. These measurements were also made spectrophotometrically, as described above. Methanol concentrations were varied in the 0−2.5 M range, and fixed substrate concentrations of 6.0 and 5.0 mM for **6** and **7**, respectively, were employed. The variation of the initial rates of reaction of **6** and **7** with methanol concentration was linear. Partition ratios of hydrolysis to methanolysis were determined from least-squares fits to the data (*21*).

Depsipeptide Aminolysis Kinetics. Spectrophotometric measurements as described above were also used to obtain initial rates of reaction of **6** and **7** in the presence of the amine D-phenylalanine. Experiments where the D-phenylalanine concentration was varied at a constant depsipeptide concentration were performed and vice versa. The data were analyzed by nonlinear least-squares fitting to the appropriate rate equation (*21*), as described in the Results and Discussion.

Peptide Hydrolysis Kinetics. Values of k_{cat}/K_m for the hydrolysis of peptides **3** and **4** in the presence of the R61 DD-peptidase were obtained spectrophotometrically by monitoring the decrease in absorbance at 230 nm (a $\Delta\epsilon$ value for **4** was determined to be 700 M^{-1} cm^{-1}; that for **3** was assumed to be the same, with **3** being such a poor substrate that only initial rates could be measured). The concentrations of **4** and the enzyme were 0.45 mM and 0.9 μM, respectively; the corresponding values for **3** were 0.40 mM and 1.8 μM, respectively.

Very slow initial rates of hydrolysis of **3** and **4** in the presence of the P99 β-lactamase were estimated from ^{1}H NMR experiments carried out in 50 mM sodium bicarbonate buffer. Concentrations of **3** and enzyme were 5 mM and 5.3 μM, respectively; the corresponding values for **4** were 5 mM and 44.5 μM, respectively. To obtain the initial rates, spectra were recorded after 4 h for **3** and after 24 h for **4**; the rates were obtained from the areas of appropriate reactant and product peaks.

The K_m values for peptide hydrolysis by the P99 β-lactamase were obtained as K_i values for **3** and **4** as inhibitors of the hydrolysis of **7**. The required k_{cat}/K_m values could then be obtained from combination of these data with the initial rates of peptide hydrolysis determined as described above.

Analysis of Products from Depsipeptide Aminolysis. Product analyses of enzyme-catalyzed aminolysis reactions were obtained from a HPLC method using a Rainin Rabbit-HPX solvent delivering system, a Machery-Nagel Nucleosil C_{18} column, and a Gilson HM/HPLC UV−vis detector. The mobile phase used in the experiments with **7** was 0.05 M aqueous sodium dihydrogen phosphate containing 30% (by volume) acetonitrile, adjusted to pH 3.5 with phosphoric acid. In the case of **6**, the mobile phase was aqueous 0.05 M sodium dihydrogen phosphate containing 15% (by volume) acetonitrile, adjusted to pH 6.5 with sodium hydroxide.

Solutions of depsipeptides **6** and **7** (9 mM) containing D-phenylalanine (5−40 mM) were prepared. Two 1 mL samples at each D-Phe concentration (one control and one test sample) were temperature equilibrated at 25 °C, and enzyme was added to the test samples to initiate the reaction. Enzyme (R61 DD-peptidase) concentrations were 0.09 and 0.02 μM for **6** and **7**, respectively. The 40 mM D-Phe sample was monitored spectrophotometrically until 10% of the reaction was complete. At that point, 100 μL aliquots were removed from each sample, both control and experimental.

Table 1: Empirical Rate Parameters for Depsipeptide Hydrolysis and Aminolysis

	P99 β-lactamase		R61 DD-peptidase	
	6[a]	**7**	**6**	**7**
k_{cat} (s^{-1})	125 ± 4	69 ± 3	1.5 ± 0.1[b]	22.2 ± 0.6
K_m (mM)	0.23 ± 0.02	2.5 ± 0.4	0.8 ± 0.1[b]	1.0 ± 0.1
k_{cat}/K_m (s^{-1} M^{-1})	$(5.4 \pm 0.5) \times 10^5$	$(2.8 \pm 0.4) \times 10^3$	$(2.0 \pm 0.3) \times 10^{3}$[b]	$(2.3 \pm 0.3) \times 10^4$
B (M^{-1})	1400 ± 400	25 ± 7	230 ± 60	110 ± 30
F (M^{-1})	0	80 ± 30	0	30 ± 20
J (M^{-1})	≤ 50	109 ± 6	41 ± 4	52 ± 9

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

These aliquots were quenched in an equal volume of acetonitrile containing 5 mM hydrocinnamic acid and 3 mM salicylic acid for **6** and **7**, respectively.

Aliquots (15 μL) of each quenched sample were passed through the HPLC column at a flow rate of 1.5 mL/min with the detector set at 215 nm. Each sample, control and test, was run at least twice. Products of the reaction of **7** had retention times of 6.9 min [(phenylacetyl)-D-alanine] and 31.9 min (**4**), and the internal standard, salicyclic acid, had a retention time of 9.75 min. For the reaction of **6**, retention times were 4.5 min [(phenylacetyl)glycine], 79.3 min (**3**), and 11.1 min (hydrocinnamic acid, internal standard). The hydrolysis and aminolysis peak areas were normalized against the internal standard in each case and converted into product ratios.

Construction of Free Energy Diagrams for Peptide Hydrolysis. The overall free energy change for hydrolysis of **3** and **4** was taken to be -3.1 kcal/mol (*27*). This value is that for hydrolysis of benzoylglycylglycine at pH 5−9 which yields benzoylglycine and glycine. Changes from glycine to alanine do not appear to significantly (± 0.2 kcal/mol) affect the values of free energies of peptide hydrolysis (*27*). The free energy of the products was then set at zero in the diagrams. Two transition states, one for enzyme acylation and the other deacylation, and the acyl−enzyme intermediate were then added. Second-order rate constants (and thus free energies of activation from eq 1, where k_E is the pseudo-first-order rate constant for the relevant step, $\ln kT/h = 29.46$, and $RT = 0.592$ kcal/mol) for enzyme acylation by a peptide (k_{cat}/K_m for peptide hydrolysis)

$$\Delta G^{\ddagger\prime} = -RT \ln \frac{k_E h}{kT} \quad (1)$$

and reaction of the acyl−enzyme intermediate with both the amine D-phenylalanine (k_3/K_2) and water ($k_6/[H_2O]$) were available (these rate constants are those of Schemes 5 and 6; see below). These were sufficient to fix the positions of both transition states and the intermediate with respect to the reactant and product ground states. All concentrations were arbitrarily taken to be 1 M in these calculations for convenience. The relative sizes of the various activation energy barriers for **3** and **4** with a given enzyme are independent of the assumed (constant) concentrations of peptide and amine.

