pH 7.5. This result therefore supports all of the negative indications from the other experiments described. It follows directly that the kinetic mechanism of Scheme I cannot be correct. This, taken with the conclusions from the steady-state kinetic data (Pazhanisamy et al., 1989), means that no simple BiBi mechanism for the aminolysis reaction can explain the data, and thus, more complex schemes need to be considered. This is done in the following paper (Pazhanisamy & Pratt, 1989).

ACKNOWLEDGMENTS

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β-Lactamase-Catalyzed Aminolysis of Depsipeptides: Peptide Inhibition and a New Kinetic Mechanism†

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ABSTRACT: The aminolysis of the depsipeptide *m*-[[(phenylacetyl)glycyl]oxy]benzoic acid (**1**) by D-phenylalanine, catalyzed by the β-lactamase of *Enterobacter cloacae* P99, is inhibited by the product of the reaction, (phenylacetyl)glycyl-D-phenylalanine (**2**), by the peptide analogue of **1**, *m*-[(phenylacetyl)-glycinamido]benzoic acid (**3**), and by (3-dansylamidophenyl)boronic acid. Analysis of the steady-state kinetics of the effect of **2** and **3** on the reaction indicated that both a competitive binding mode and a noncompetitive binding mode existed for each peptide. Thus, there probably are two distinct binding sites (sites 1 and 2) that **2** and **3**, and by implication **1**, are able to simultaneously occupy on the enzyme surface. Given this information, it was possible to devise a new kinetic mechanism for the aminolysis reaction which yielded the experimentally observed empirical rate equation [Pazhanisamy, S., Govardhan, C. P., & Pratt, R. F. (1989) *Biochemistry* (first of three papers in this issue)] but did not involve initial binding of D-phenylalanine to the free enzyme, which has been shown not to occur [Pazhanisamy, S., & Pratt, R. F. (1989) *Biochemistry* (second of three papers in this issue)]. The mechanism requires two different 1:1 enzyme/**1** complexes, only one of which leads to the hydrolysis and aminolysis reactions (**1** in site 1), and a 1:2 enzyme/**1** complex (**1** in both sites), which leads only to hydrolysis. The dansyl boronate inhibits by binding competitively with **1** in site 1. It is suggested that this scheme also applies to the analogous transpeptidase reactions of small model peptides catalyzed by the bacterial cell wall DD-peptidases, where similar steady-state kinetics have been observed. The relevance of these results to the evolution of β-lactamases is also discussed.

The previous papers in this series (Pazhanisamy et al., 1989; Pazhanisamy & Pratt, 1989) demonstrated that the kinetic mechanism of the aminolysis of the depsipeptide **1** by D-phenylalanine (eq 1), catalyzed by the β-lactamase of *Enterobacter cloacae* P99, does not involve an ordered sequence with the D-phenylalanine binding to the enzyme first, which is the simplest mechanism compatible with the steady-state kinetics. In this paper we describe the steady-state kinetics of inhibition of this reaction by the peptide product, **2**, by the substrate analogue **3**, and by (3-dansylamidophenyl)boronic

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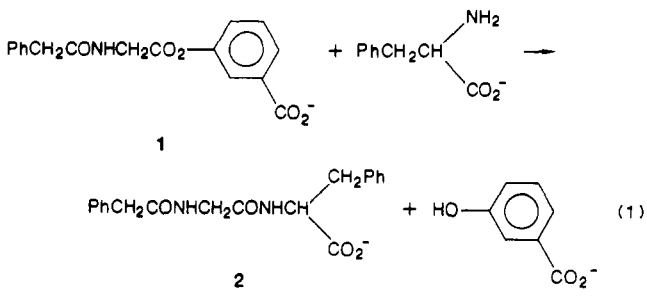
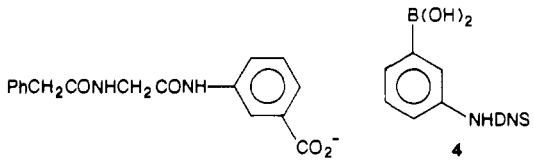


1

2

(1)

acid (**4**). The patterns of inhibition observed prove that the



3 4

region of the active site of the β-lactamase must be able to accommodate two peptides or depsipeptides analogous to **1**–**3**, simultaneously. On the basis of this discovery, a new mechanism for the aminolysis is proposed (Scheme III), which is kinetically equivalent to the one referred to above but which does not require the binding of the amine (D-phenylalanine) to free enzyme. It is likely that this mechanism also applies to the analogous reaction catalyzed by DD-peptidases, where there has been difficulty in reconciling the kinetics with other data (Frere & Joris, 1985; Nguyen-Disteche et al., 1986). The relevance of the new scheme to β-lactamase evolution is also discussed.

Experimental Procedures

m-[[(Phenylacetyl)glycyl]oxy]benzoic acid (**1**), (phenylacetyl)glycyl-D-phenylalanine (**2**), and *m*-[(phenylacetyl)-glycinamido]benzoic acid (**3**) were prepared as described previously (Govardhan & Pratt, 1987). (3-Dansylamidophenyl)boronic acid (**4**) was prepared as part of the present work (Pazhanisamy et al., 1989). The β-lactamase of *Enterobacter cloacae* P99 was, as for our previous studies, obtained from the Centre for Applied Microbiology and Research, Porton Down, England.

Instrumentation and general analytical and steady-state kinetic methods were as previous described (Pazhanisamy et al., 1989; Pazhanisamy & Pratt, 1989). Steady-state parameters were determined by the method of Wilkinson (1961), and all other least-squares data analysis was performed by means of the computer program of Johnson et al. (1976).

