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β-Lactamase-Catalyzed Aminolysis of Depsipeptides: Amine Specificity and Steady-State Kinetics†

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ABSTRACT: β-Lactamases catalyze not only the hydrolysis but also the aminolysis of certain depsipeptides [Pratt, R. F., & Govardhan, C. P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1302–1306]. This paper explores further the specificity of the aminolysis reaction with respect to the structure of the amine and also the steady-state kinetics of the reaction. The amines preferred by the class C β-lactamase of *Enterobacter cloacae* P99 appear to be aromatic D-α-amino acids. The general order of substrate effectiveness at pH 7.5 appears to be aromatic D-α-amino acids > large aliphatic D-α-amino acids > small aliphatic D-α-amino acids ~ small aliphatic L-α-amino acids > large L-α-amino acids. Charges on the aliphatic side chains seem unimportant. Ineffective as acyl acceptors were β-amino acids, α-amino phosphonic acids, and, in general, amines, including amino acid carboxyl derivatives and peptides. There is thus strong evidence for specific interaction between the amine and the enzyme. A detailed kinetics study was made of the P99 β-lactamase-catalyzed aminolysis of *m*-[[(phenylacetyl)glycyl]oxy]benzoic acid by D-phenylalanine. The steady-state kinetics were complex because of the presence of parallel enzyme-catalyzed hydrolysis and aminolysis reactions. An empirical rate equation was obtained for the total reaction. This has important elements in common with that previously found for the aminolysis of specific peptides by the DD-peptidases of various *Streptomyces* strains [e.g., Frere, J.-M., Ghuysen, J.-M., Perkins, H. R., & Nieto, M. (1973) *Biochem. J. 135*, 483–492]. Thus there is further evidence for the close functional similarity between the β-lactamase and DD-peptidase active sites, which supports an evolutionary relationship between the two groups of enzymes. A classical Ping Pong BiBi mechanism does not fit the kinetic data. An ordered BiBi mechanism, with the amino acid binding first, does fit the data, as it did the DD-peptidase results, but is in need of further confirmation.

The major biological role of the β-lactamases is generally believed to be protection of bacteria from β-lactam antibiotics, and indeed, these enzymes do efficiently catalyze the hydrolysis of many such antibiotics. We have shown however that β-lactamases can also catalyze the hydrolysis and aminolysis by D-α-amino acids of certain depsipeptides of general structure **1** (Pratt & Govardhan, 1984). This discovery was significant on two counts. First, it expanded our knowledge of the

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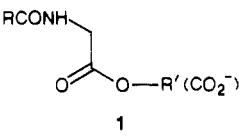

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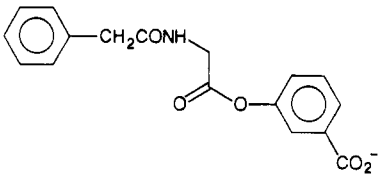
chemical capability of the β-lactamase active site, thus creating the potential for novel probe and inhibitor design, and second, it provided further evidence for a close relationship between the β-lactamases and the bacterial cell wall DD-peptidases. The latter enzymes are, of course, the primary targets of β-lactam antibiotics. The existence of an evolutionary relationship between these two groups of enzymes was originally proposed on the basis of their shared affinity for β-lactam antibiotics and the structural resemblance between penicillins and *N*-acyl-D-alanyl-D-alanine peptides (Tipper & Strominger, 1965). More recently, this proposal has also been put on a stronger structural footing by the results of X-ray crystallographic studies of the relevant proteins (Kelly et al., 1986; Samraoui et al., 1986) and detailed amino acid sequence analysis (Joris et al., 1988).

An investigation of the kinetics of the β-lactamase-catalyzed hydrolysis and methanolysis of **1** suggested that a double-displacement (Ping Pong) mechanism obtained, with an acyl-enzyme intermediate (Govardhan & Pratt, 1987). In this and the accompanying papers (Pazhanisamy & Pratt, 1989a,b), we present the results of our analysis of the kinetics of the β-lactamase-catalyzed aminolysis reaction (Scheme I). This reaction is important because of its close analogy to the transpeptidation reaction catalyzed by the DD-peptidases, where the natural substrates are the more chemically inert peptides rather than depsipeptides, a distinction probably important in β-lactamase evolution (Pratt & Govardhan, 1984).

The substrate and enzyme chosen for detailed examination in the present study were the depsipeptide **2** and the class C β-lactamase from *Enterobacter cloacae* P99, respectively.



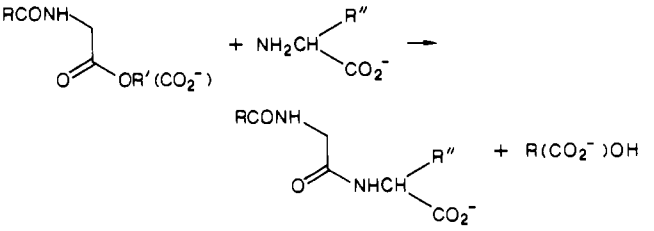
2

This combination had been shown to demonstrate the most effective "depsipeptidase" activity of those yet investigated, with the further advantage that the acyl-transfer reactions of **2** can be conveniently followed spectrophotometrically (Govardhan & Pratt, 1987). In this paper, the specificity of the reaction of Scheme I with respect to the structure of the amino acid is further investigated, and an empirical steady-state kinetic equation for the reaction is obtained. The latter is discussed in terms of kinetic mechanisms which have precedent from studies of the DD-peptidases in other laboratories. The following papers (Pazhanisamy & Pratt, 1989a,b) further test these models and examine alternatives for the reaction sequence, one of which appears to better accommodate all of the available data and which may be applicable to all of the above-mentioned enzymes.