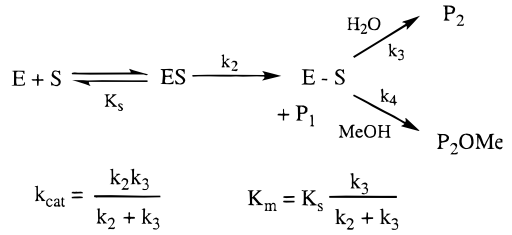
Computational Methods. The computations were set up essentially as previously described (*28*) and run on an IBM 3CT computer with INSIGHT II 97.0 (Biosym/MSI, San Diego, CA). The starting point for the P99 β-lactamase structural simulations was the crystal structure of the enzyme with a phosphonate inhibitor covalently attached to the active site serine residue (PDB file 1bls; *29*). This was transformed into the peptide tetrahedral intermediate by means of the Builder module of INSIGHT II. MNDO charges were employed for the peptide-derived ligand, while charges on the protein were assigned by INSIGHT II. Crystallographic water molecules were retained. The enzyme−peptide complex was then hydrated by a 15 Å sphere of water centered at the active site serine O_γ. Likely conformations of the side chain and leaving group were then assessed from the results of molecular dynamics runs of up to 40 ps at temperatures of 300 and 1000 K. A CV force field within the Discover program (version 2.98) was employed for all simulations and energy calculations. During the dynamics runs, the ligand, water, and the following residues were permitted to move: Ser 64, Lys 67, Tyr 150, Asn 152, and Lys 315. In these calculations, Lys 67 and Lys 315 were cationic, Tyr 150 was neutral, and the tetrahedral intermediate was dianionic (carboxylate and oxyanion). An 8 ps dynamics run at 300 K, where the entire system was allowed to move, did not reveal any further ligand conformations or interactions between ligand and protein. Significantly populated conformations were chosen from these runs, and the energy of each was minimized in the CV force field by means of 1000 steps by the method of steepest descents followed by 2000 steps of conjugate gradients. In this minimization procedure, the entire system, protein, ligand, and water, was freed from constraint. The final derivative of energy with respect to structural perturbation was then in the range of 0.02−0.03 kcal/Å. Bond distances and interaction energies, including those of all nonbonded interactions, could then be obtained directly from the minimized structures by means of the Discover program. Residues included in the E_{int} calculations (*28*) were those mentioned above and also Thr 316, Gly 317, and Ser 318.

The R61 DD-peptidase computations were similarly performed where the initial structure (including crystallographic water) was derived from that of a cephalothin complex of the enzyme (PDB file 1ceg; *9*). Residues involved in the energy calculations were Ser 62, Lys 65, Tyr 159, Asn 161, Arg 285, His 298, Thr 299, Gly 300, and Thr 301. In these calculations, Lys 65, Arg 285, and His 298 were cationic, Tyr 159 was neutral, and the tetrahedral intermediate was dianionic.

RESULTS AND DISCUSSION

The steady-state rate parameters for hydrolysis of the depsipeptides **6** and **7** by the P99 β-lactamase and the R61 DD-peptidase are shown in Table 1. Previous work in this laboratory has established that Scheme 4 is sufficient for explaining the hydrolysis of depsipeptides by these enzymes (*19*, *21*, *22*). The participation of the alternative nucleophile methanol is also shown. In Scheme 4, ES represents the noncovalent Michaelis complex of enzyme and depsipeptide,

Scheme 4



E−S the covalent acyl−enzyme intermediate, P_1 the initially released product (m-hydroxybenzoate), P_2 the carboxylic acid hydrolysis product, and P_2 OMe, released in the presence of methanol, the methyl ester of P_2.

Methanolysis kinetics, where the effect of methanol concentration on k_{cat} was determined, have indicated that under conditions of substrate saturation, enzyme deacylation is rate-determining for turnover of **6** by the P99 β-lactamase; i.e., $k_{cat} = k_3$ (*19*, *20*, *21*). Similarly, methanol (0−1.5 M) was observed to linearly accelerate the hydrolysis of **7** when catalyzed by the P99 β-lactamase (data not shown). Presumably, $k_{cat} = k_3$ in this case also. The extent of methanol acceleration yielded a k_4/k_3 value of 25.9 ± 1.2, which is similar to the value of this ratio for **6**, viz. 28.1 ± 1.2 (*20*). Methanol (0−2.5 M) did not unambiguously increase the rate of reaction of either **6** or **7** in the presence of the R61 DD-peptidase. Nor did a significant amount of methanolysis of **4** occur in the presence of 2.5 M methanol, as indicated by a ^{1}H NMR experiment (*19*). Methanolysis experiments therefore are uninformative with regard to the meaning of k_{cat} for the R61 DD-peptidase. Apparently, methanol is less able to productively replace water at the active site of the R61 DD-peptidase than at that of the β-lactamase. Nonetheless, k_{cat} for the R61 enzyme (and for the P99 β-lactamase) is increased by D-phenylalanine (see below) in an aminolysis reaction, an observation that certainly suggests that deacylation is rate-determining under k_{cat} conditions. Similar data with other combinations of ester substrates and amine nucleophiles with this enzyme have also been interpreted in this way (*30*, *31*). It will therefore be assumed in all that follows, for both enzymes and both substrates, **6** and **7**, that $k_{cat} = k_3$ (i.e., $k_3 \leq 0.1k_2$) and thus $K_m = K_s k_3/k_2$.

^{1}H NMR studies showed that reaction of **6** and **7** in the presence of D-phenylalanine and either enzyme yielded a mixture of hydrolysis and aminolysis products. In all cases, the initial rates of *m*-hydroxybenzoate formation also increase with D-phenylalanine concentration. Representative data for **7** with the DD-peptidase are given in Figure 1. Similar observations have been previously made for both enzymes with various substrates (*20*, *30*, *31*). Data for the P99 β-lactamase and **6** are available from earlier work in this laboratory (*21*).

Analysis of the effect of substrate and D-phenylalanine concentrations on the rates of reaction and on product distribution in the case of **6** and the P99 β-lactamase led to Scheme 5 (*21*, *32*). The rate constants in this scheme are numbered differently from those in Scheme 4 but are in accord with our previous usage (*21*, *32*); all rate constants mentioned below refer to Scheme 5 and the derived Schemes 6 and 7. In this scheme, ED_1 represents the noncovalent Michaelis complex of enzyme and depsipeptide. Both free enzyme, E, and ED_1 also bind D at a second site, leading to ED_2 and ED_1D_2, respectively. Both ED_1 and ED_1D_2 proceed