Initial Rates of Depsipeptide (**1**) *Hydrolysis in the Presence of Peptides* **2** *and* **3** *and the Boronate* **4**. Spectrophotometric initial rates at 300 nm were measured on addition of 10 μL of a P99 β-lactamase solution (final concentration 15 nM) to 0.8-mL samples of MOPS buffer, pH 7.5, 25 °C, containing fixed concentrations of **2** (0–15 mM) and varied concentrations of **1** (0.2–3 mM). Thus, the effect of **2** on the steady-state parameters of hydrolysis of **1** could be determined. The effects of **3** on these parameters were determined similarly, except that the reactions were followed at 307 nm (because of strong absorption at 300 nm by **3**) and the concentrations of **1** ranged to 4.5 mM. The steady-state kinetics of inhibition by **4** of the hydrolysis of **1** were also similarly obtained spectrophotometrically at 300 nm; the concentrations of **1** employed ranged to 10 mM while those of **4** were varied up to 7.5 μM.

Measurement of Ratio (V_H/V_A) *of Initial Rates of Hydrolysis of* **1** *to Initial Rates of Aminolysis by* D-*Phenylalanine in the Presence of Peptides* **2** *and* **3**. As described previously (Pazhanisamy et al., 1989), the rate of the total reaction (hydrolysis plus aminolysis) could be determined spectrophotometrically, while that of aminolysis alone could be determined polarimetrically. Hence, the V_H/V_A ratio could be obtained. These experiments were carried out at fixed concentrations of depsipeptide (1.0 mM) and D-phenylalanine (3.75 mM) while the concentrations of the peptides were varied (0–15 mM).

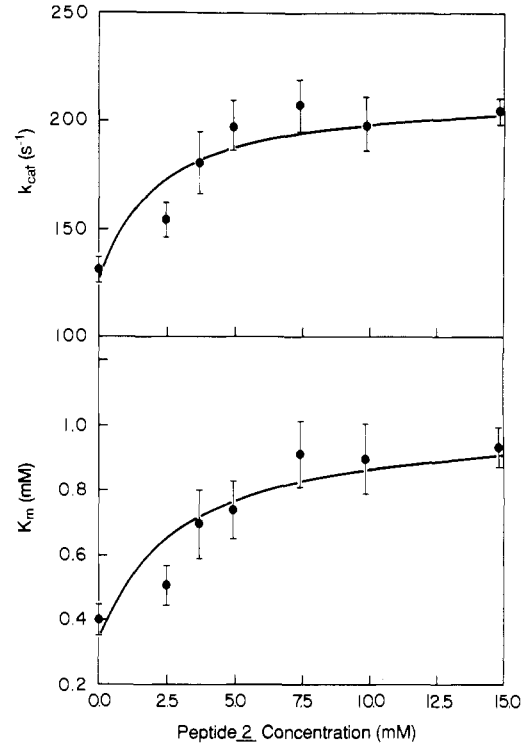


FIGURE 1: Effect of the peptide **2** on the steady-state kinetic parameters for the P99 β-lactamase- (15 nM) catalyzed hydrolysis of the depsipeptide **1** (3 mM). The points are experimental, and the lines are calculated from eq 2 as described in the text.

Inhibition by the Boronate **4** *of Aminolysis of* **1** *by* D-*Phenylalanine*. Initial rates of the total reaction were measured spectrophotometrically as described above. In one set of experiments the rates were determined at fixed concentrations of depsipeptide (2.25 and 4.13 mM) and dansyl boronate (0–7.5 μM) and varying D-phenylalanine concentrations (0–75 mM). Additional measurements were made under conditions of saturating D-phenylalanine where the depsipeptide concentrations were 1.0–4.9 mM and those of the dansyl boronate were 0–21 μM. The latter experiments were possible at achievable D-phenylalanine concentrations because the dansyl boronate lowers the apparent K_M of D-phenylalanine (see Results).

Results

Effect of Product Peptide **2** *on Depsipeptide Hydrolysis*. Preliminary experiments suggested that the hydrolysis of **1** (5 mM) was *accelerated* by the presence of **2** (10 mM). The D-amino acid oxidase/peroxidase coupled assay (Pazhanisamy & Pratt, 1989) showed that **2** was not contaminated with D-phenylalanine. The peptide **2** is not a substrate of the P99 β-lactamase (Govardhan & Pratt, 1987), and hence, D-phenylalanine could not arise from that source either. The acceleration was thus deemed to be nonartifactual and was pursued further. In order to do this, the apparent K_M and k_{cat} for depsipeptide hydrolysis were obtained at various fixed concentrations of **2**. As shown in Figure 1, both K_M and k_{cat}

Scheme I

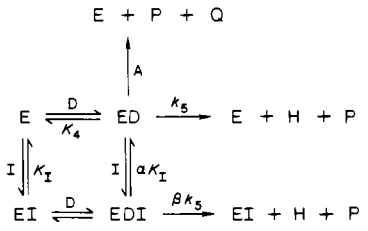


Table I: Steady-State Kinetic Parameters Derived from P99 β-Lactamase-Catalyzed Hydrolysis and Aminolysis by D-Phenylalanine of Depsipeptide **1** in the Absence and Presence of Peptides **2** and **3**

	hydrolysis		aminolysis	
		2	**2**	**3**
parameter[b]	Scheme III, eq 3–6[a]	Scheme I,[c] eq 2, Figure 1	Scheme V,[c] eq 9, Figure 2	Scheme V, eq 9, Figure 3
K_I (mM)	5.5 ± 2.0		4.0	4.0
k_3/K_2 (s^{-1} M^{-1})[f]	(1.1 ± 0.3) × 10^5		0.8 × 10^5	(0.76 ± 0.25) × 10^5
k_4 (s^{-1} M^{-1})	(3.0 ± 0.3) × 10^5		3.0 × 10^5	3.0 × 10^5
k_{-4} (s^{-1})	20 ± 60			
K_4 (mM)	0.42 ± 0.13	0.34 ± 0.08	0.4	0.4
k_5 (s^{-1})	105 ± 19	126 ± 9	105	105
K_I (mM)		1.0 ± 0.7	0.7 ± 0.3	2.5 ± 1.5
α		2.8 ± 0.7	2.8 ± 1.2	1.0
β		1.7 ± 0.3	1.7 ± 0.3	1.0
γ		24 ± 14[d]	11.8 ± 2.4[e]	2.3 ± 1.0