Experimental Procedures

Materials. The β-lactamase of *Enterobacter cloacae* P99 was obtained from the Centre for Applied Microbiology and Research (Porton Down, England) and, as previously (Govardhan & Pratt, 1987), used as supplied. Benzylpenicillin was purchased from Sigma Chemical Co. The substrate *m*-[[(phenylacetyl)glycyl]oxy]benzoic acid (**2**), the product of aminolysis of **2** by D-phenylalanine [(phenylacetyl)glycyl-

Scheme I



D-phenylalanine (**3**)], and the other depsipeptides employed were prepared as previously described (Govardhan & Pratt, 1987). Amines and amino acids were, in general, commercial products and used as obtained; D-phenylalanine, in particular, was obtained from Sigma Chemical Co. D,L-(1-Aminoethyl)phosphonate and D,L-(1-amino-2-phenylethyl)-phosphonate were gifts from Dr. Max Tishler.

Analytical Methods. Absorption spectra and spectrophotometric steady-state reaction rates were measured by means of a Perkin-Elmer Lambda 4B spectrophotometer. Polarimetric rates were measured with a Perkin-Elmer 241 polarimeter, equipped with a thermostated 10-cm path-length microcell. Measurements of optical rotation were recorded on a strip-chart recorder with a full-scale output of 2 mV, corresponding to a rotation of 20 mdeg at maximum sensitivity. β-Lactamase activity was routinely checked by the spectrophotometric method of Waley (1974). The β-lactamase concentrations were determined spectrophotometrically by employment of the extinction coefficient 7.10×10^4 M^{-1} cm^{-1} at 280 nm (Joris et al., 1985). NMR spectra were obtained by means of a Varian XL-400 spectrometer.

Steady-State Kinetics. (A) Total Rate Measurements. The net initial rate (hydrolysis plus aminolysis) of reaction of **2** was determined by spectrophotometric observation of the release of *m*-hydroxybenzoate. Since D-phenylalanine, at the high concentrations employed, did absorb somewhat at the absorption maximum of *m*-hydroxybenzoate (286 nm), rate measurements were made at slightly longer wavelengths, and generally at 300 nm. Care was taken to determine the extinction coefficient change (ca. 930 M^{-1} cm^{-1} at 300 nm) at that wavelength on each occasion that rates were measured.

In a typical kinetic run at pH 7.5, appropriate volumes of stock solutions of D-phenylalanine (or an alternative acceptor) and **2** (both in buffer at the required pH) were mixed with buffer to a total volume of 0.80 mL in a cuvette and held in the thermostated cell compartment of the spectrophotometer at 25 °C for 10 min. The reaction was then initiated by addition of 10 μL of a β-lactamase solution. The data were acquired and initial rates determined by means of Perkin-Elmer software. The buffer employed at pH 7.5 was 20 mM MOPS.

At pH 9.0, the nonenzymic hydrolysis of **2** was quite significant, such that a slightly different procedure was necessary. Stock solutions of **2** were prepared in water with 2–4 equiv of sodium bicarbonate and stored on ice. The carbonate buffer was correspondingly prepared at concentrations higher than 0.1 M such that, on addition of all the reaction components, the final buffer at pH 9.0 was 0.10 M in carbonate/bicarbonate. D-Phenylalanine (or an alternative acceptor) stock solutions were prepared in 0.10 M carbonate buffer, and the pH was adjusted to 9.0. The acceptor plus buffer solutions (>0.6 mL) were thermostated in the spectrophotometer to 25 °C. An appropriate volume of the stock solution of **2** (also thermostated to 25 °C) was then added to bring the total volume to 0.80 mL. The reaction was thereafter immediately initiated by addition of 10 μL of an enzyme stock solution.

(*B*) *Aminolysis Rate Measurements.* These were determined polarimetrically at 365 nm in 20 mM MOPS buffer at pH 7.5 at 25 °C. Under these conditions, both D-phenylalanine and the aminolysis product, (phenylacetyl)-glycyl-D-phenylalanine (**3**), contributed to the measured optical rotation of reaction mixtures, and therefore, the molar rotations of these compounds (+15.8° and −47.5°, respectively) were determined from measurements on separate solutions. These molar rotations then allowed conversion of the initial rates into M s^{-1} units. Measured rotation changes for complete reaction agreed with those calculated from the molar rotations of the components. A typical kinetics run was performed by mixing appropriate volumes of D-phenylalanine and **2** stock solutions, both in the required buffer, with buffer to a final volume of 1.6 mL. This reaction mixture was temperature equilibrated in a vial held in a water bath at 25 °C for 10 min, before the reaction was initiated by addition of 10 μL of an enzyme stock solution. It was then quickly transferred to the polarimeter cell which had been thermostated at the same temperature and the optical rotation recorded as a function of time. Initial rates of aminolysis were determined from the initial, essentially linear, parts of the curves.

Initial rates of hydrolysis, in the presence of amino acid acceptors, were determined by subtraction of the polarimetrically determined aminolysis rates from the spectrophotometrically determined total (aminolysis plus hydrolysis) rates. Under conditions of saturating amino acid concentrations, where product analysis by ^1H NMR indicated complete aminolysis, the polarimetric and spectrophotometric rates were identical. Linear and nonlinear (Johnson et al., 1976) least-squares computer programs were used to fit the kinetic data to appropriate equations.

Product Identification by ^1H NMR. As described previously (Pratt & Govardhan, 1984; Govardhan & Pratt, 1987), ^1H NMR spectroscopy could be used to detect and determine the products of β-lactamase-catalyzed acyl-transfer reactions of **2**. In typical experiments, ca. 0.1-mg samples of the P99 β-lactamase were added to NMR tubes containing **2** (10 mM) and amines or amino acids (50 mM) in 0.5 mL of ^2H_2O; also present were $NaHCO_3$ (0.1 M) as a buffer and sodium (trimethylsilyl)propanesulfonate as an internal standard. Spectra were then recorded until the depsipeptide substrate had completely reacted. Aminolysis products could be identified by their spectra. In particular, a glycyl methylene group with resonance between that of the starting material at 4.28 ppm and the hydrolysis product at 3.74 ppm was diagnostic of, and could be used to quantitate, an amide product; for example, that of **3** occurred at 3.80 ppm as an AB quartet. The lower limit of detection by this method was around 2% of total reaction.