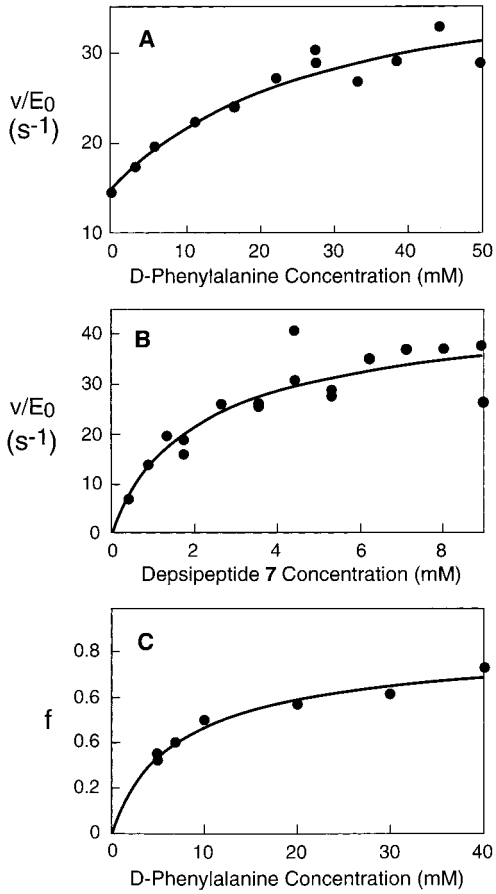
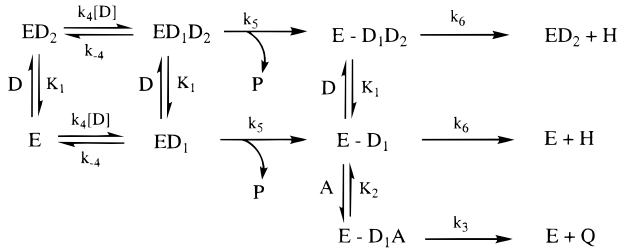


FIGURE 1: (A) Initial rates of total reaction (hydrolysis and aminolysis) of the depsipeptide **7** (9 mM) in the presence of D-phenylalanine and the R61 DD-peptidase. (B) Initial rates of total reaction (hydrolysis and aminolysis) of the depsipeptide in the presence of D-phenylalanine (20 mM) and the R61 DD-peptidase. (C) Fraction of aminolysis product during the initial stages of reaction of the depsipeptide **7** (9 mM) in the presence of D-phenylalanine and the R61 DD-peptidase.

Scheme 5

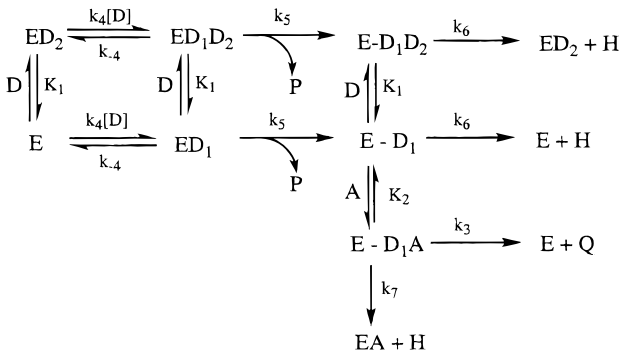


to hydrolysis products P and H, while the former may also bind a D-amino acid A and proceed to aminolysis products P and Q. The symmetry of rate constants is required to explain the simple hydrolysis kinetics of Scheme 1 (*32*). Scheme 5 yields the steady-state rate equation (eq 2) (*21*)

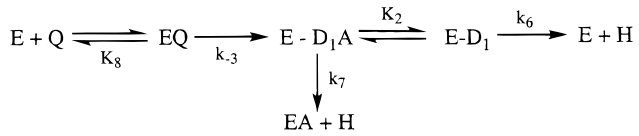
$$v/E_0 = \frac{k_{cat}(K_1 + [D])[D] + k_{cat}K_1B[A][D]}{K_1K_m + (K_1 + K_m)[D] + [D]^2 + K_1K_mB[A] + C[A][D]} \quad (2)$$

where k_{cat} [$=k_5k_6(k_5 + k_6)$] and K_m [$=k_4k_6/(k_5 + k_6)$] are the steady-state parameters for hydrolysis (Table 1), $B = k_3/k_6K_2$, and $C = K_1(k_3 + k_5)/K_2(k_5 + k_6)$.

Scheme 6



Scheme 7



Since plots of initial rates versus depsipeptide concentration (e.g., Figure 1B) showed no sign of a decrease in rate at high D concentrations, it will be assumed that $K_1 > [D]$ at all D concentrations that were employed. This should be an acceptable approximation but perhaps not a very good one. It certainly would not be a good approximation in the case of **6** with the P99 β-lactamase (*20*, *21*) where the effect of the $[D]^2$ term in the denominator of eq 2 is discernible at [D] values below 9 mM. The latter concentration was the highest employed in this work because of the limited solubility of **7**. In any event, given the assumption that $K_1 > [D]$ (and therefore $K_1 > K_m$), eq 2 can be reduced to eq 3

$$v/E_0 = \frac{k_{cat}[D]}{K_m + [D][(1 + J[A])/(1 + B[A])]} \quad (3)$$

where $J = C/K_1 = (k_3 + k_5)/K_2(k_5 + k_6)$. The fraction of aminolysis product Q formed at constant depsipeptide concentration as a function of D-phenylalanine ([A]) concentration can be obtained directly from eq 3 and is given by eq 4. The parameter B (k_3/k_6K_2) represents the partition ratio of ED_1 between aminolysis and hydrolysis, and, as seen from eq 4, is the slope of a plot of f versus [A] at low A concentrations. Also, according to eq 4,

$$f = \frac{[Q]}{[H] + [Q]} = \frac{B[A]}{1 + B[A]} \quad (4)$$

f should approach 1.0 (total aminolysis) at high A concentrations. Inspection of the experimentally determined plots of f versus [A] (e.g., Figure 1C) suggested that this is not true for the β-lactamase and **7**, and probably not for the DD-peptidase and **7** either. Consequently, Scheme 5 and eq 3 cannot be complete representations of the reactions occurring in these cases. Apparently, even at saturating A concentrations, a significant amount of depsipeptide is hydrolyzed rather than aminolyzed. Scheme 6 and the derived (with the assumptions used to derive eq 3) eq 5 are able to accommodate this result and represent a more general situation than that of Scheme 5 and eq 3.

$$v/E_0 = \frac{k_{cat}[D]}{K_m + [D][(1 + J[A])/(1 + B[A] + F[A])]} \quad (5)$$

In eq 5, $F = k_7/k_6K_2$, the partition ratio of ED_1 favoring hydrolysis through $E-D_1A$. The fraction of aminolysis is then given by eq 6, which predicts, as apparently required by the data in the instances noted above, that f will increase with [A] to a value of <1.0, viz. $B/(B + F)$, at saturating A concentrations. The reaction of **6** with both enzymes led to f values that could not be distinguished from unity, and thus, in these cases, $k_7 \ll k_3$.

$$f = \frac{B[A]}{1 + (B + F)[A]} \quad (6)$$

It should be noted that Jamin et al. (*31*) also required an amine-dependent hydrolysis pathway in fitting their data for the R61 DD-peptidase-catalyzed hydrolysis of a thioester substrate. Their data however appeared to require the binding of a second thioester molecule to $E-D_1A$ prior to hydrolysis. Although such a quaternary complex was not observed in the kinetics of hydrolysis of **6** by the P99 β-lactamase, it might occur in the present cases, in which case F would be a function of [D]. Since analysis of F was not one of the goals of the this paper, however, distinction between Scheme 6 and the scheme of Jamin et al. (*31*) will not be further pursued here.