[a] The data from Pazhanisamy et al. (1989) were fitted. [b] Parameters without error limits were constrained to the given values. [c] The values reported here assume $\gamma = \infty$, except where noted. [d] With K_I, k_3/K_2, k_4, K_4, and k_5 fixed, optimization yielded this value of γ with K_I = 1.56 ± 0.47 mM, α = 3.3 ± 0.6, and β = 2.2 ± 0.3. [e] All other values as in footnote *d*. [f] As described in the text, lower limits to k_3 and K_2 are 1500 s^{-1} and 15 mM, respectively.

increased, and appeared to saturate, with increasing concentrations of **2**. The activation of hydrolysis called for a productive ternary EDI complex, while the increase in K_M requires the presence of a binary EI complex. Hence, a minimal mechanism accommodating these observations and involving a noncompetitive interaction between the depsipeptide D (**1**) and the peptide I (**2**) is that of Scheme I, where P and H represent the hydrolysis products, *m*-hydroxybenzoate and (phenylacetyl)glycine, respectively; Q represents the aminolysis product, **2**, obtained when D-phenylalanine (A) is present (see below). It should be noted here, however, and we thank a reviewer for pointing this out, that if the binding of D is not treated as a fast equilibrium [i.e., $k_5 > k_{-4}$, as is likely (see below)], the existence of EI is not required to explain the effects of **2** on the hydrolysis of **1**. We add it here however since it is needed to interpret the results obtained with peptide **3**, as described in the next sections. The inclusion of the nonequilibrium binding situation into Scheme I is not necessary at this stage.

The experimental data of Figure 1 could be fitted to Scheme I via derived eq 2, whose parameters are defined by the scheme. The values for these parameters yielding the best fit to eq 2 are given in Table I. The solid lines in Figure 1 were then calculated from these values.

$$V/E_0 = \frac{\dfrac{k_5(1 + \beta[I]/\alpha K_I)[D]}{1 + [I]/\alpha K_I}}{\dfrac{K_4(1 + [I]/K_I)}{1 + [I]/\alpha K_I} + [D]} \qquad (2)$$

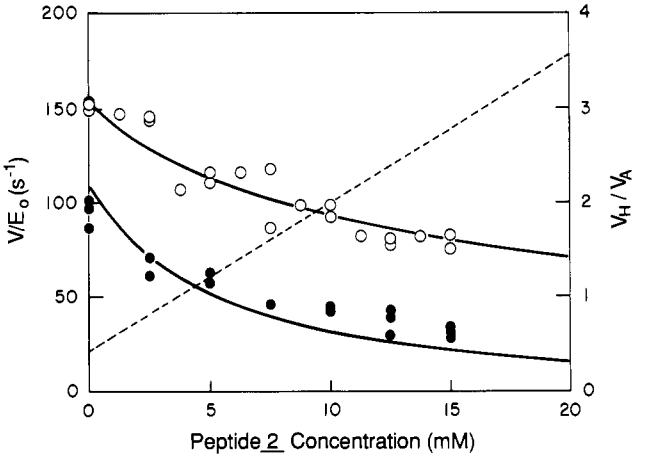


FIGURE 2: Effects of the peptide **2** on the initial rates of the total reaction (○) and of aminolysis by D-phenylalanine (3.75 mM) (●) of the depsipeptide **1** (1.0 mM), catalyzed by the P99 β-lactamase (15 nM). The points are experimental, and the lines are calculated from eq 9 as described in the text. Also shown (– –) is the ratio of the rates (V_H/V_A), calculated from these lines, as a function of peptide concentration.

Inhibition of Depsipeptide Hydrolysis by Substrate Analogue Peptide **3**. In the presence of this peptide, which, like **2**, is not a substrate of the P99 β-lactamase (Govardhan & Pratt, 1987), only inhibition of the hydrolysis of **1** was observed. Since k_{cat} was not affected but K_M rose linearly with the concentration of **2** to 15 mM (data not shown), the inhibition appeared to be purely competitive, with K_I = 5.7 ± 2.7 mM. Although the simplest explanation of this result is that EDI does not exist when I is **3**, i.e., α is very large, an alternative possibility is that $\beta \simeq 1$, i.e., that the ED and EDI complexes are comparably productive; the latter case would still require $\alpha \geq 5$ however to explain the linearity of K_M with peptide concentration up to 15 mM.

Effect of Peptides **2** *and* **3** *on Ratio of Hydrolysis to Aminolysis Rates* (V_H/V_A). In the preceding paper of this series (Pazhanisamy & Pratt, 1989) it was established that the amine (A), D-phenylalanine, did not bind to free enzyme, and hence must presumably react with some sort of ED complex in aminolysis. The hydrolysis data above suggest that, in the presence of **2**, an EDI complex is formed which can break down, yielding the depsipeptide hydrolysis products. If this complex could not lead to depsipeptide aminolysis (or, at least, could aminolyze at a different rate than the productive ED complex), then the V_H/V_A ratio would increase (or at least change) with the concentration of **2**. Under circumstances of competing hydrolysis and aminolysis the rate ratio V_H/V_A is a very sensitive indicator of kinetic mechanism (Frere, 1973; Pazhanisamy et al., 1989). As shown in Figure 2, V_H/V_A increases linearly with the concentration of **2**, indicating that the presence of **2** favors hydrolysis rather than aminolysis by D-phenylalanine; one interpretation of this would be that EDI can lead to depsipeptide hydrolysis but not aminolysis. These data could be accommodated, for example, by Scheme I where only ED leads to aminolysis, giving rise to P and the peptide product Q, which of course would be **2**.

