Kinetics and Mechanism of the Hydrolysis of Depsipeptides Catalyzed by the β-Lactamase of *Enterobacter cloacae* P99[†]

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ABSTRACT: The steady-state kinetics and mechanism of the hydrolysis and aminolysis of a series of acyclic depsipeptides, catalyzed by the class C β-lactamase of *Enterobacter cloacae* P99, have been studied in order to more firmly establish the nature of the transition states involved. The class C β-lactamase of *Enterobacter cloacae* P99 was employed. The depsipeptide substrates contained a constant acyl group, (phenylacetyl)glycyl, and chemically different leaving groups, *m*-carboxyphenoxide, *m*-carboxythiophenoxide, 3-carboxy-4-nitrophenoxide, lactate, and thiolactate. Evaluation of the steady-state kinetic parameters and the effect of the alternative nucleophile methanol on these parameters and on the product distribution showed that deacylation was largely rate-determining to turnover of the aryl esters under conditions of substrate saturation, while acylation was rate-determining to the alkyl esters. The earlier conclusion [Govardhan & Pratt (1987) *Biochemistry 26*, 3385−3395] that acylation largely limited the turnover of the aryl esters was shown to be an artifact of phosphate buffer inhibition. The aminolysis of both the aryl and alkyl esters by D-phenylalanine was influenced by binding of the substrate at a second binding site on the acyl-enzyme intermediate. A study of inhibition of the hydrolysis of (phenylacetyl)-glycyl-D-thiolactate by the aminolysis product (phenylacetyl)glycyl-D-phenylalanine indicated that the second binding site is also available for ligands to bind to the free enzyme and to the noncovalent Michaelis complex with this substrate. It is likely that penicillin-recognizing enzymes in general, both β-lactamases and DD-peptidases, possess an extended substrate-binding site into which a variety of small ligands may bind at any point along the reaction coordinate and, to a greater or lesser extent depending on circumstances, affect catalysis.

Acyclic depsipeptides and thiodepsipeptides have been shown to be β-lactamase substrates (Pratt & Govardhan, 1984; Govardhan & Pratt, 1987; Adam et al., 1990). Since these compounds are also substrates of the structurally related bacterial DD-peptidases (Rasmussen & Strominger, 1978; Pratt & Govardhan, 1984; Adam et al., 1990, 1991), the targets of β-lactam antibiotics, they provide useful comparative probes of the active sites of these two classes of enzyme (Xu et al., 1994; Xu & Pratt, 1994, Damblon et al., 1995). Functional comparisons such as these are needed to complement the considerable amount of X-ray crystallographic structural data at atomic resolution that has been obtained on these enzymes in recent years. Both functional and structural information is needed to understand the different functions of β-lactamases and DD-peptidases and to design specific inhibitors for them.

It is now generally agreed that the turnover of depsipeptides (D) by serine β-lactamases and DD-peptidases (E) involves a double displacement (Ping Pong) mechanism (Scheme 1) with an acyl-enzyme intermediate (E-D) (Rasmussen & Strominger, 1978; Nguyen-Distèche et al., 1986; Govardhan & Pratt, 1987; Jamin et al., 1991). The second step, involving acylation of the enzyme and release of the product P, derived from the leaving group of the substrate, can be treated as irreversible under the initial velocity conditions of steady-state kinetics. The final step, involving hydrolysis of the acyl-enzyme and release of the carboxylate product H, is effectively irreversible at neutral pH. Thus the steady-state kinetic parameters k_{cat}/K_m and k_{cat} can be expressed in terms of rate constants in the way shown below Scheme 1.

Scheme 1

$$E + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ED \xrightarrow{k_2} E - D + P \xrightarrow{k_3} E + H$$

$$k_{cat}/K_m = k_1k_2/(k_{-1} + k_2) \qquad k_{cat} = k_2k_3 / (k_2 + k_3)$$

A recent comparative study from this laboratory of β-lactamase and DD-peptidase kinetics with depsipeptide substrates (Xu & Pratt, 1994) emphasized the need for, but absence of, information on the nature of the transition states that correspond to the above-mentioned steady-state kinetic parameters. It is important to be able to decide in any given case whether these constants primarily reflect acylation or deacylation transition states and whether they reflect the covalent chemistry of acyl transfer or some necessary concomitant physical process. This and the accompanying paper (Adediran et al., 1996) demonstrate how this problem has been approached with one particular β-lactamase.