The fitting of eqs 5 and 6 to the aminolysis data, e.g., as shown in Figure 1, led to the empirical parameters B, F, and J presented in Table 1. These, from the expressions for B, F, and J given above, lead to the kinetic constants of Scheme 6 presented in Table 2.

Kinetic studies of peptide hydrolysis led to k_{cat}/K_m values of 2.0 and 0.12 s^{-1} M^{-1} for **3** and **4** in the presence of the P99 β-lactamase and 0.34 and 40 s^{-1} M^{-1} in the presence of the R61 DD-peptidase, respectively. These were interpreted in terms of Scheme 7 where the majority of the symbols are those used in Scheme 6; k_3 is omitted since, under steady-state initial rate conditions, an acyl−enzyme intermediate

Table 2: Derived Rate Parameters for Depsipeptide Hydrolysis and Aminolysis[a]

	P99 β-lactamase		R61 DD-peptidase	
	6	**7**	**6**	**7**
k_6 (s^{-1})	125 ± 4	69 ± 3	1.5 ± 0.1	22.2 ± 0.6
k_5/K_4 (s^{-1} M^{-1})	(5.4 ± 0.5) × 10^5	(2.8 ± 0.4) × 10^3	(2.0 ± 0.3) × 10^3	(2.3 ± 0.3) × 10^4
k_3/K_2 (s^{-1} M^{-1})	(1.8 ± 0.5) × 10^5	(1.7 ± 0.5) × 10^3	(3.5 ± 0.9) × 10^2	(2.5 ± 0.6) × 10^3
k_7/K_2 (s^{-1} M^{-1})	0	(5 ± 2) × 10^3	0	(6 ± 4) × 10^2
k_3 (s^{-1})	≥1000	16 ± 4	9 ± 2	49 ± 15
k_7 (s^{-1})	0	50 ± 30	0	12 ± 8
K_2 (mM)	≥6	9 ± 4	24 ± 9	19 ± 7

[a] The rate coustants in this table refer to Scheme 6.

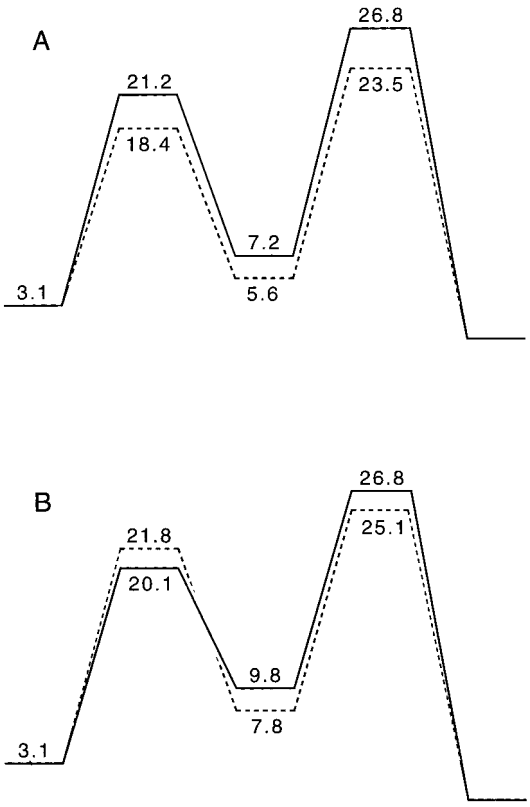


FIGURE 2: Free energy−reaction coordinate diagrams for reactions of the enzymes, the R61 DD-peptidase (A) and P99 β-lactamase (B), in the presence of D-phenylalanine and water (1 M each) and each peptide, **3** (solid lines) and **4** (dashed lines). These diagrams represent comparisons between the two substrates with a fixed enzyme.

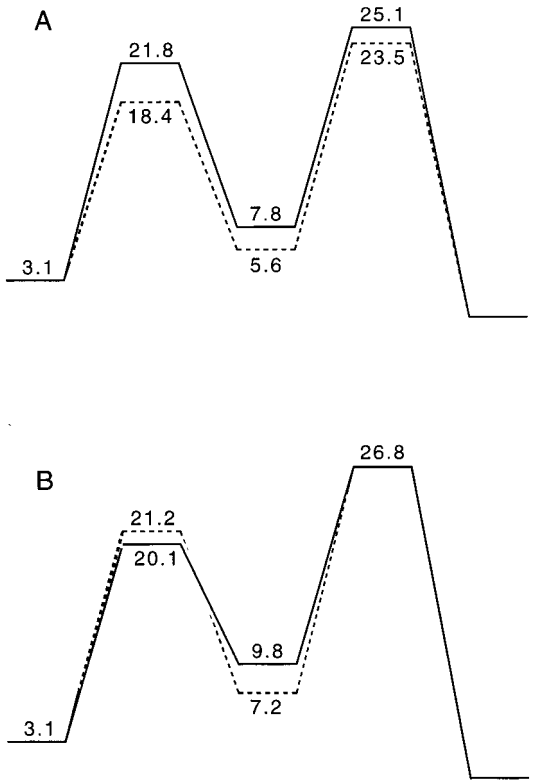


FIGURE 3: Free energy−reaction coordinate diagrams for reactions of the peptides **4** (A) and **3** (B) in the presence of D-phenylalanine and water (1 M each) and each enzyme, the P99 β-lactamase (solid lines) and the R61 DD-peptidase (dashed lines). These diagrams represent comparisons between the two enzymes with a fixed substrate.

does not accumulate during peptide hydrolysis, i.e., ($k_7 + k_6K_2$) $\gg k_3$. A noncovalent complex of enzyme and peptide, EQ, has been included. In terms of Scheme 7, k_{cat}/K_m for peptide hydrolysis would represent k_{-3}/K_8.

Free Energy Profiles. Given the above-described rate constants, the free energy profiles of Figures 2 and 3 for peptide hydrolysis were constructed as described in Experimental Procedures. These profiles will be considered in turn below. As drawn, the profiles indicate that in all cases, D-phenylalanine is a better nucleophile for deacylation of the acyl−enzyme intermediate than is water. This result would of course be strongly influenced by the relative concentrations of water and amine nucleophile. In free aqueous solution, hydrolysis often competes strongly with aminolysis (Figure 1C), but in vivo, where a DD-peptidase active site would presumably be found directly adjacent to the growing cell wall, the competition may well favor aminolysis. The noncovalent binding of D-amino acid nucleophiles is weak for both enzymes [Table 2 (*31*, *32*)], but this too may not be important in vivo for the same essentially entropic reason. It is interesting that the D-alanyl, but not the glycyl, substrates allow water to leak into acyl−enzyme intermediate−amine complexes and cause substrate hydrolysis, i.e., the existence of the E−D_1A → EA + H step in Scheme 6 for the D-alanyl substrates (see k_7 values in Table 2). One could speculate that this would be useful in allowing peptide hydrolysis (thereby limiting cell wall cross-linking) in the presence of an adjacent, and difficult to dissociate, amine nucleophile, as again might be present in the in vivo situation. It should probably be mentioned here that although the R61 DD-peptidase is clearly a DD-peptidase, its actual in vivo role does not seem to be known; it is, unusually for a DD-peptidase, apparently not membrane-bound in vivo, but is excreted into the extracellular medium. The profiles of Figure 2 suggest that deacylation would be rate-determining with respect to the turnover of peptides, but that is a consequence of the assumed concentrations. At lower concentrations of peptide and amine and a higher concentration of water, acylation would in fact be rate-determining, as observed at a saturating peptide concentration, zero initial amine concentration, and 55.5 M water. Differential effects however do not vary with concentration, as long as the same concentration is chosen for both sides of the comparison.