The situation with respect to **3**, although originally appearing simpler than that of **2** when only hydrolysis was considered, became more complicated when aminolysis was examined, since it showed that **3**, like **2**, had a significant effect on the V_H/V_A ratio (Figure 3). This suggested, as for **2**, that on increase of the concentration of **3** an EDI complex forms which can undergo depsipeptide hydrolysis much more readily than aminolysis by D-phenylalanine. The observed change in

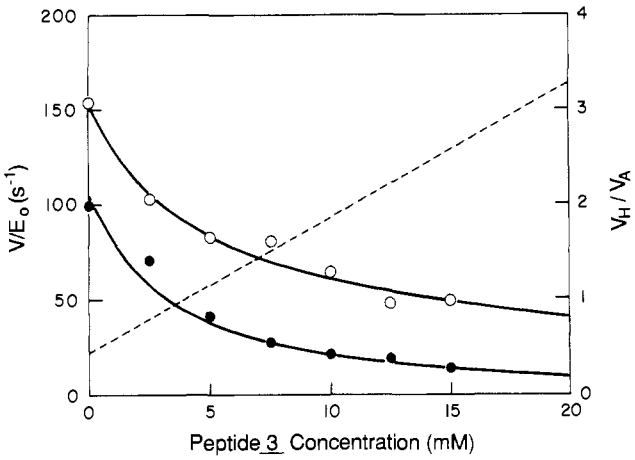
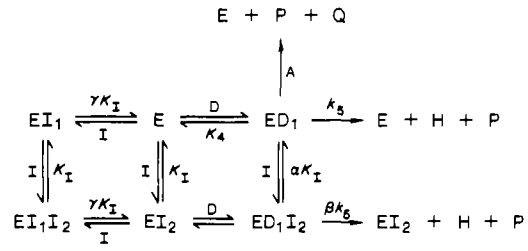


FIGURE 3: Effects of the peptide **3** on the initial rates of the total reaction (○) and of aminolysis by D-phenylalanine (3.75 mM) (●) of the depsipeptide **1** (1.0 mM), catalyzed by the P99 β-lactamase (15 nM). The points are experimental, and the lines are calculated from eq 9 as described in the text. Also shown (– –) is the ratio of the rates (V_H/V_A), calculated from these lines, as a function of peptide concentration.

Scheme II



V_H/V_A, from 0.4 in the absence of **3** to nearly 2.7 in the presence of 15 mM **3**, must reflect a comparable change in the amounts of ED and EDI in solution. Yet the effect of **3** on the hydrolysis reaction alone, where strictly competitive inhibition was observed, could be interpreted most simply (above) to indicate that very little EDI can be present. This dilemma can be resolved only by expansion of Scheme I, for example to Scheme II.

In Scheme II it is proposed that there are two peptide binding sites, one of which is probably also the depsipeptide binding site. The latter seems reasonable given the structural similarity between **1** and the peptides. The depsipeptide **1** binds preferentially to the first of the two sites, and hence, the productive ED complex is denoted ED_1. The peptide **3**, which is close to isosteric with **1**, also binds to this site, forming EI_1. The results from the hydrolysis of **1** in the presence of **2** require the existence of ED_1I_2, as do those from the aminolysis of **1** in the presence of **3**, as discussed above. In order to retain the strictly competitive inhibition of hydrolysis by **3**, EI_2 and EI_1I_2 complexes are added whose dissociation constants are arranged in symmetrical fashion as shown in the scheme. For the latter reason also it is necessary to impose the restriction $\alpha \approx \beta \approx 1.0$ for **3**. The observed competitive inhibition constant for **3** in depsipeptide hydrolysis (5.7 mM) would then correspond to γK_I in terms of Scheme II. The symmetry of Scheme II with respect to **3** binding, only broken by superimposition of the aminolysis pathway, implies little interaction between **1** and **3** in the two binding sites. The symmetry is also broken by **2** since in this case both α and β must be significantly different from unity. Apparently, according to the interpretation provided by Scheme II, the binding of **2** to ED_1 is weaker than that to E and the hydrolytic reactivity of ED_1I_2 is greater than that of ED_1, both implying interaction

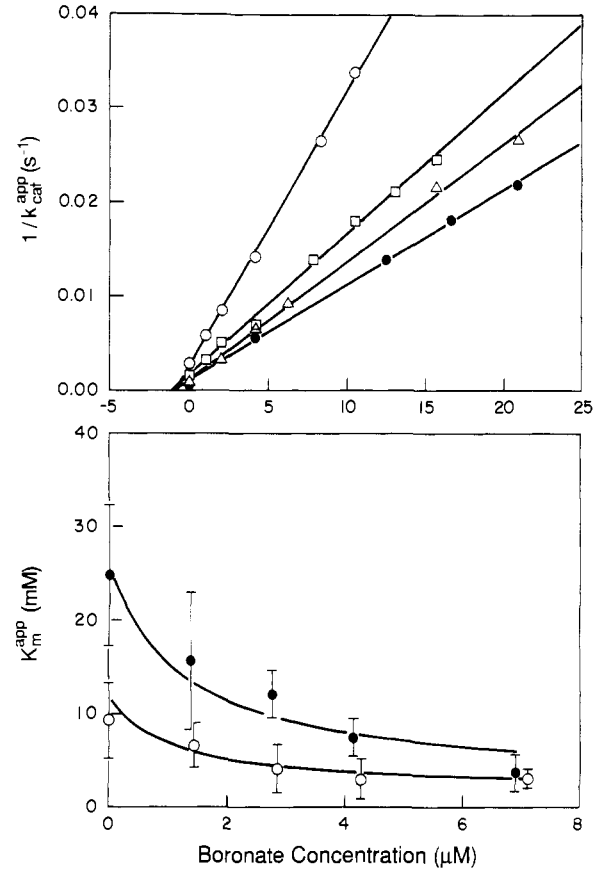


FIGURE 4: Effect of the dansyl boronate **4** on k_{cat}^{app} (shown as a reciprocal plot) and K_M^{app} for the aminolysis of the depsipeptide **1** by D-phenylalanine in the presence of the P99 β-lactamase (15 nM). The depsipeptide concentrations in the lower panel were 2.25 (○) and 4.13 mM (●) while those in the upper panel were 1.23 (○), 2.45 (□), 3.68, (△) and 4.90 mM (●). The points are experimental, and the lines are calculated from eq 7 and 8 as described in the text.

between bound **1** and **2**. The existence of significant binding of **2** to site 1, i.e., the existence of EI_1 and EI_1I_2 when I is **2**, is not required, but also not precluded, by the data presented (see below for further discussion relating to this point).