Results

Structural Specificity of Amine (i.e., Acyl Group Acceptor). Our previous work (Pratt & Govardhan, 1984) had suggested that the P99 β-lactamase exhibited considerable specificity with respect to the structure of the amine acceptor of the acyl group in the depsipeptide aminolysis reaction, although the range of compounds examined was not large. D-Amino acids appeared to be the preferred acceptors, while L-amino acids and peptides were much poorer or completely ineffective. The question of this specificity has been pursued further, and under the conditions specified under Experimental Procedures ($NaHCO_3$ solution, pH 7–8), ^1H NMR spectra demonstrated the following:

(1) No aminolysis occurred with the following compounds: *N*-methyl-D-phenylalanine, α-methyl-D-phenylalanine, D-phenylalaninol, DL-phenylalaninamide, β-alanine, DL-3-aminobutyrate, D-proline, D,L-(1-aminoethyl)phosphonate, D,L-(1-amino-2-phenylethyl)phosphonate, ammonia, butylamine, ethanolamine, and 2-phenylethylamine. On the other hand, benzylamine produced almost complete aminolysis.

(2) All L-amino acids tested (phenylalanine, tryptophan, leucine, valine, glutamic acid, and lysine) except alanine gave no or small amounts of peptide products (≤10% of total reaction).

(3) All D-amino acids tested (alanine, phenylalanine, phenylglycine, valine, leucine, lysine, tryptophan, aspartic acid, glutamic acid, and tyrosine), as well as glycine, gave much higher yields of peptide products (>10%) than the L-amino acids (with the exception of L-alanine, which seemed to have similar reactivity as the D-isomer).

(4) Amino acid amides (peptides) produced no aminolysis products (glycinamide, DL-phenylalaninamide, glycylglycine, DL-alanyl-DL-phenylalanine, D-phenylalanyl-DL-alanine, D-phenylalanyl-D-phenylalanine, DL-alanyl-DL-valine).

At pH 9 (carbonate buffer) there was evidence of reduced specificity, in that the L-amino acids (e.g., L-phenylalanine) and amines (ammonia, 2-phenylethylamine) gave greater yields of amide products than at pH 7.5. This observation could reflect the greater proportion of the neutral amine in solution at this pH. It should be noted that no spontaneous aminolysis, i.e., without enzyme catalysis, occurred over the time intervals of these experiments.

Some further detail on the question of specificity is available in Table I where quantitative comparisons of the performance of a variety of D-amino acids are given. As is described more fully below, at constant **2** concentration, the initial rates of reaction increased with D-amino acid concentration to a saturating value. From such data apparent binding (K_m^{app}) and rate (k_{cat}^{app}) parameters could be obtained, and these are given in Table I. These data were collected at pH 9.0, although qualitatively and quantitatively similar results could be obtained at pH 7.5.

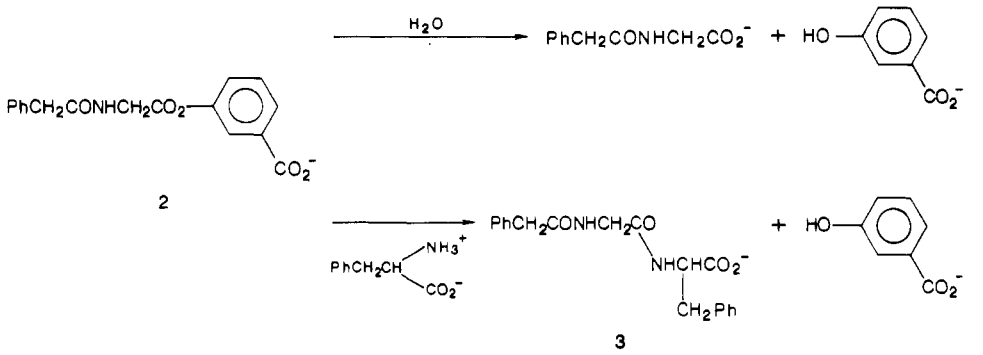
Table I: Steady-State Kinetic Parameters for the P99 β-Lactamase-Catalyzed Reaction of D-Amino Acids with the Depsipeptide **2**[a]

amino acid	k_{cat}^{app} (s^{-1})[b]	K_M^{app} (mM)[b]
D-phenylalanine	76	1.5
D-homophenylalanine	79	1.3
p-iodo-D-phenylalanine	147	0.65
3-(1-naphthyl)-D-alanine	≥205	≥1.6[c]
3-(2-naphthyl)-D-alanine	146	1.25
D-tryptophan	130	1.5
D-tyrosine	138	1.4
DL-2-amino-3-phenylbutyrate	130	3.5
D-2-aminobutyrate	85	5.7
D-phenylglycine	≥50	≥8.0
D-leucine	97	5.8
D-norleucine	125	3.2
D-lysine	69	13.2
N^{ϵ}-acetyl-D-lysine	90	12.2
DL-α-aminopimelic acid	87	14.4
DL-α,ε-diaminopimelic acid	≥25	≥40
glycine	98	34
D-alanine	114	80

[a] 1 mM **2**, 0.1 M carbonate buffer, pH 9.0, and 25 °C. [b] Standard deviations <20%. [c] Limited by solubility.