First, the profile for turnover of the peptides by the R61 DD-peptidase (Figure 2A) reveals a clear preference for the D-alanine peptide **4** at all stages of catalysis sampled in these experiments. This suggests uniform occupancy of the active site along the reaction coordinate by the methyl group, and an enzyme firmly evolved to prefer a penultimate D-alanine rather than glycine residue in the substrate.

On the other hand, the P99 β-lactamase profile (Figure 2B) indicates a specific effect at one point along the reaction coordinate. A glycine peptide is preferred (or, perhaps more importantly, alanine is disfavored) in the acylation step, while alanine is preferred at the acyl−enzyme stage and during deacylation. This suggests an enzyme that has been subjected to selective pressure to reject D-alanine substrates as much as possible at the first step, before any covalent reaction occurs. No further selective pressure however seems to have been applied to influence the fate of the peptide after

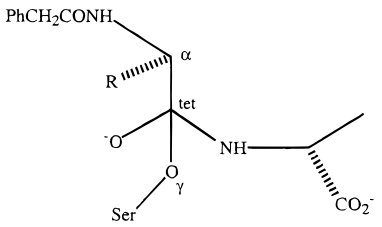
acylation, and deacylation would occur essentially as rapidly for a D-alanyl peptide as a glycyl. Selective destabilization of the D-alanyl acyl−enzyme intermediate, which would lead to more facile reformation of the peptide and, possibly, more rapid deacylation, has not occurred. It seems likely that the presence of the leaving group as well as the methyl group is required to bring about the observed differential effect in the acylation transition state. The glycine preference in acylation of course can be seen to correlate with the specificity of the enzyme for 6-β-amido β-lactams (*3*, *4*).

Alternatively, the results can be assessed by a complementary set of comparisons. Figure 3A shows the free energy profiles for turnover of the D-alanine-containing peptide **4** by the two enzymes. The interaction with the DD-peptidase appears stronger at all three points of the reaction coordinate that were sampled. If one were to imagine evolution of the β-lactamase from the DD-peptidase, then a mechanism of uniform dissociation by the β-lactamase [the inverse of uniform binding, the term of Albery and Knowles (*33*) for positive selection] has been approximated. Figure 3B, the corresponding plot for the glycine substrate, exhibits the differential effect whereby acylation of the β-lactamase is favored over that of the DD-peptidase.

These results therefore tend to confirm earlier indications (*5*, *15*) that the major selectivity for or against the methyl group of D-alanyl peptide substrates would occur in the acylation transition state. The presence of the leaving group must be an important factor in this process.

Finally, in consideration of the data in Table 2, comparison of the D-methyl group effect on depsipeptide hydrolysis with that on peptide hydrolysis might be made. Previous results suggest that the effect on depsipeptide hydrolysis may be smaller than that on peptide hydrolysis (*15*). This point is not so obvious with the present compounds, where the DD-peptidase has a clear preference for the D-alanine substrate **7**, both in k_{cat} and in k_{cat}/K_m, and in k_{cat}/K_m at least, the β-lactamase has a clear preference for the glycine derivative **6**. It seems likely that these distinctions may be leaving group-dependent (as concluded above for peptide hydrolysis). A side chain dependence has already been noted (*15*).

Structural Interpretation. To understand the structural basis of the penultimate D-alanine methyl group, as revealed in the above kinetic data, computational models of the tetrahedral intermediates (**8**), involved in the acylation of the two enzymes by D-alanyl and glycyl peptides, were constructed.



8a R = H
8b R = CH_3

D-Alanine rather than D-phenylalanine was chosen as the leaving group to reduce the number of conformational possibilities. It is clear that the methyl effect is also observed in terminal D-alanine peptides (*15*, *16*). The most likely conformations of side chain and leaving groups were assessed from molecular dynamics runs, and typical snapshots of these were subjected to energy minimization. In general, two classes of ligand conformation appeared to be populated during these simulations, representing those with the D-methyl (or hydrogen) substituent of the penultimate D-alanine (glycine) residue directed either into the enzyme (conformation A; see Figure 4A, for example) or out, more toward solvent (conformation B; see Figure 5A, for example). These two conformations are characterized by Ser $O_\gamma-C_{tet}-C_\alpha-R$ dihedral angles (see **8**) of from 40−60° and around −60°, respectively. The most populated conformations of the leaving group were those where the carboxylate interacted with the side chains of two active site residues, one cationic, Lys 315 and Arg 285 in the P99 β-lactamase and R61 DD-peptidase, respectively, and the other neutral, Thr 316 and the homologous Thr 299 in the two enzymes, respectively. The energy-minimized structures which yielded the strongest interactions between peptide ligand and enzyme [as indicated by the E_{int} parameter which takes into account direct enzyme−ligand interactions as well as enzyme−enzyme and ligand−ligand interactions (*28*)] for each of the combinations of enzyme and ligand are shown in Figures 4 and 5, the former for the R61 DD-peptidase and the latter for the P99 β-lactamase. δE_{int} values for these conformations are presented in Table 3.

First, the DD-peptidase situation will be discussed. Figure 4 shows that the conformations of both the D-alanyl and glycyl ligands most strongly interacting with the enzyme adopt the same conformation (A) of the penultimate residue and the structures are very similar. Hydrogen-bonding interactions in particular are essentially identical. These include occupation by the tetrahedral oxyanion of the oxyanion hole (Ser 62 NH and Thr 301 NH), interaction of Lys 65 and Tyr 159 with Ser O_γ [both of these interactions are also present in the original phosphonate transition-state analogue crystal structure (*29*)], and interaction of the leaving group carboxylate with Arg 285 and Thr 299. The former of these carboxylate interactions is interesting in comparison with the situation with the β-lactamase (see below) where the cognate interaction of the carboxylate is with Lys 315. The sequence analogue of the latter residue in the DD-peptidase would appear to be His 298, but the side chain of this residue is shorter than that of lysine and apparently is therefore less able to directly interact with the leaving group carboxylate. The place of the Lys 315 cation in β-lactamases is apparently taken by Arg 285 in the R61 DD-peptidase. His 298, in the protonated form, would still provide positive charge to the active site that could help stabilize the oxyanion intermediate [energies for interaction between Arg 285 and His 298 and the D-alanyl ligand (excluding the leaving group) were calculated to be −101.6 and −62.8 kcal/mol, respectively]. Mutation of His 298 to a neutral residue considerably reduces the rate of acylation of the enzyme by peptides and β-lactams (*34*).