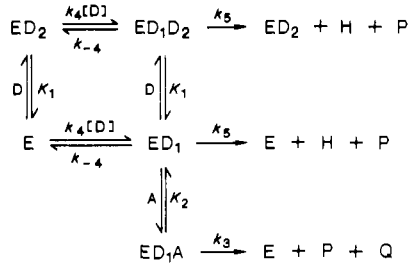
Inhibition of Hydrolysis and Aminolysis by the Dansyl Boronate **4**. As indicated in the previous paper of this series (Pazhanisamy & Pratt, 1989), fluorescence titration shows the dansyl boronate **4** forms a complex with the P99 β-lactamase with a dissociation constant of 2.0 ± 0.5 μM. The boronate appears also to be a purely competitive inhibitor of **1** hydrolysis with an inhibition constant of 2.5 ± 1.0 μM.

Aminolysis of **1** by D-phenylalanine is also affected by **4**. Figure 4 shows that k_{cat}^{app} and K_M^{app}, the parameters describing the response of the total rate at fixed concentrations of **1** to increasing D-phenylalanine concentrations (Pazhanisamy et al., 1989), both decrease with increasing concentrations of **4**; a linear reciprocal plot is obtained in the case of k_{cat}^{app}.

SYNTHESIS

The aim of this section is to assemble the diverse elements of the aminolysis reaction, described in this and the previous papers of this series (Pazhanisamy et al., 1989; Pazhanisamy & Pratt, 1989), into a kinetic mechanism that accommodates all of them. The present paper describes the properties of the peptides **2** and **3** as modulators of the hydrolysis and aminolysis of **1**, catalyzed by the P99 β-lactamase, eq 1. It should perhaps be noted again that **2** is the product of the aminolysis of **1** by D-phenylalanine and that **3** is the peptide analogue of **1**; both peptides therefore might be expected to directly compete with

Scheme III



1 in binding to the enzyme. The major finding of the experiments reported above however is that it is necessary to postulate *two* separate peptide binding sites, which can be occupied simultaneously. Occupation of one of these (site 1) is apparently competitive with the binding of **1** in the hydrolysis (and probably aminolysis) reaction, while binding to the other site (site 2) is noncompetitive with respect to hydrolysis. These findings can be most simply accommodated, qualitatively and quantitatively, by Scheme II.

It is necessary now to impose on Scheme II the constraints derived from the kinetic studies of the hydrolysis and aminolysis reactions in the absence of peptide inhibitors. Scheme II, as it stands, is insufficient, since it does not include all of the required features, viz., separate hydrolysis and aminolysis pathways, two enzyme forms which can bind **1**, competition for an enzyme form between A and D, and no binding of D-phenylalanine to the free enzyme (Pazhanisamy et al., 1989; Pazhanisamy & Pratt, 1989). Scheme II, however, does require two separate binding sites for **2** and **3**, and thus we may suppose that two sites of binding of **1** also exist. If only one of these is productive with respect to both hydrolysis and aminolysis, Scheme III is immediately indicated. Scheme III *does* have the necessary features referred to above. The symmetry of the binding and rate constants of Scheme III is necessary to explain the simple Michaelis–Menten hydrolysis kinetics—any significant asymmetry would have been detected there and led to Scheme III much earlier. This symmetry is supported by the indication of equivalent binding, presumably to the same site, of the nearly isosteric (to **1**) **3** to E and EI_2 (Scheme II). In neither case, apparently, is there any interaction between the two bound molecules, in ED_1D_2 on one hand and EI_1I_2 on the other.

A steady-state rate equation of the required form can be derived from Scheme III, provided that it is assumed that the binding of **1** to the reactive site 1 is not at equilibrium with respect to subsequent aminolysis, i.e., $k_3/K_2 \geq k_{-4}$. Since, in the absence of this assumption, the binding of **1** to site 2 may be either fast or slow and not yield an equation of the correct form, the simplest scheme fitting the kinetic data leaves this binding of **1** to E and ED_1 as fast equilibria. Under these conditions, eq 3 may be derived for the rate of the total reaction, V_T. From eq 3 it follows that the previously used diagnostic kinetic parameters V_{MAX}^{app}, K_M^{app}, and V_H/V_A are given in terms of Scheme III by eq 4–6. These will be of the

$$V_T/E_0 = \{k_5K_1K_2[D] + k_5K_2[D]^2 + k_3K_1[A][D]\}/ \{K_1K_2(k_{-4} + k_5)/k_4 + K_2[K_1 + (k_{-4} + k_5)/k_4][D] + k_2[D]^2 + (k_3/k_4)K_1[A] + K_1[A][D]\} \quad (3)$$

$$V_{MAX}^{app}/E_0 = k_3k_4[D]/(k_3 + k_4[D]) \quad (4)$$

$$K_M^{app} = K_2(1 + [D]/K_1)\frac{k_4[D]}{k_3 + k_4[D]}\frac{(k_{-4} + k_5)/k_4 + [D]}{[D]} \quad (5)$$

$$V_H/V_A = k_5K_2(1 + [D]/K_1)/k_3[A] \quad (6)$$

form required by experiment if $k_3 \gg k_4[D]$ at all [D]. If the latter were true, the term in [A][D] disappears from the denominator of eq 3, which is then of the same mathematical form as the empirical kinetic equation [eq 10 in Pazhanisamy et al. (1989)], and thus, Scheme III of the present paper fits the experimental data as well as Scheme V of the earlier paper. K_M^H, the apparent dissociation constant of D for hydrolysis alone, is given by $(k_{-4} + k_5)/k_4$.

Other variations of Scheme III, also incorporating the existence of an ED_1D_2 complex, were considered, but we are unaware of any other that fits the data as well, without more arbitrary asumptions. In particular, the variant where the complex ED_2 is required for the aminolysis reaction while hydrolysis proceeds through ED_1 and ED_1D_2 was ruled out, since this should give more complex kinetics than observed. Similarly, no scheme involving enzyme isomerization to yield different ED species could be found to fit the data. We conclude that Scheme III is the simplest kinetic mechanism that accommodates all of the information at hand, and therefore, all further discussion will assume that it represents the true situation.