These results appear to support the previous indications that the optimal acceptor is a primary D-α-amino acid. This structural selectivity and stereoselectivity seem to correlate well with the fact that the peptide bridges of Gram-negative bacteria are formed from nucleophilic attack by the D center of a *meso*-diaminopimelate residue of a peptide chain of one peptidoglycan strand onto a terminal peptide bond of a chain

Scheme II



from an adjacent strand (Rogers et al., 1980). In the present case, small L-amino acids such as L-alanine are also acceptors, suggesting that the α-H and α-R groups are interchangeable in an amino acid binding site when R is small. In the natural *meso*-diaminopimelate acceptors of Gram-negative bacteria, the R group is of course much larger than that of alanine. The α-carboxylate group on the acceptor appears to be important, in view of the impotence of D-phenylalanine carboxyl derivatives, the α-amino phosphonates, the β-amino acids, the peptides, and the amines (although the curious result with benzylamine, which was quite reproducible, suggests that these indications of the structural specificity of acyl group acceptors may still be incomplete). The negative results with *N*-methyl-D-phenylalanine and D-proline show that a primary amino group is necessary. The more quantitative results of Table I indicate that D-amino acids with large hydrophobic side chains are preferred, where the aromatic compounds are superior to the naturally occurring aliphatics. A single positive (D-lysine) or negative (D-α-aminopimelate) charge on an aliphatic side chain appears to exert little effect. On the basis of these results, D-phenylalanine was chosen for the detailed kinetics studies described below. An important parameter in this choice was the solubility of the amino acid since, as seen below, K_M^{app} increased markedly with the concentration of **2** and high concentrations of the acyl acceptor were thus needed to approach saturation.

Structural Specificity of Depsipeptide Leaving Group. The extent of aminolysis as a function of the leaving group of the depsipeptide was examined by ^{1}H NMR experiments in 0.1 M sodium bicarbonate buffer. The acyl moiety of the depsipeptide in each case was the (phenylacetyl)glycyl group, and the initial concentrations of depsipeptide and the acyl acceptor, D-phenylalanine, were 10 mM and 20 mM, respectively. The percentages of the total reaction leading to aminolysis were, for various leaving groups, as follows: D-lactate, 26; D-mandelate, 30; *o*-hydroxybenzoate, 10; *m*-hydroxybenzoate, 8; *p*-hydroxybenzoate, 13. The estimated uncertainty in these values is ±5%. The conclusions derived from these data are, first, that all of the depsipeptides are aminolyzed to some extent and, second and more importantly, that the extent of aminolysis is not the same for all depsipeptides despite their common acyl group. This means that, unlike for the P99 β-lactamase-catalyzed methanolysis (Govardhan & Pratt, 1987), a simple Ping Pong mechanism, where the leaving group departs prior to interaction of the acyl acceptor with the enzyme, cannot apply for the aminolysis of all of the above depsipeptides. In certain of the depsipeptides at least, the amino acid must be able to detect the presence of the leaving group at the enzyme active site. This result, reinforced by the indications of significant specificity in the structure of the D-amino acid, was suggestive of a D-amino acid binding site separate from the depsipeptide site. This possibility encouraged us to pursue the detailed kinetics studies described below.

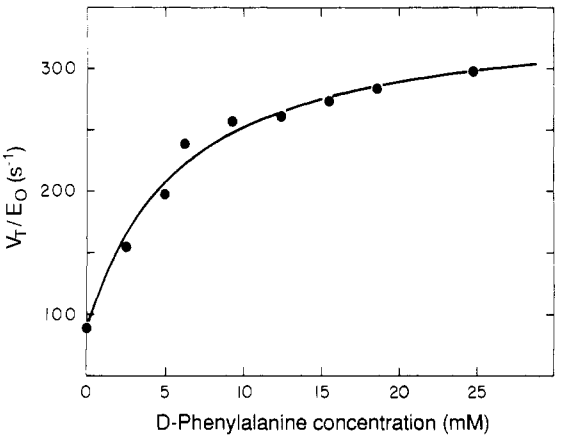


FIGURE 1: Initial total velocity (V_T) of the reaction of **2** (1.0 mM) with varying concentrations of D-phenylalanine in the presence of the P99 β-lactamase (0.015 μM). The points are experimental, and the line is calculated from eq 1 as described in the text.

Steady-State Kinetics of Aminolysis Reaction. The *E. cloacae* P99 β-lactamase catalyzes both hydrolysis and aminolysis of depsipeptides such as **2**. As the concentration of amino acid rises, so too does the proportion of reaction leading to aminolysis; both the proportion of the reaction leading to hydrolysis and the absolute rate of hydrolysis decrease (see below). Since it was not possible, with the amino acids thus far investigated, to obtain all kinetic parameters for the aminolysis reaction under conditions of complete aminolysis, it was necessary to perform experiments under conditions where both hydrolysis and aminolysis reactions were proceeding. For the reaction of **2** in the presence of D-phenylalanine (Scheme II), initial rates of the total reaction (V_T) were determined spectrophotometrically via hydroxybenzoate appearance and the initial rates of aminolysis (V_A) polarimetrically via disappearance of D-phenylalanine and appearance of (phenylacetyl)glycyl-D-phenylalaninate (**3**); initial rates of hydrolysis (V_H) could then be determined by difference.

At a fixed depsipeptide concentration, V_T increased with D-phenylalanine concentration to a saturation value, as shown for example in Figure 1. Such a rate increase suggests that aminolysis proceeds by a route separate from hydrolysis or that the amino acid intercepts an intermediate in the hydrolysis pathway whose breakdown to products is rate determining. Since acylation appears to be rate determining to hydrolysis (Govardhan & Pratt, 1987), the former possibility would seem to be more likely. The other D-amino acids of Table I, L-alanine, and benzylamine also increased the total rate observed and thus presumably participate in the same aminolysis pathway.