The calculated energies (Table 3) show that the D-alanyl ligand does interact more strongly with the active site than does the glycyl analogue, in agreement with the experimental results. The calculated difference is quite small, however (although comparable to the experimental result), and cannot be attributed to interaction of the ligand with any particular active site residue. It is interesting to note however that if Tyr 159 is constrained to strongly hydrogen bond with the amine nitrogen of the leaving group [as would occur in

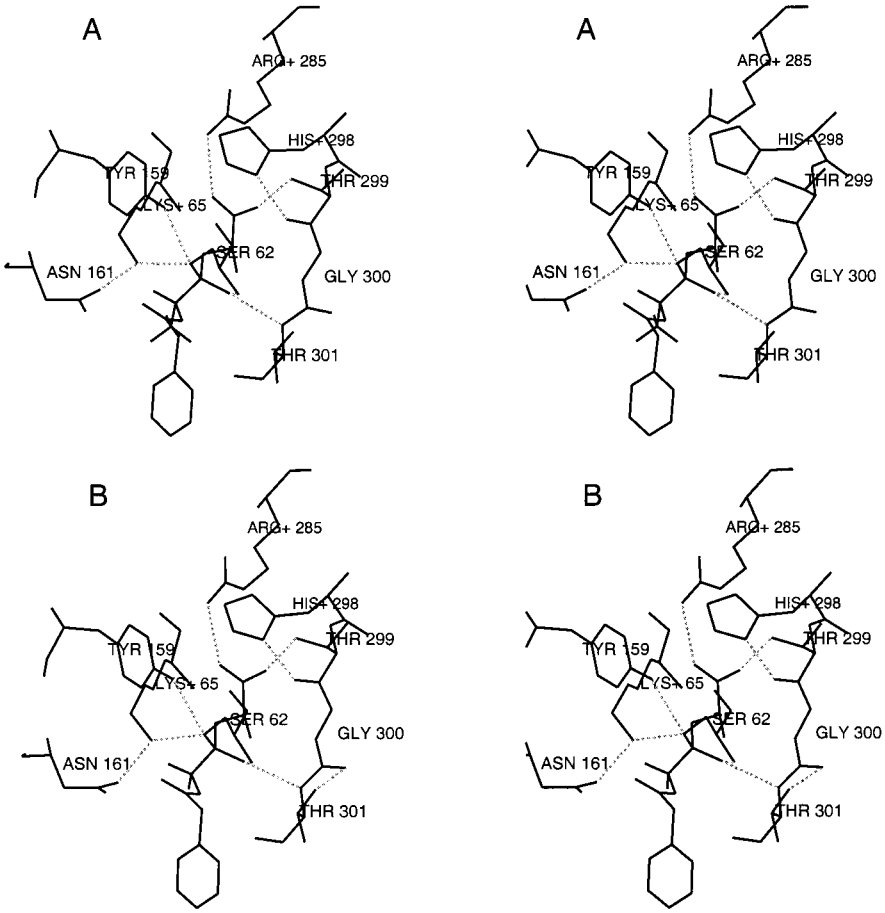


FIGURE 4: (A) Optimized tetrahedral intermediate structure formed on interaction of the R61 DD-peptidase with *N*-(phenylacetyl)-D-alanyl-D-alanine. (B) The analogous structure for the R61 DD-peptidase and *N*-(phenylacetyl)glycyl-D-alanine. In both diagrams, the fainter lines represent possible hydrogen bonds (heavy atom distances of $\leq$3.0 Å). Hydrogen atoms are only shown on the α-carbon and methyl group of the penultimate residue.

general acid-catalyzed breakdown of the tetrahedral intermediate, as most proposed mechanisms envisage (*29*, *35*)], the energetic preference for D-alanine, as measured by δE_{int}, increased. The hydrophobic effect, most of which is not included in these calculations, could also contribute to the preference for D-alanine of anything up to a few kilocalories per mole (*36*).

In contrast to the situation of the DD-peptidase, Figure 5 shows that for the β-lactamase, the strongest-interacting conformation is different for the D-alanyl and glycyl compounds, with the latter preferring conformation A and the former B. The glycyl compound (Figure 5B) seems nicely hydrogen-bonded into the active site in a fashion similar to that seen with the DD-peptidase. The carboxylate of the leaving group is hydrogen-bonded to the side chains of Lys 315 and Thr 316. The situation of the D-alanyl derivative (Figure 5A) seems to be less optimal however. The position of the penultimate D-alanine methyl group appears to prevent the amido group of the side chain from taking its normal conformation where hydrogen bonding to Ser 318 NH and the Asn 152 side chain NH is possible. The methyl group may also prevent the catalytic Tyr 150 hydroxyl group from easily hydrogen bonding to Ser 62 O_γ or to the leaving group nitrogen; Tyr 150 in the minimized structure is only hydrogen-bonded to the leaving group carboxylate, probably not a catalytically productive position. In terms of energy, the δE_{int} value (Table 3) supports the visual impression, and suggests a much weaker interaction between the D-alanyl compound and the active site than with the glycyl compound. The calculated difference might be reduced by the hydrophobic effect to a value closer to the experimental value. These results therefore also seem to be in good accord with experiment.

An interesting question is why the D-alanyl compound does not take up conformation A with the methyl group directed into the β-lactamase, which was found to be its optimal position in the DD-peptidase. To understand the issues relating to this question, a D-methyl group was added to the glycyl structure in the minimized position, and energy minimization of the resulting structure was carried out in the usual way. The methyl group did not move into conformation B during the minimization procedure, and the Ser $O_\gamma-C_{tet}-C_\alpha-R$ dihedral angle only changed from 40.3 to 48.6°. The interaction energy however was considerably less favorable than in the optimal D-alanyl position (δE_{int} = 17.1 kcal/mol, cf. 9.3 kcal/mol).

A crucial point involved the position of the D-methyl group, which, as can be seen in Figure 6, is in van der Waals contact with the side chain of Tyr 221. It seems likely that this steric interaction precludes the D-alanyl compound from fitting productively into the active site of the β-lactamase. Tyr 221 lies on a small helix at one end of the broad loop beneath the active site (*17*). There is no such problem with the methyl group in the R61 DD-peptidase since the closest residue (Trp 233) is further away. The methyl group in conformation A in the β-lactamase, jammed up against Tyr