Values for the kinetic parameters of Scheme III, derived from application of eq 3 to the kinetic data at pH 7.5 (Pazhanisamy et al., 1989), are reported in Table I. In terms of Scheme III, the productive binding of the depsipeptide to form ED_1 is too slow to represent only a diffusion-controlled combination process and may thus include rate-limiting conformational accommodation by the enzyme. Comparably slow binding of β-lactam substrates has not been observed, but since k_{cat}/K_m values for good β-lactam substrates of this enzyme are commonly in excess of 10^6 s^{-1} M^{-1} (Govardhan & Pratt, 1987; Galleni & Frere, 1988; Galleni et al., 1988a), the binding of such substrates must be faster than that of **1**. Our attempts to observe a lag in appearance of *m*-hydroxybenzoate during the P99 β-lactamase-catalyzed hydrolysis of **1** using spectrophotometric stopped-flow methods were unsuccessful, as in fact was predicted by computer simulations using the parameters of Table I (the combination of a small time constant and a small amplitude of the lag phase precluded its observation with the Durrum D110 apparatus).

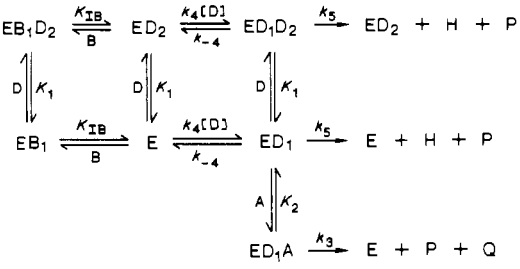
The data of Table I also indicate that the binding of D-phenylalanine to ED_1, to form the complex productive in aminolysis, is weak. As noted above, the linearity of V_{MAX}^{app} with [D] requires $k_3 > k_4[D]$ at all [D], which places a lower limit of 1500 s^{-1} on k_3. The k_3/K_2 value from Table I then gives a lower limit of 15 mM for K_2. D-Phenylalanine can bind only weakly to the free enzyme also (Pazhanisamy & Pratt, 1989).

Supporting evidence for Scheme III was obtained from the dansyl boronate **4** inhibition of aminolysis. Although **4** appears to be purely competitive with respect to **1** as an inhibitor of hydrolysis, it inhibits aminolysis in a mixed fashion with respect to A, i.e., k_{cat}^{app} and K_M^{app} are both diminished (Figure 4). This observation requires that **4** and D-phenylalanine bind to separate enzyme forms (K_M^{app} effect) and that the rate-determining step in aminolysis occurs before D-phenylalanine binding (k_{cat}^{app} effect), as would be provided by Scheme IV, an extension of Scheme III, where B represents **4**. A steady-state rate equation for Scheme IV, with the same assumption as for the kinetics in the absence of inhibitor, namely, $k_3 \gg k_4[D]$, yields eq 7 and 8 for k_{cat}^{app} and K_M^{app}, which indeed describe a decrease

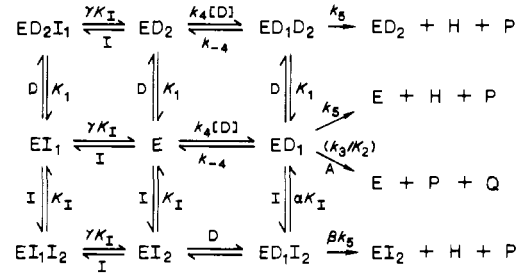
$$k_{cat}^{app} = k_4[D]/(1 + [B]/K_{IB}) \quad (7)$$

$$K_M^{app} = [k_4K_2(1 + [D]/K_1)/k_3][(k_{-4} + k_5)/k_4 + [D]/(1 + [B]/K_{IB})] \quad (8)$$

Scheme IV



Scheme V



in both parameters as [B] increases. The slope of reciprocal plots of $1/k_{cat}^{app}$ vs [B] at fixed [D] is given by eq 7 as $1/k_4K_{IB}[D]$. A plot of this slope vs $1/[D]$ (not shown) can be accommodated by a straight line through the origin as therefore expected. This plot yields a K_{IB} value of 1.1 ± 0.2 μM, while the [B] axis intercepts of the former plots yield an average K_{IB} value of 1.0 ± 0.9 μM. These details eliminate two other possible binding schemes for **4**, one where **4** binds to site 2, i.e., to E and ED_1, which predicts that K_M^{app} should be independent of the concentration of the inhibitor, and another where **4** binds only to E, i.e., not to ED_2, which predicts that the plot of slope vs $1/[D]$ be distinctly nonlinear in the region of [D] covered. The K_M^{app} data of Figure 4 could be fitted (solid lines) by employment of a K_{IB} value of 0.9 ± 0.5 μM and the other parameters of Table I.

The effects of **4** on the total reaction therefore appear consistent with Scheme III and require that **4** binds in site 1. The latter seems intuitively reasonable since **4**, as a boronate, would be expected to covalently bind to the (β-lactamase) active site serine hydroxyl group, which, one would imagine, would be also required for the hydrolysis and aminolysis of **1** (see below for further discussion on this point).

If Scheme III is the correct reaction pathway for the reactions of **1** in the absence of the peptide inhibitors, then the complete scheme incorporating the inhibitor binding must be that of Scheme V. The symmetry of the the scheme is necessary to explain the (relatively) simple kinetics observed (see Results). Although some of the equalities of rate or binding constants shown in Scheme V may not be exactly so, making them otherwise does not improve the fit to the data currently collected, to a statistically significant degree. The total rate V_T, according to Scheme V, is given by

$$V_T/E_0 = HJ/(JL + MN) \quad (9)$$

where

$$H = \alpha k_5 K_I(K_1 + [D]) + \beta k_5 K_1[I] + \alpha(k_3/K_2)K_1K_I[A]$$

$$J = \gamma K_I(K_1K_I + K_I[D] + K_1[I])[D]$$

$$L = \alpha K_1K_I + \alpha K_I[D] + K_1[I] + \alpha K_1K_I[A]/K_2$$

$$M = J/[D] + K_I[I][D] + K_1K_I[I] + K_1[I]^2$$

$$N = \alpha K_I(K_1 + [D])[(k_{-4} + k_5)/k_4] + K_1[I][(\alpha k_{-4} + \beta k_5)/k_4] + \alpha K_1K_I[A](k_3/k_4K_2)$$