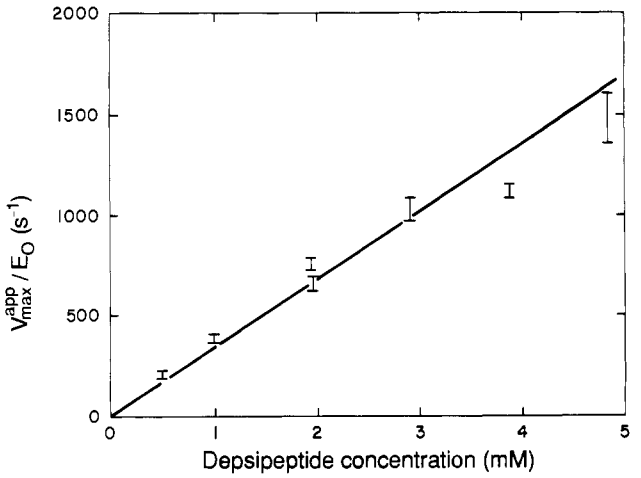


FIGURE 2: Plot of V_{MAX}^{app}/E_0 as a function of the depsipeptide (D, **2**) concentration. The points are experimental, and the line is calculated from eq 15 as described in the text.

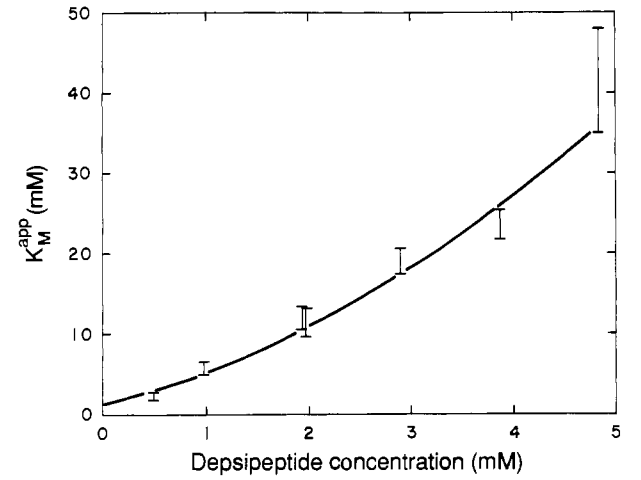


FIGURE 3: Plot of K_M^{app} as a function of the depsipeptide (D, **2**) concentration. The points are experimental, and the line is calculated from eq 16 as described in the text.

These curves, from several different depsipeptide concentrations, were fitted by a nonlinear least-squares procedure to eq 1, where V_0 is the (hydrolysis) rate at that depsipeptide

$$V_T = (V_0K_M^{app} + V_{MAX}^{app}[A])/(K_M^{app} + [A]) \quad (1)$$

concentration in the absence of D-phenylalanine (A). V_{MAX}^{app} is the rate at saturating D-phenylalanine concentration and K_M^{app} the D-phenylalanine concentration at half the maximal rate, i.e., the apparent dissociation constant of D-phenylalanine. Both V_M^{app} and K_M^{app} increased with depsipeptide (D) concentration as shown in Figures 2 and 3, respectively. The ratio V_H/V_A, determined at fixed [D], increased linearly with 1/[A] (Figure 4) and, at fixed [A], increased linearly with [D] (at least at low [A]) (Figure 4). Comparable data were obtained at pH 9.0, although here the dependence of V_{MAX}^{app} on [D] was not linear, showing evidence of saturation in [D], and V_H/V_A did not appear to show variation with [D] at 5.0 mM D-phenylalanine (data not shown).

Empirical eq 2–4, where a_1 to a_6 are concentration independent parameters, were then fitted to the data of Figures 2–4, and the solid lines in these diagrams are calculated on the basis of these equations (see also below).

$$V_{MAX}^{app} = a_1[D] \quad (2)$$

$$K_M^{app} = a_2 + a_3[D] + a_4[D]^2 \quad (3)$$

$$V_H/V_A = (a_5 + a_6[D])/[A] \quad (4)$$

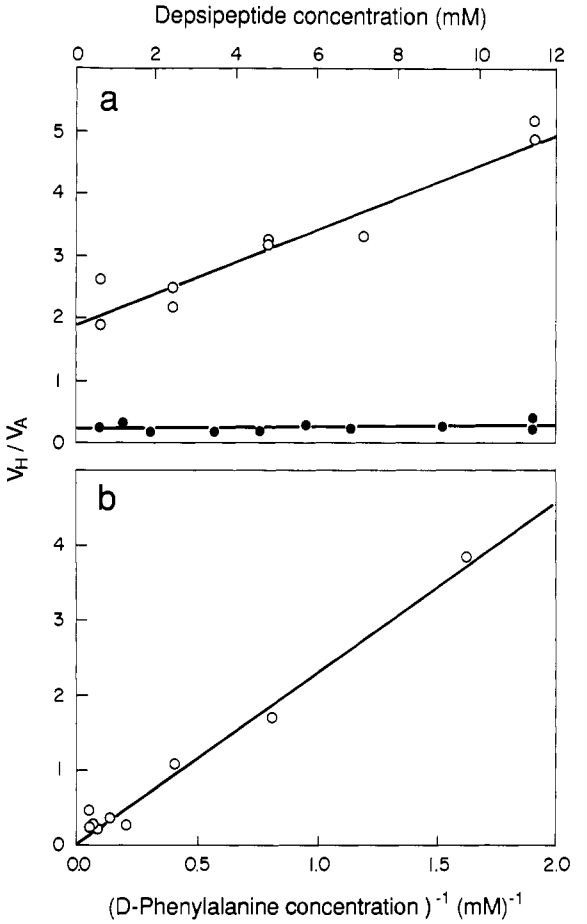


FIGURE 4: Plots of V_H/V_A as a function of (a) the depsipeptide (D, **2**) concentration at constant D-phenylalanine concentration, 1.0 (○) and 16.0 mM (●), and (b) the reciprocal of the D-phenylalanine (A) concentration at constant depsipeptide concentration (0.54 mM). The points are experimental, and the lines are calculated from eq 14 as described in the text.

The increase in K_M^{app} with [D] suggested that the aminolysis reaction somehow competed with hydrolysis, for example, as if the binding of A competed with the binding of D. This is also suggested by eq 4. Further, the $[D]^2$ term in eq 3 suggests that D binds to two distinct enzyme forms. These equations therefore appear to support the proposition of two separate pathways for hydrolysis and aminolysis.