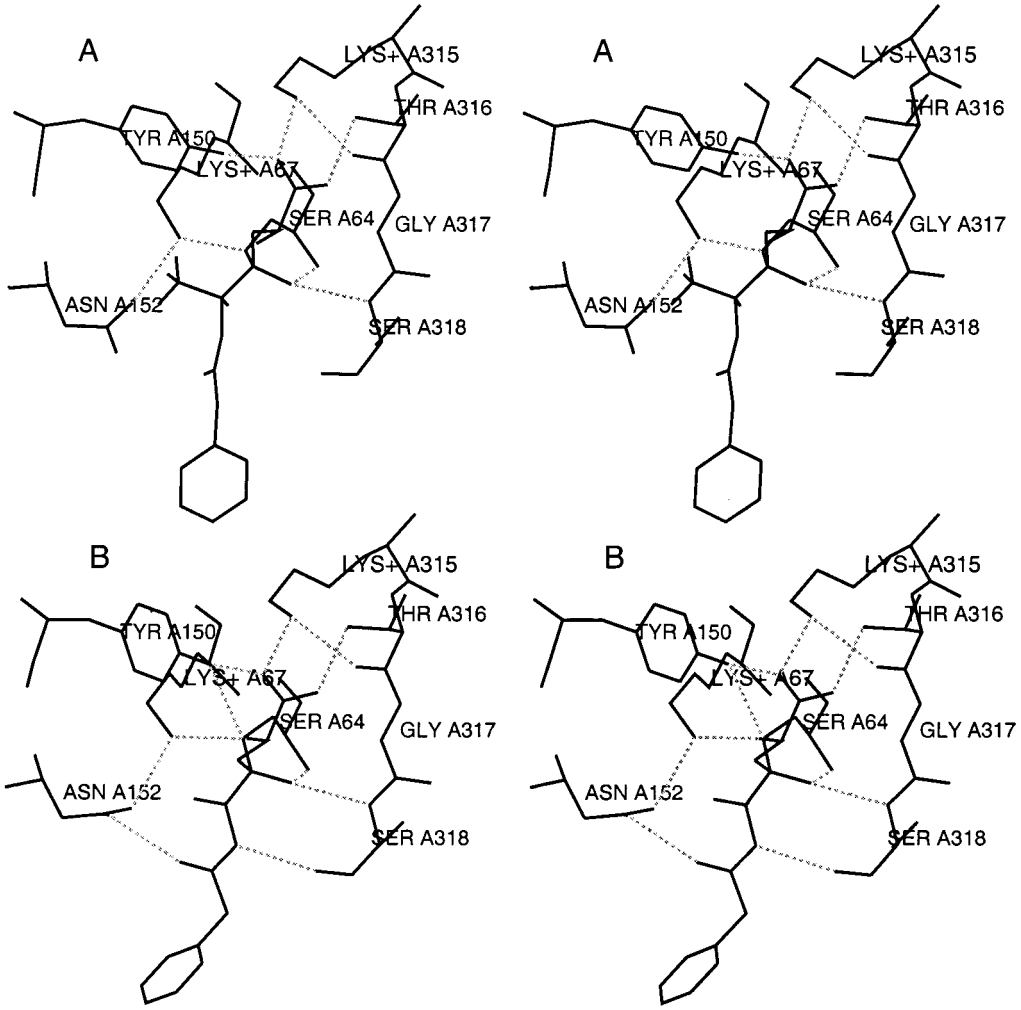


FIGURE 5: (A) Optimized tetrahedral intermediate structure formed on interaction of the P99 β-lactamase with *N*-(phenylacetyl)-D-alanyl-D-alanine. (B) The analogous structure for the P99 β-lactamase and *N*-(phenylacetyl)glycyl-D-alanine. Likely hydrogen bonds are represented as they are in Figure 4. Hydrogen atoms are only shown on the α-carbon and methyl group of the penultimate residue.

Table 3: Energies for Interaction between Tetrahedral Intermediate Ligands **8** and the Enzyme Active Sites

	δE_{int} (kcal/mol)[a]	
R	P99 β-lactamase	R61 DD-peptidase
H	0	0
Me	9.3	−1.2

[a] E_{int} values when R was H were −361.3 and −534.7 kcal/mol for the P99 β-lactamase and the R61 DD-peptidase, respectively; the larger value for the latter largely reflects the inclusion of an additional positively charged residue, Arg 285, in the calculation (see the text).

221, appears to push the phenylacetamido side chain further out of the active site. Energies for the interaction between the ligand and both the oxyanion hole and lysines 67 and 315 are smaller than for the glycyl compound whose positioning in the A conformation is not impeded by interaction with Tyr 221.

General Discussion and Conclusions. The kinetic results for peptide hydrolysis (Figure 2) indicate that the DD-peptidase prefers a D-alanyl substrate to glycyl at each of the kinetically significant points on the free energy profile, the acyl−enzyme and the acylation and deacylation transition states. This is a vital distinction for the DD-peptidase, not because there are glycyl peptides available in vivo against which it must discriminate, but because of the resemblance of the glycyl peptide to inhibitory β-lactams (*3*, *4*) which the enzyme should attack as slowly as possible for the bacterium to survive. It is observed that this preference for D-alanine is even greater in substrates with side chains closer in structure to bacterial cell wall peptides, e.g., *N*,*N*′-diacetyl-L-lysyl-D-alanyl derivatives (*15*), where further specific interactions of the substrate with the active site are possible. A cooperative "zippering" effect of the latter may enhance the free energy of interaction of the D-alanyl methyl group with the enzyme (*37*). Evolution of a β-lactamase from this DD-peptidase, or rather, from an ancestor of it, has involved discrimination against the D-alanyl peptide at the acylation step, to reduce to a minimum any covalent interaction between the β-lactamase and bacterial cell wall peptides. Conversely, the reactivity for glycyl peptides, and thus β-lactams, has been selected for.

The structural basis for these kinetic results has been investigated by molecular modeling of the tetrahedral intermediate of *N*-(phenylacetyl)-D-alanyl/glycyl-D-alanine hydrolysis. Although a clear and simple interpretation of the preference for the D-alanyl peptide by the DD-peptidase was not evident on inspection of the energy-minimized models (see below, however), energy calculations did favor this substrate. On the other hand, the structural basis for the preference of a glycyl substate by the β-lactamase was much

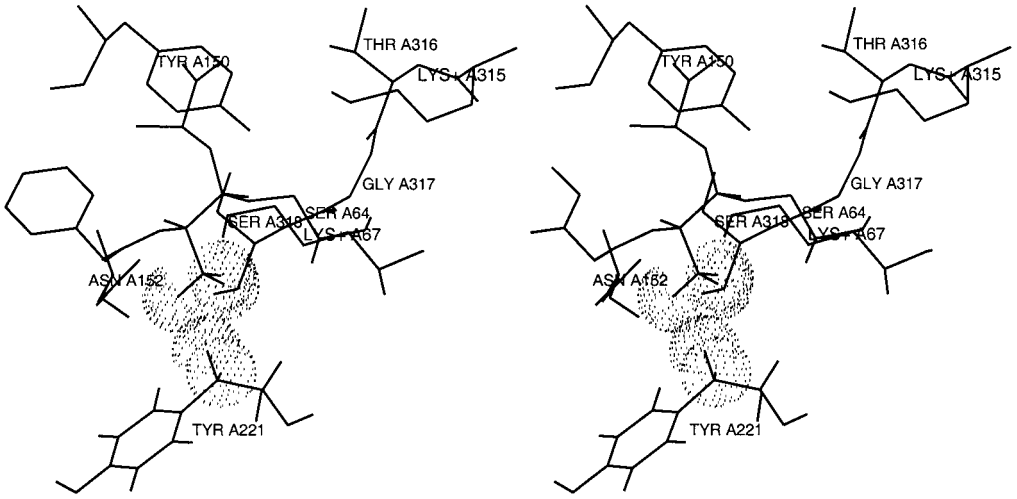


FIGURE 6: Interaction between the methyl group of the penultimate D-alanine residue of *N*-(phenylacetyl)-D-alanyl-D-alanine and Tyr 221 of the P99 β-lactamase in the energy-minimized tetrahedral intermediate structure with the ligand in the energetically disfavored conformation A (see the text). Hydrogen atoms are only displayed on the D-alanyl methyl group and the β-methylene group of Tyr 221; van der Waals surfaces of these hydrogen atoms are represented by the dotted spheres.