Previous publications from this laboratory have described in detail the kinetics and mechanism of hydrolysis and aminolysis by specific amino acids of the depsipeptide

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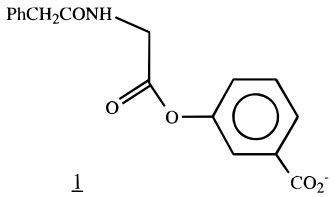
1, catalyzed by the class C β-lactamase of *Enterobacter*



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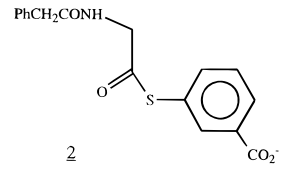
cloacae P99 (Govardhan & Pratt, 1987; Pazhanisamy et al., 1989; Pazhanisamy & Pratt, 1989a,b). A study of the effect of the alternative nucleophile methanol on the rate of solvolysis of **1** led to the conclusion that $k_{cat} = k_2$ (i.e., $k_3 \gg k_2$) for hydrolysis of this substrate in the presence of the P99 β-lactamase (Govardhan & Pratt, 1989). Subsequent investigations of the depsipeptides **2**−**5**, however,revealed some



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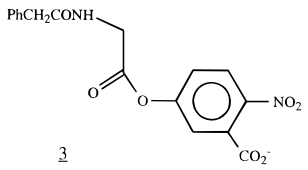

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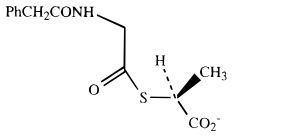

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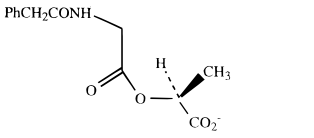

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inconsistencies with the earlier conclusions with respect to **1**. This paper describes the new results obtained with these substrates and presents a self-consistent kinetic model to accommodate all of the data, past and present. It is concluded that, for substrates **1**−**3**, $k_2 > k_3$, and thus *deacylation* of the enzyme is largely rate-determining under conditions of substrate saturation, while for **4** and **5** the converse is true and an acylation-associated step is rate-determining. These results are discussed in further detail in the accompanying paper (Adediran et al., 1996) where the β-secondary deuterium kinetic isotope effects on these reaction steps are described.

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. *m*-[[(Phenylacetyl)glycyl]oxy]benzoic acid (**1**), (phenylacetyl)glycyl-D-lactic acid (**5**), (phenylacetyl)glycyl-D-thiolactic acid (**4**), methyl (phenylacetyl)glycinate, and (phenylacetyl)glycyl-D-phenylalanine (**6**) were prepared as previously described (Govardhan & Pratt, 1987; Xu et al., 1994).

*m-[[(Phenylacetyl)glycyl]thio]benzoic Acid (**2**)*. In order to prepare this compound, *m*-dithiobenzoic acid was first prepared from benzoic acid by the procedure of Smiles and Stewart (1921), in 50% yield after recrystallization from acetic acid; mp 243−248 °C [lit. mp 246 °C (Smiles & Stewart, 1921)]. The diacid was then converted into its bis-(*p*-methoxybenzyl) ester by the method of Arnoldi et al. (1985); after recrystallization from diisopropyl ether, its melting point was 84−86 °C. The diester was quantitatively reduced to *p*-methoxybenzyl *m*-mercaptobenzoate by sodium borohydride. Thus, bis(*p*-methoxybenzyl) *m*-dithiobenzoate (6.0 g, 0.011 mol) was dissolved in a mixture (30 mL each) of 2-propanol and methylene chloride under a nitrogen atmosphere. Sodium borohydride (2.289, 0.06 mol) was added to the magnetically stirred solution and the reaction allowed to proceed for 2 h at room temperature. Solvents were then removed by means of a rotary evaporator, and the residue was partitioned between ethyl acetate and 1 M HCl. The ethyl acetate layer, pooled with two further ethyl acetate extracts, was washed with water and dried over $MgSO_4$. Evaporation then quantitatively yielded the thiol [IR absorption (film) $\gamma_{SH} = 2567$ cm^{-1}] as a yellow oil.