Application of this equation to the data of Figures 2 and 3 gave the fits represented by the solid lines in those diagrams and the numerical values for the various parameters given in Table I. The agreement between the parameters of Table I, derived from the effects of the peptide **2** on the hydrolysis rates on one hand and from the total reaction on the other, appears convincing. As described in detail under Results, it is necessary to permit the peptide **3** to bind to both sites, with no interaction between two bound molecules of **3** or between **3** and **1**. The binding of **2** to site 1, although not necessary to fit the hydrolysis data (Figure 1, Scheme II), does allow a statistically better (likelihood ratio test; LR = 9.4, 46 degrees of freedom, >99% confidence) fit to the data of Figure 3. Parameters for fits with and without site 1 binding are reported in Table I. The solid lines in Figure 2 are those that include site 1 binding. At any event, it is clear that **2** binds preferentially to site 2 and that its binding to site 1 is much weaker than that of **3** to that site.

Discussion

Perhaps the most important conclusion that can be derived from this identification of Scheme III as best representing the kinetic mechanism for the P99 β-lactamase-catalysed hydrolysis and aminolysis of the depsipeptide **1**, and reinforced by the peptide inhibition studies culminating in Scheme V, is that of the existence of an extended peptide binding area on the surface of the enzyme. Although the spatial relationship between the depsipeptide and peptide binding sites identified in this work, and the β-lactam binding site, has not yet been explicitly defined, there are indications that the two overlap, at least to some degree. For example, 6β-bromopenicillanate and the boronate **4**, both of which almost certainly covalently bind to the Ser-64 hydroxyl group (Joris et al., 1985; Beesley et al., 1983; Crompton et al., 1988), inhibit depsipeptide hydrolysis and aminolysis, and the hydrolysis of **1**, like that of β-lactams, appears to proceed via acylation of the enzyme (Govardhan & Pratt, 1987), presumably at the same serine. [The amino acid numbering scheme used in this paper is that of Galleni et al. (1988b).]

The extended binding site can apparently accommodate two peptides/depsipeptides of the size of **1**–**3** and the amine acceptor. Binding of the latter, however weak, seems necessary to explain the specificity of the reaction with respect to amine structure (Pazhanisamy et al., 1989). Since there is no evidence for a quaternary EADD complex, it seems likely that the amine binding site overlaps the second, not chemically reactive, (depsi-) peptide site, site 2. It may be, for example, that D-phenylalanine binds in the same region of the active site as the C-terminal D-phenylalanine residue of **2**. The latter would then bind "tail to tail" with respect to **1**. It is possible that the carboxylate of the favored D-amino acid acyl acceptor would interact with one conserved lysine of the β-lactamase active site (perhaps Lys-67) while the carboxylate of **1**, like that of β-lactam substrates, would interact with the other [Lys-315 according to the sequence alignment of Joris et al. (1988)]. The reactive amine group of the D-amino acid, if it bound in the ammonium form, could interact with the conserved carboxylate ion [probably Asp-217 in this case (Joris et al., 1988)]. The importance of the C-terminal D-phenylalanyl residue to the observed effects of **2** on depsipeptide hydrolysis, which support its presence close to **1**, seems confirmed by our observation that the effects of *N*-acetyl-D-phenylalanine (73 mM) on the rates of hydrolysis of **1** are very

similar to those of **2**—inhibition at low (1.0 mM) concentration of **1** and acceleration at high (5.0 mM) concentration.

This situation is reminiscent of that existing during early studies of carboxypeptidase A, where the kinetics of hydrolysis of dipeptide substrates were found to be very complex. Eventually they were rationalized by a scheme analogous to Scheme III, but without the symmetry (Auld & Vallee, 1987). Only after more extended substrates, tri- and tetrapeptides, were investigated were simple kinetics achieved. Such a situation is expected, in general, when small substrates are used to probe the active site of an enzyme whose natural substrates are polymeric. Up until now of course, β-lactamases would not have been thought of in this regard.

Another important issue that is raised by the present work is that of the existence of an acyl-enzyme intermediate in the aminolysis reaction. Scheme III indicates that the pathways of hydrolysis and aminolysis of **1** diverge beyond ED_1. A previous investigation of the P99 β-lactamase-catalyzed hydrolysis of **1** indicated that the rate-determining step of the hydrolysis reaction under saturating conditions, with rate constant k_5 associated with it, involved acylation of the enzyme (Govardhan & Pratt, 1987). Thus ED_1 cannot be an acyl-enzyme common to the two pathways, and the question of the existence of an acyl-enzyme intermediate in aminolysis is moot. An acyl-enzyme certainly could form subsequent to a conformational change, or to amine binding, but there is at present no evidence one way or the other.

The relationship between our present findings and the mechanism of action of the DD-peptidases should also be discussed. As suggested in the first two papers of this series (Pazhanisamy et al., 1989; Pazhanisamy & Pratt, 1989), the kinetics of the P99 β-lactamase-catalyzed aminolysis of **1** by D-phenylalanine are probably the same as those of the aminolysis of *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine by various amino acids and peptides, catalyzed by the DD-peptidases of *Streptomyces* R61 (Frere et al., 1973) and *Streptomyces* K15 (Nguyen-Disteche et al., 1986). These latter enzymes represent the best studied and closest models of the bacterial cell wall transpeptidases. Although the kinetics in these cases have been interpreted in terms of an ordered reaction sequence with the amine binding first, the current result suggests that, as with the P99 β-lactamase, the kinetically equivalent Scheme III may well apply. There are in fact many observations in the work of Ghuysen, Frere, and co-workers which point in this direction. First, it is well-known that these DD-peptidases specifically bind and catalyze the reactions of quite large peptides as acyl donors and acceptors (Ghuysen et al., 1979; Frere & Joris, 1985), which clearly points to an extended binding site. To the same point, peptide amines have been shown to inhibit the aminolysis of *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine in a way that is best interpreted as demonstrating an additional binding site for the peptide amine, i.e., the existence of an EAAD species (Frere et al., 1973; Perkins et al., 1973; Ghuysen et al., 1974). Despite this however and despite the probable competitive nature of the interaction between peptide substrates and β-lactam antibiotics (Frere & Joris, 1985), specific amino acids and peptide amines which are efficient acyl group acceptors in the aminolysis do not affect the rate or equilibrium constant of β-lactam binding (Nieto et al., 1973; Fuad et al., 1976; Leyh-Bouille et al., 1986). D-Alanine, a good acyl group acceptor for the R61 enzyme, did not, at high concentration, affect the thermodynamics of thermal denaturation of the R61 enzyme (Nieto et al., 1973). These of course are precisely the same types of observations, implying a weak interaction between amines and the free enzymes, that led to our pursuit of Scheme III (Pazhanisamy et al., 1989; Pazhanisamy & Pratt, 1989). It thus seems likely that Scheme III will also apply to the DD-peptidases. This realization may resolve the problems of reconciliation of the DD-peptidase kinetics with other work on these enzymes (Frere & Joris, 1985; Nguyen-Disteche et al., 1986).