Equation 5 follows from eq 1, if it is assumed that no hydrolysis terms contain [A]. Simultaneous solution of eq 1–5, eliminating V_0 and V_H/V_A, yielded eq 6 as an empirical equation, which should describe the total reaction.

$$V_H/V_A = V_0K_M^{app}/V_{MAX}^{app}[A] \quad (5)$$

$$V_T = \frac{a_1(a_5 + a_6[D])[D] + a_1[A][D]}{a_2 + a_3[D] + a_4[D]^2 + [A]} \quad (6)$$

If this were so, then in the absence of A, the initial velocity (of hydrolysis) should be given by eq 7.

$$V_0 = \frac{a_1(a_5 + a_6[D])[D]}{a_2 + a_3[D] + a_4[D]^2} \quad (7)$$

By prior experiment however (Govardhan & Pratt, 1987), and confirmed in this work, the hydrolysis rates follow eq 8 within the limits of experimental uncertainty. From measurements of hydrolysis rates in the absence of A, under the conditions employed for these aminolysis studies (20 mM MOPS, pH

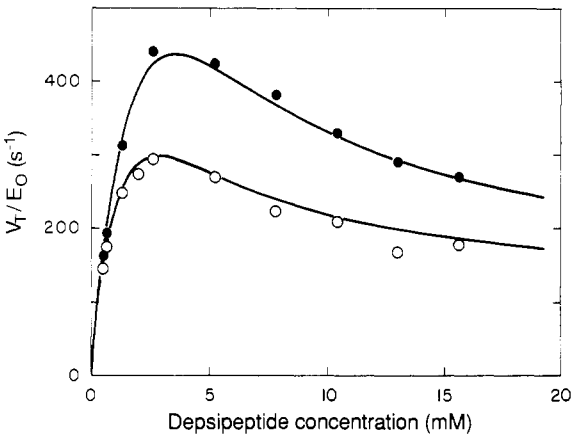
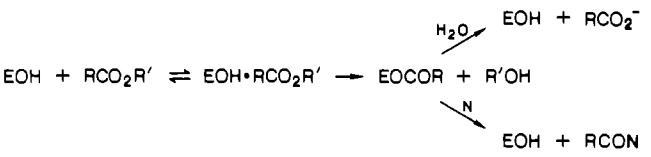


FIGURE 5: Initial total velocity (V_T) of the reaction of D-phenylalanine at 7.5 (○) and 14.8 mM (●) concentration with varying concentrations of **2** in the presence of the P99 β-lactamase. The points are experimental, and the lines are calculated from eq 11 as described in the text.

Scheme III



7.5, 25 °C), $k_{cat}^H = V_{MAX}^H/E_0 = 105 \pm 19\ s^{-1}$ and $K_M^H = 0.42 \pm 0.13$ mM.

$$V_0 = V_{MAX}^H[D]/(K_M^H + [D]) \quad (8)$$

In order to reduce eq 7 to eq 8, we could, in principle, have $a_5 \gg a_6[D]$ and $(a_2 + a_3[D]) \gg a_4[D]^2$ or have $a_6[D] \gg a_5$ and $(a_3[D] + a_4[D]^2) \gg a_2$, but the thereby eliminated terms are needed to explain the aminolysis data at similar depsipeptide concentrations. Therefore, the simplicity of eq 8 probably arises from the cancellation of terms, as in eq 9.

$$V_0 = \frac{V_{MAX}^H[D](b_1 + b_2[D])}{(K_M^H + [D])(b_1 + b_2[D])} = \frac{b_1V_{MAX}^H[D] + b_2V_{MAX}^H[D]^2}{b_1K_M^H + (b_1 + b_2K_M^H)[D] + b_2[D]^2} \quad (9)$$

From equation of eq 7 with eq 9 and elimination of a_2 ($=b_1K_M^H$), a_3 ($=b_1 + b_2K_M^H$), a_4 ($=b_2$), a_5 ($=b_1V_{MAX}^H/a_1$), and a_6 ($=b_2V_{MAX}^H/a_1$), eq 10, the final empirical equation for V_T, is obtained. This equation predicts a complex dependence

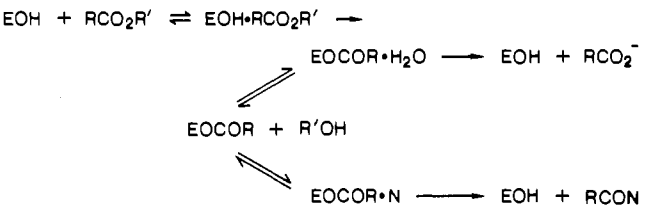
$$V_T = \frac{b_1V_{MAX}^H[D] + b_2V_{MAX}^H[D]^2 + a_1[A][D]}{b_1K_M^H + (b_1 + b_2K_M^H)[D] + b_2[D]^2 + [A]} \quad (10)$$

of V_T on [D] at fixed [A], and this is indeed observed, both qualitatively (Figure 5) and quantitatively [the solid lines of Figure 5 are calculated from the parameters of eq 2–4 and 8 (see below)]. Thus, there is good reason to believe that eq 12 represents at least a good approximation to the empirical rate equation.

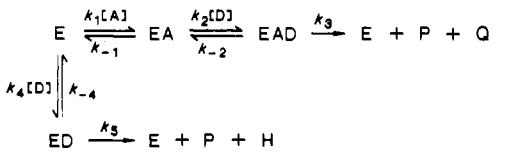
Discussion

In view of our previously published studies of the β-lactamase-catalyzed methanolysis of the depsipeptide **2**, where a classical Ping Pong kinetic mechanism (Scheme III; N is an alternative nucleophile to water) was established, it was reasonable at first to carefully assess the kinetic data in terms of such a model. Such a model is of course also well established for the aminolysis of esters catalyzed by serine and cysteine proteinases (Fruton, 1982) although, in certain cases, specific binding of the nucleophile to the acyl-enzyme prior to deacylation (Scheme IV) has also been demonstrated (Fink & Bender, 1969; Wynne & Shalitin, 1972; Kasche et al., 1984; Breddam & Ottesen, 1984; Riechmann & Kasche, 1984, 1985). The present data however firmly reject Schemes III and IV. Neither V_{MAX}^{app} nor K_M^{app} should increase with depsipeptide (D) concentration, when $[D] \gg K_M^H$ (cf. Figures 2 and 3). Nor, in either case, should V_H/V_A be a function of [D] (Figure 4).