The crude thiol (5.84 g, 0.021 mol) was then condensed with (phenylacetyl)glycine (5.20 g, 0.027 mol) as described for the preparation of **5** (Xu et al., 1994). The product, *p*-methoxybenzyl *m*-[[(phenylacetyl)glycyl]thio]benzoate, was purified by flash chromatography on silica gel using 3/1 (v/v) hexane/acetone as eluant and then recrystallized from diisopropyl ether; the purified thiol ester, 1.09 g (12%), had an appropriate ^{1}H NMR spectrum: (C^2HCl_3) δ 3.56 (2H, s, PhCH_2), 4.29 (2H, d, $J = 9$ Hz, NHCH_2), 5.98 (1H, br t, NH), 7.0−8.0 (9H, m, ArH).

The synthesis of **2** was completed by removal of the *p*-methoxybenzyl protecting group by trifluoroacetic acid. Thus the ester (1.09 g, 2.42 mmol) was added to a stirred solution of anisole (0.60 g, 5.6 mmol) and distilled trifluoracetic acid (4.3 g, 0.038 mol) under nitrogen. After 15 min, the volatiles were removed by rotary evaporation. The product was then recrystallized from benzene. Its yield from the final step was 0.78 g (97%), mp 162−165 °C dec, and ^{1}H NMR: (C^2HCl_3) δ 3.70 (2H, s, PhCH_2), 4.29 (2H, d, $J = 9$ Hz, NHCH_2), 5.98 (1H, br t, NH), 7.0−8.0 (9H, m, ArH). Anal. Calcd for $C_{17}H_{15}NO_4S$: C, 61.99; H, 4.59; N, 4.25; S, 9.73. Found: C, 62.08; H, 4.42; N, 4.01; S. 9.95.

*2-Nitro-5-[[(phenylacetyl)glycyl]oxy]benzoic Acid (**3**)*. 5-Hydroxy-2-nitrobenzoic acid was prepared by alkaline hydrolysis of 5-chloro-2-nitrobenzoic acid (Tilley et al., 1981). The *p*-methoxybenzyl ester of this acid was then synthesized by the method of Baldwin et al. (1984) and coupled to (phenylacetyl)glycine by the method employed for **1** (Govardhan & Pratt, 1987). Finally, the *p*-methoxybenzyl protecting group was removed by trifluoroacetic acid treatment as described for **2** above, and the product, after trituration with benzene, was recrystallized from 1/5 (v/v) ethyl acetate/benzene. The essentially colorless crystals (1.0 g, overall yield from 10 g of 5-chloro-2-nitrobenzoic acid, 5.5%), mp 160.5 °C dec, yielded the expected ^{1}H NMR spectrum: (C^2HCl_3, [$^2H_6$]DMSO) δ 3.57 (2H, s, PhCH_2), 4.16 (2H, d, $J = 6$ Hz, NHCH_2), 7.01 (1H, br t, NH), 7.2−7.9 (8H, m, ArH). Anal. Calcd for $C_{17}H_{14}N_2O_7$: C, 56.98; H, 3.91; N, 7.82. Found: C, 57.19; H, 3.74; N, 7.73.

Analytical Methods. Absorption spectra and spectrophotometric reaction rates were measured by means of Perkin-Elmer Lambda 4B and Hewlett-Packard 8452A spectrophotometers. The concentrations of stock solutions of the P99 β-lactamase were obtained using a published extinction coefficient (Joris et al., 1985).