Finally, what do the present results contribute to the ongoing discussion about the evolution of β-lactamases from DD-peptidases, for which there is now so much evidence (Tipper & Strominger, 1965; Waxman & Strominger, 1983; Pratt & Govardhan, 1984; Kelly et al., 1986; Samraoui et al., 1986; Joris et al., 1988)? They clearly indicate that the P99 β-lactamase possesses an extended binding site, capable of accommodating two dipeptides at least and capable of catalyzing an acyl-transfer reaction that is completely analogous to that catalyzed by the DD-peptidases, except in the respect that the acyl donor must be an ester rather than an amide. That the binding site and so much of the catalytic machinery of the DD-peptidase are still present in the β-lactamase suggests that the great difference in amino acid sequence generally found between β-lactamases and DD-peptidases (Joris et al., 1988) may more reflect DD-peptidase evolution than β-lactamase evolution and that the change needed to convert a DD-peptidase into a β-lactamase may be rather minor and perhaps readily accomplished. It may require, for example, only some subtle relative motion between the elements of secondary structure to permit access of water to the acyl-enzyme (e.g., to produce a site for an occluded water molecule) which is necessary for acyl-enzyme hydrolysis and to restrict access of planar peptides to the general acid catalyst required for amide and β-lactam hydrolysis (Pratt et al., 1980; Pratt & Govardhan, 1984; Govardhan & Pratt, 1987). Such a change could conceivably be produced by a small number of amino acid mutations and thus, in principle, be readily achieved artifically by site-specific mutagenesis.

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Proteolytic Modification of Calcium-Dependent Protease 1 in Erythrocytes Treated with Ionomycin and Calcium†

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ABSTRACT: In vitro, limited proteolytic cleavage of the subunits of the purified calcium-dependent proteases [also known as calpains (EC 3.4.22.17) or calcium-activated neutral proteinases (CANPs)] appears to be required for enzyme activity. It has not yet been demonstrated if similar processing of the protease subunits occurs in vivo. To directly assess proteolytic modification of these proteases in cells, we have measured the loss of the proenzyme form of the regulatory subunit (a 26-kDa protein) and/or the appearance of the modified regulatory subunit (a 17-kDa protein) by densitometric analysis of immunoblots. In rat erythrocytes, proteolytic modification of the endogenous calcium-dependent protease (calcium-dependent protease 1, μCANP) occurs in vivo in response to ionomycin and calcium. The extent of enzyme modification was dependent on time, ionomycin concentration, and calcium concentration, suggesting that in this cellular model Ca^{2+} regulates proteolytic modification of the enzyme.

Recently it has become clear that the two intracellular calcium-dependent proteases [also known as calpains or calcium-activated neutral protease (CANP, EC 3.4.22.17)] are inactive proenzymes (DeMartino et al., 1986; Suzuki et al., 1987; Pontremoli & Melloni, 1986). Each proenzyme is a heterodimer composed of a unique catalytic subunit (80 kDa) and a common regulatory subunit (26 kDa). Studies of the purified proteases have demonstrated proteolytic processing of each of the protease subunits under conditions where proteolytic activity is measured (DeMartino et al., 1986; Suzuki, 1987; Suzuki et al., 1987; Mellgren et al., 1982; Coolican et al., 1986; Pontremoli & Melloni, 1986; Inomata et al., 1985; Imajoh et al., 1986). The autoproteolyzed enzymes are active against protein substrates and have reduced calcium requirements for this activity (Suzuki et al., 1987; Coolican et al., 1984; Coolican & Hathaway, 1986; Mellgren et al., 1982; DeMartino et al., 1986; Inomata et al., 1985). Proteolysis of the larger catalytic subunit is sometimes (Croall & DeMartino, 1984; Inomata et al., 1984, 1985; Pontremoli & Melloni, 1986; Samis et al., 1987) but not always detectable by denaturing gel electrophoresis (Inomata et al., 1984, 1985; DeMartino et al., 1986; Suzuki et al., 1987; Mellgren et al., 1982) depending on the type and source of calcium-dependent protease. Modification of the regulatory subunit of each protease changes the 26-kDa protein, through several intermediates, to a 17-kDa form (Mellgren et al., 1982; Coolican et al., 1986; DeMartino et al., 1986; Suzuki, 1987; Suzuki et al., 1987). There is currently some controversy as to the exact correlations between specific cleavage events and protease function(s) (Coolican & Hathaway, 1986; Mellgren et al., 1982; Suzuki et al., 1985, 1987; DeMartino et al., 1986). However, the enzyme proteins are proteolytically modified if substrates are hydrolyzed or if the enzymes are exposed to conditions appropriate for enzyme activity in vitro. Because proteolytic modification of the enzyme subunits is a unidirectional process, the presence of the modified enzyme subunits provides evidence that the enzymes are, or have been, active. If similar proteolytic modification occurs in vivo, the change in protease composition would provide direct evidence for enzyme acti-

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