Scheme IV



Scheme V



Frere (1973) has shown that in cases like this one, where parallel paths of acyl transfer, hydrolysis and aminolysis, occur, V_H/V_A is a sensitive indicator of kinetic mechanism. In particular, he has shown that in BiBi mechanisms V_H/V_A will vary with [D] *only* when the other substrate, the amino acid A, D-phenylalanine in this case, binds first to the enzyme. The mechanism of Scheme V is thereby indicated, where P represents the common aminolysis and hydrolysis product *m*-hydroxybenzoate, H the hydrolysis product (phenylacetyl)-glycine, and Q the aminolysis product **3**. This scheme does carry with it the motifs recognized under Results, viz., separate hydrolysis and aminolysis pathways, competition (for E) between A and D, and the binding of D to two distinct enzyme forms (E and EA). The steady-state kinetic equation derived from this scheme is simpler of course if certain binding steps are represented as rapid equilibria. Examination of these possibilities shows that the only one which *cannot* be thus represented and still retain the V_H/V_A dependence of [D] is step 1. One cannot assume therefore that $k_{-1} \gg k_2k_3[D]/(k_{-2} + k_3)$.

Under the assumption that steps 2 and 4 represent fast equilibria, with dissociation constants K_2 and K_4, respectively, eq 11, as the simplest possible equation fitting the data, can be derived from Scheme V. It follows from this equation that

$$V_T/E_0 = [k_5k_{-1}K_2[D] + k_3k_5[D]^2 + k_1k_3K_4[A][D]]/[k_{-1}K_2K_4 + (k_{-1}K_2 + k_3K_4)[D] + k_3[D]^2 + k_1K_4K_2[A] + k_1K_4[A][D]] \quad (11)$$

under conditions of fixed [D] and variable [A], eq 12–14 apply.

$$V_{MAX}^{app}/E_0 = k_3[D]/(K_2 + [D]) \quad (12)$$

$$K_M^{app} = \frac{k_{-1}K_2K_4 + (k_{-1}K_2 + k_3K_4)[D] + k_3[D]^2}{k_1K_4(K_2 + [D])} \quad (13)$$

$$V_H/V_A = \frac{k_5[k_{-1} + (k_3/K_2)[D]]}{k_1K_4(k_3/K_2)[A]} \quad (14)$$

If it is assumed that $K_2 \gg [D]$, i.e., that the binding of D to

Table II: Kinetic Parameters Derived from the Fitting of the Experimental Data to Scheme V

parameter	source: V_{MAX}^{app}, K_M^{app} (Figures 2 and 3)	V_H/V_A (Figure 4)	V_T (Figure 5)	V_{MAX}^{app}, K_M^{app} (pH 9.0)
K_1 (mM)	1.29 ± 0.33	2.24 ± 0.87	1.36 ± 0.81	0.35 ± 0.08
$k_1 \times 10^{-5}$ (s^{-1} M^{-1})	8.5 ± 3.3	9.6 ± 3.6	5.0 ± 0.4	150 ± 150
k_{-1} (s^{-1})	1010 ± 480	2140 ± 1150	680 ± 400	5500 ± 5500
$k_3/K_2 \times 10^{-5}$ (s^{-1} M^{-1})	2.95 ± 0.34	2.95[a]	3.7 ± 0.5	2.3 ± 0.9
k_3 (s^{-1})	>1500[b]			1900 ± 400
K_2 (mM)	>5[b]			8.2 ± 2.6
K_4 (mM)	0.42 ± 0.13[a]	0.42[a]	0.42[a]	0.49 ± 0.08[a]
k_5 (s^{-1})	105 ± 19[a]	105[a]	105[a]	46 ± 2[a]

[a] Assumed from the hydrolysis data. [b] At pH 7.5, V_{MAX}^{app} varied linearly with [D] to 5 mM, and thus, only lower limits to k_3 and K_2 could be estimated.

EA is weak, then eq 12 and 13 become eq 15 and 16, respectively, and the term in [A][D] disappears from the denominator of eq 11. Under those circumstances, eq 11 and

$$k_{cat}^{app} = V_{MAX}^{app}/E_0 = (k_3/K_2)[D] \quad (15)$$

$$K_M^{app} = [k_{-1}K_2K_4 + (k_{-1}K_2 + k_3K_4)[D] + k_3[D]^2]/k_1K_2K_4 \quad (16)$$

14–16 become identical in functional form with empirical eq 2–4 and 10, and thus Scheme V is consistent with the kinetic data.

The least-squares fitting of eq 11 and 14–16 to the experimental data of Figures 2–4 yields the lines shown in the figures and the values for the rate and equilibrium parameters given in Table II. To obtain the values reported, the data of Figures 2 and 3 were analyzed separately from that of Figures 4 and 5. In fitting the data of Figures 2–4, the hydrolysis parameters k_5 ($=V_{MAX}^{H}/E_0$) and K_4 ($=K_M^H$) were fixed at the values that fitted the independent hydrolysis data. The agreement between the values obtained from the different experiments appears to be acceptable. The most accurate estimates of the kinetic parameters however are believed to come from the V_{MAX}^{app} and K_M^{app} data.