Steady-State Hydrolysis Kinetics. Steady-state kinetic parameters were determined by the method of Wilkinson (1961) from spectrophotometric initial velocity measurements. The wavelengths thus employed were 300 nm ($\Delta\epsilon = 950$ M^{-1} cm^{-1}) or 286 nm ($\Delta\epsilon = 1770$ M^{-1} cm^{-1}) for **1**, 335 nm ($\Delta\epsilon = 607$ M^{-1} cm^{-1}) or 318 nm ($\Delta\epsilon = 698$ M^{-1} cm^{-1}) for **2**, and 440 nm ($\Delta\epsilon = 2530$ M^{-1} cm^{-1}) or 420 nm

($\Delta\epsilon$ 8360 M^{-1} cm^{-1}) for **3**. Initial rates of hydrolysis of **4** were determined, as previously described (Xu et al., 1994), employing 4,4′-dipyridyl disulfide as a chromophoric thiol trap. The hydrolysis of **5** was monitored by an acid−base indicator method (Xu & Pratt, 1994). All kinetics experiments were performed at 25 °C in 20 mM MOPS buffer, pH 7.5, unless otherwise stated. Enzyme concentrations were generally of the order of 5−10 nM. The stock solutions of depsipeptides were prepared in aqueous buffer except for **3**, whose more rapid background hydrolysis precluded this option. Stock solutions of **3** were prepared in 95/5 (v/v) acetonitrile/DMSO. Cosolvent composition of the reaction mixtures containing **3** was kept below 5% and its effect on the kinetics noted (see Results). Background hydrolysis rates of **3** were subtracted from the observed initial rates in the presence of the enzyme.

Methanolysis Kinetics. The effects of methanol on the initial rates of solvolysis of **1**−**4** in the presence of the P99 β-lactamase were determined spectrophotometrically, as described above, in aqueous methanol/MOPS buffers. Methanol concentrations were varied up to 3.0 M; previous experience (Govardhan & Pratt, 1987) showed that the P99 β-lactamase was unaffected by these levels of methanol over the time period of the experiments. Substrate concentrations of at least three times K_m were employed. The effect of methanol (0−3.0 M) on the solvolysis rate of **1** at low substrate concentrations, 0.02 mM (pseudo-first-order or k_{cat}/K_m conditions), was also checked. Further, the solvolysis rate of **1** was examined in 2.5 M methanol as a function of substrate concentrations (0−5 mM). Finally, the methanolysis of **1** was studied in 0.1 M phosphate buffer for comparison with previous results (Govardhan & Pratt, 1987).

Aminolysis Kinetics. The net initial rates (hydrolysis plus aminolysis) of reaction of **1**−**3** in D-phenylalanine solutions, catalyzed by the P99 β-lactamase, were also measured spectrophotometrically. Two experimental protocols were employed, one with fixed substrate concentration (1.04 mM **1**, 3.23 mM **2** 0.11 and 0.34 mM **3**) and varying D-phenylalanine concentration (0−40 mM), and the other with fixed D-phenylalanine concentration (15 mM for **1**, 20 mM for **2**) and varying substrate concentation (0−30 mM). Aminolysis of **4** was also monitored spectrophotometrically with 4,4′-dipyridyl disulfide as described above. The reactions of a fixed concentration of **4** (7.3 mM) with variable concentrations of D-phenylalanine (0−40 mM) were studied.

Inhibition Kinetics. The inhibition by phosphate ion of the hydrolysis of **1** catalyzed by the P99 β-lactamase was measured in the presence and absence of 3.0 M methanol. The fixed concentrations of phosphate were 10, 50, and 100 mM, and the varied concentrations of **1** were 0−2.5 mM. The inhibition of the hydrolysis of **4** by the D-phenylalanine aminolysis product (phenylacetyl)glycyl-D-phenylalanine (**6**) was also investigated. Fixed concentrations of the peptide were 0, 5.0, 10.0, and 20.0 mM, and the concentration of **4** was varied between 0 and 10 mM.

Alkaline Hydrolysis Kinetics. The alkaline hydrolyses of **2** and **4** were monitored spectrophotometrically (270 and 250 nm, respectively) in 1 and 5 mM sodium hydroxide solutions, respectively (μ = 1.0 with KCl). The alkaline hydrolysis of **3** in 10 and 20 mM sodium hydroxide solutions (μ = 1.0 with KCl) was monitored spectrophotometrically at 400 nm by means of a Durrum D-110 stopped-flow spectrophotometer. In all cases, pseudo-first-order rate constants were converted to second-order constants by division by hydroxide ion concentrations.