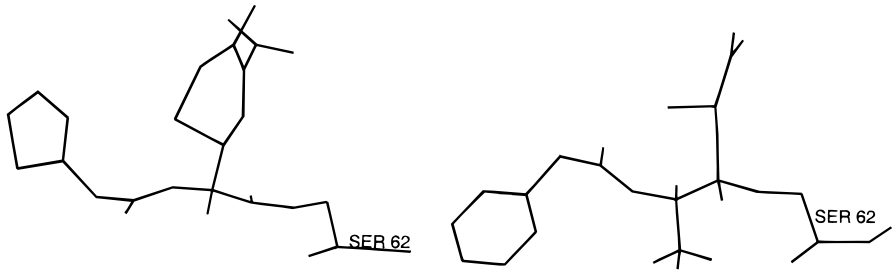


FIGURE 7: Conformation of the ligand in complexes of the R61 DD-peptidase with the tetrahedral intermediate generated from *N*-(phenylacetyl)-D-alanyl-D-alanine (taken from Figure 4B and shown on the right) and the acyl−enzyme intermediate generated from the reaction with cephalothin [taken from the crystal structure (*9*) and shown on the left]. Hydrogen atoms are shown only on the α-carbon and methyl group in the former case and on the α-carbon in the latter.

clearer. Steric hindrance by Tyr 221, at the C-terminus of the Ω loop (*17*), precluded optimal binding of the D-alanyl tetrahedral intermediate. A direct analogue of this residue is not present in the DD-peptidase. It would be interesting to see if a Tyr 221 Gly mutant of the β-lactamase would have enhanced D-Ala peptidase activity.

In neither enzyme would a D-substituent much larger than methyl be easily accommodated in D-alanine-favored conformation A. It is known that such substrates are hydrolyzed only very slowly by the R61 DD-peptidase (*16*, *38*). Penultimate L-amino acid derivatives (L-X) also are nonsubstrates of the R61 DD-peptidase (*16*). One factor in this result may be the ground state conformation of the substrate (*39*), but interactions between the substrate and enzyme must also be important. Inspection of the energy-minimized model of the D-Ala derivative (Figure 4A) indicates that an L-methyl group placed in conformation A would sterically interact with Asn 161 and Tyr 150. Presumably, conformational accommodation would result from these interactions, changing the position of the substrate with respect to the catalytic functional groups and thus affecting catalysis, probably negatively. Such L-X peptides would of course include bicyclic β-lactams where the heterocyclic substituent would have the L-configuration. It would seem possible however to accommodate L-X compounds in a conformation close to that of B where the L-substituent would protrude into the active site cavity and away from the protein surface. Such a position of a bulky substituent [which is actually observed in the crystal structure of the inert acyl−enzyme complex of cephalothin and the R61 DD-peptidase (Figure 7)], however, could well interfere with deacylation, as observed in the action of β-lactams as antibiotics. The other consequence of the difference between conformations A and B is that the amido side chains are aligned differently (Figure 7). This has been proposed earlier on the basis of substrate specificity studies and conformational analysis (*15*, *40*, *41*). The observation of the different specificities of nucleophiles toward acyl enzymes of the R61 DD-peptidase generated from peptides on one hand and from cleavage of penicilloyl derivatives on the other has also been interpreted in terms of different side chain orientations between peptides and β-lactams (*42*).

Interestingly, Damblon et al. (*38*) have shown that a penultimate L-Phe depsipeptide is a better (k_{cat}/K_m) substrate of the P99 β-lactamase than its D-Phe analogue. This result would suggest, in terms of the models presented above, that the tetrahedral intermediate of the L-Phe analogue could perhaps achieve something close to the A conformation with hydrogen in the methyl pocket and the benzyl side chain, flexible at the β-methylene carbon, protruding into the active site. The A conformation would be unavailable to the D-Phe analogue because of the size of the benzyl substituent. The broader expanse of the β-lactamase active site has been proposed to facilitate deacylation (*5*).

Molecular modeling studies showed that the D-amino acid leaving group in peptide hydrolysis, or, equivalently, the

nucleophile in aminolysis of the acyl−enzyme intermediate, interacted strongly with the active site at the tetrahedral intermediate stage. In the case of the P99 β-lactamase, the leaving group carboxylate strongly hydrogen bonds to the side chains of Lys 315 and Thr 316, whereas in that of the R61 DD-peptidase, the corresponding residues are Arg 285 and Thr 299. Arg 285 does not seem to have been previously identified as an important residue at the active site of the R61 DD-peptidase, although it does lie within the broad area that has been suggested to be the acyl acceptor binding site (*10*, *38*). The catalytic consequences of its mutation would clearly be interesting. It is noteworthy, however, that the crystal structures of inert covalent complexes of the R61 DD-peptidase with the β-lactams cephalothin and cefotaxime (*9*) suggest that the carboxylate group of these β-lactams interacts only with the side chains of Thr 299 and Thr 301. Interaction of these carboxylates with Arg 285 seems to be precluded by the C3′ exomethylene substituent of the thiazine ring.

As noted above, His 298, the direct analogue in the R61 DD-peptidase of Lys 315 of the β-lactamase, does not interact directly with the leaving group in the optimized complexes. The imidazole side chain of His 298 is hydrogen-bonded to the hydroxyl group of Tyr 280 in the crystal structure (*9*), and on the basis of the results of mutagenesis experiments, this pair has been implicated in the R61 DD-peptidase-catalyzed aminolysis of depsipeptides by D-amino acids (*43*). It is not known, however, if the His 298 Tyr 280 pair is neutral or zwitterionic. If the latter, then an electrostatic contribution to the reaction is still possible. If not, then the effect noted in the mutagenesis experiments seems more likely to be structural.

The results of this study confirm in both a structural and functional sense the importance of the D-methyl group rather than hydrogen at the penultimate position of a peptide substrate in both DD-peptidase and β-lactamase evolution and catalysis. The D-methyl group has presumably been selected for in DD-peptidase evolution and against in that of the β-lactamase. The biological reasons for these preferences are clear. An understanding of the importance of this methyl group may elucidate the ongoing process of ligand and inhibitor design for these enzymes.

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