At pH 9, unlike at pH 7.5, the plot of V_{MAX}^{app}/E_0 vs [D] (data not shown) shows some evidence of saturation behavior, which is also accommodated by Scheme V (eq 12); thus at pH 9.0, estimates of k_3 and K_2 separately could be made (see Table II). At pH 9.0 also, k_{-1} is much larger with respect to k_3/K_2 than at pH 7.5, which makes the variation of V_H/V_A with [D] at fixed [A] more difficult to observe.

Scheme V is significant in that it is identical with that proposed by Ghuysen, Frere, and co-workers (Frere et al., 1973; Ghuysen et al., 1979) to best explain the kinetics of parallel acyl transfer from specific peptide substrates to water and to specific amino acid and peptide acceptors, catalyzed by the cell wall DD-peptidases of various *Streptomyces* strains. As mentioned above, the dependence of V_H/V_A on acyl-donor concentration was a crucial observation leading them to this model. In more recent times however, they have commonly represented the reaction as a classical Ping Pong (Scheme III), for example, with respect to the *Streptomyces* K15 DD-peptidase (Nguyen-Disteche et al., 1986), and Ghuysen (1987) has emphasized the similarities between the DD-peptidases and serine proteinases. The kinetics however do not seem to have been reconciled with this new view. Indeed, the dependence of V_H/V_A on acyl-donor concentration is shown in the same paper as the Ping Pong scheme is presented (Nguyen-Disteche et al., 1986). The recent resurgence of the Ping Pong model appears to stem from the decisive experiments of Strominger and co-workers (Yocum et al., 1979, 1980), who showed that an acyl-enzyme intermediate was involved in the DD-peptidase-catalyzed hydrolysis of depsipeptides and that the acylated serine therein was the same serine acylated by β-lactam antibiotics. (It should be noted however that the existence of an acyl-enzyme intermediate in *aminolysis* does not seem to have yet been demonstrated.) On the basis of these and other results, Strominger and co-workers have generally advocated a simple Ping Pong mechanism (Waxman & Strominger, 1983). Also probably pertinent was the final realization that the kinetics of protection by peptide substrates of DD-peptidases from inactivation by β-lactam antibiotics was most likely competitive rather than noncompetitive in nature (Frere & Joris, 1985). These findings tend to constrict rather than expand one's conception of the dimensions of these active sites.

It is important that this uncertainty about the reality of the mechanism of Scheme V be resolved, because otherwise any attempts to understand the structure–activity relationships involved in the interactions of these enzymes and their substrates may be seriously compromised. This of course has implications for future β-lactam antibiotic design. Our suspicions as to the validity of Scheme V, in our particular case at least, were aroused when we found that D-phenylalanine at concentrations up to 30 mM (when, according to the dissociation constant K_1 of Table II, the enzyme should be essentially all present as EA) had no effect on the rate of hydrolysis of benzylpenicillin catalyzed by the P99 β-lactamase. This lack of interaction seemed possible, but rather unlikely. In view of all of the above we felt it necessary to more stringently test Scheme V. The results of this investigation are described in the accompanying papers (Pazhanisamy & Pratt, 1989a,b), where it is in fact concluded that Scheme V cannot be correct and a new mechanism is proposed.

Irrespective of the interpretation of the kinetics however, it is clearly significant that a β-lactamase catalyzes acyl transfer from a depsipeptide to a specific amino acid acceptor with the same curious kinetics as does a DD-peptidase. This provides further strong evidence for the proposition that β-lactamases have indeed evolved from DD-transpeptidases and, further, that the differences between their active sites may not be very great.

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β-Lactamase-Catalyzed Aminolysis of Depsipeptides: Proof of the Nonexistence of a Specific D-Phenylalanine/Enzyme Complex by Double-Label Isotope Trapping†

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ABSTRACT: The steady-state kinetics of the *Enterobacter cloacae* P99 β-lactamase-catalyzed aminolysis of the depsipeptide *m*-[[(phenylacetyl)glycyl]oxy]benzoic acid by D-phenylalanine were consistent with an ordered sequential mechanism with D-phenylalanine binding first [Pazhanisamy, S., Govardhan, C. P., & Pratt, R. F. (1989) *Biochemistry* (first of three papers in this issue)]. In terms of this mechanism, the kinetics data required that in 20 mM MOPS buffer, pH 7.5, the dissociation constant of the initially formed enzyme/D-phenylalanine complex be around 1.3 mM; at pH 9.0 in 0.1 M carbonate buffer, the complex should be somewhat more stable. Attempts to detect this complex in a binary mixture by spectroscopic methods (fluorescence, circular dichroic, and nuclear magnetic resonance spectra) failed. Kinetic methods were also unsuccessful—the presence of 20 mM D-phenylalanine did not appear to affect β-lactamase activity nor inhibition of the enzyme by phenylmethanesulfonyl fluoride, phenylboronic acid, or (3-dansylamidophenyl)boronic acid. Equilibrium dialysis experiments appeared to indicate that the dissociation constant of any binary enzyme/D-phenylalanine complex must be somewhat higher than the kinetics allowed (>2 mM). Since the kinetics also required that, at high depsipeptide concentrations, and again with the assumption of the ordered sequential mechanism, the reaction of the enzyme/D-phenylalanine complex to aminolysis products be faster than its reversion to enzyme and D-phenylalanine, a double-label isotope-trapping experiment was performed. The results of this experiment, which employed [U-^{14}C]-D-phenylalanine in the pulse and [2-^{3}H]-D-phenylalanine in the chase, unambiguously demonstrated that an enzyme/D-phenylalanine complex with the properties required by the steady-state kinetics cannot exist under the relevant conditions. Thus the simple ordered sequential kinetic mechanism cannot be correct, and a more complex reaction scheme must be sought.

The steady-state kinetics of the *Enterobacter cloacae* P99 β-lactamase-catalyzed aminolysis of the depsipeptide **1** by D-phenylalanine have been shown to be consistent with only one simple BiBi mechanism, that of Scheme I (Pazhanisamy et al., 1989). As written in Scheme I, D represents the depsipeptide, A the amino acid (here D-phenylalanine), and Q the

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