Methanolysis and Aminolysis Product Ratios. The relative amounts of hydrolysis and methanolysis products from the solvolysis of **1**, **2**, and **4** under specified conditions were determined by HPLC using a Rainin Rabbit-HPX system with a Gilson UV−vis detector. Separation was achieved on a Macherey-Nagel Nucleosil 5 C_{18} column with a mobile phase of 10% v/v acetonitrile in an aqueous solution containing 50 mM NaH_2PO_4 at pH 3.5. A flow rate of 0.75 mL/min was employed, and the effluent was monitored at 220 nm. Under these conditions, retention times of (phenylacetyl)glycine, the hydrolysis product, methyl (phenylacetyl)glycinate, the methanolysis product, and 2,6-dimethoxybenzoic acid, employed as an internal standard, were 17.2, 44.4, and 24.4 min, respectively. Standard samples at known concentration were used to reduce HPLC absorption peak area ratios to concentration ratios.

Reaction mixtures contained around 1 mM substrate in order to limit binding at the second site (Pazhanisamy & Pratt, 1989b). Aliquots were quenched by addition of an equal volume of acetonitrile (to limit any slow enzyme-catalyzed hydrolysis of the methyl ester) and held on ice until the HPLC analysis was performed. Reactions were generally allowed to run essentially to completion although it did not seem that the ratio of hydrolysis to methanolysis varied with the extent of reaction.

A similar procedure was employed for the aminolysis studies. With the same HPLC column, a mobile phase of 20% aqueous acetonitrile (v/v) containing 50 mM KCl and 8 mM HCl (pH 2.2), and a flow rate of 1.0 mL/min, allowed ready separation of (phenylacetyl)glycine and *N*-(phenylacetyl)glycyl-D-phenylalanine with retention times of 5.25 and 31.1 min, respectively. Reaction mixtures contained **4** and D-phenylalanine where the ratio of their concentrations was held at 1:2 as the concentration of **4** was increased in separate runs. This procedure was adopted to minimize the effects of depletion of D-phenylalanine during the reaction—high concentrations of D-phenylalanine could not be used because essentially only aminolysis would be observed. In each run, a series of points were taken by quenching aliquots of the reaction mixture at times corresponding to extents of reaction up to 10%.

RESULTS AND DISCUSSION

Steady-state kinetic parameters for the hydrolysis of depsipeptides **1**−**5**, as catalyzed by the *E. cloacae* P99 β-lactamase, are shown in Table 1. These substrates were chosen of course to represent a series with the same acyl group, (phenylacetyl)glycyl—including the phenylacetamido side chain of good β-lactam substrates—and chemically different leaving groups.

If deacylation were rate-determining under substrate saturation conditions, k_{cat} values for **1**−**5** would be identical according to Scheme 1. If, on the other hand, acylation were rate-determining, the observed order of k_{cat} values should be, if they followed the order of chemical reactivity, **5** ≅ **4** < **1** ≅ **2** < **3**, where the difference between **4** and **1** might be some 30-fold and between **2** and **3** some 5- to 10-fold. These estimates are based on the rates of alkaline hydrolysis of analogous acetate esters (Kirsch & Jencks, 1964; Douglas et al., 1981). This order reflects the fact that nucleophilic

Table 1: Steady-State Kinetic Parameters for the P99 β-Lactamase-Catalyzed Hydrolyses of **1**−**5**

	kinetic parameter			
substrate	k_{cat} (s^{-1})[a]	K_m (mM)[a]	k_{cat}/K_m (s^{-1} M^{-1})[a]	k_{OH^-} (s^{-1} M^{-1})[b]
1	125	0.23	5.43×10^5	26.0[g]
1	89[d]	0.24[d]	3.77×10^5	
1	101[c,e]	1.9[c,e]	5.4×10^4	
2	153	0.89	1.72×10^5	310
3	86[d]	0.11[d]	7.59×10^5	1680
4	22.3[f]	3.3[f]	6670	0.9
5	27.3[f]	8.1[f]	3390	0.3[h]

[a] Reaction conditions: 20 mM MOPS buffer, pH 7.5, 25 °C except where noted otherwise. Standard deviations in k_{cat} and K_m were less than 10%. [b] Reaction conditions: 25 °C, $\mu = 1.0$ (KCl). [c] Govardhan and Pratt (1987). [d] The substrate was dissolved in 95/5 (v/v) acetonitrile/DMSO (see text). [e] Reaction conditions: 0.1 M phosphate buffer, pH 7.5, 25 °C. [f] Xu and Pratt (1994). [g] Adediran et al. (1996). [h] Murphy and Pratt (1991).

attack by hydroxide, i.e., formation of the tetrahedral intermediate, is completely or largely rate-determining to these hydrolyses (Guthrie, 1973, 1978; Hupe & Jencks, 1977). It is worth noting in passing, however, that in serine proteinases the dependence of k_{cat}/K_m (representing acylation) on leaving group ability in specific substrates is less than observed in alkaline hydrolysis, perhaps because of electrophilic assistance by the active site (Williams, 1970: Williams & Bender, 1971; Morgenstern et al., 1987).

If, contrary to the situation with the alkaline hydrolysis of acetate esters, breakdown of the tetrahedral intermediate were rate-determining to the β-lactamase-catalyzed reaction, the leaving group effect would be larger. Precedent from model systems would suggest the order **5** < **4** < **1** < **2** < **3**, where the difference between **4** and **5** and between **1** and **3** would be some 100-fold or perhaps larger (Guthrie, 1973, 1978; Hupe & Jencks, 1977; Jensen & Jencks, 1979). This rate-determining step may in fact obtain for the alkaline hydrolysis of **2** and **3** where it is likely that intramolecular nucleophilic catalysis by the side chain amide occurs (de Jersey et al., 1969). Such reactions are known to exhibit higher leaving group dependence (Williams, 1975), and this fact is revealed in the alkaline hydrolysis rates of **2** and **3** in comparison with **1** (Table 1).

Taking the above into account, probably all that can be safely concluded from the k_{cat} values of Table 1 is that deacylation is not rate-determining for all **1**−**5**. The very similar k_{cat} values for the aryl esters (taking into account the organic cosolvent effect in the case of **3**) suggest that these may have a common rate-determining step under substrate saturation conditions, which may be deacylation. Further evidence on this matter is presented below.

Methanolysis. The P99 β-lactamase also catalyzes methanolysis of depsipeptides (Govardhan & Pratt, 1987). The extents of methanolysis of **1**, **2**, and **4** in solutions containing 2 M methanol were determined by the HPLC method described above. This experiment was not carried out for **3** because of its high spontaneous solvolysis rates. The hydrolysis to methanolysis product ratios for **1**, **2**, and **4**, shown in Table 2, were converted by eq 1 to a ratio of

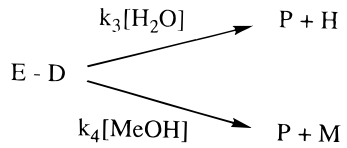
$$k_3/k_4 = [H][MeOH]/[M][H_2O] \quad (1)$$

second-order rate constants assuming the partitioning of a common intermediate [Scheme 2; P and H represent the hydrolysis products of Scheme 1, and M represents the methanolysis product, methyl (phenylacetyl)glycinate. The rate constant ratios calculated in this way are also shown in Table 2. These are in good agreement with those reported earlier for **1** and **5** from NMR measurements in $^2H_2O/^2HCO_3^-$ (Govardhan & Pratt, 1987). These results, as previously, can be interpreted in terms of solvolysis of a common intermediate, presumably subsequent to leaving group (P) departure and likely to be, most rationally, as shown in Scheme 2, the acyl-enzyme E-D.

The effect of methanol concentration on the rates of solvolysis of **1**−**4** (at concentrations ≥3 K_m) is shown in Figure 1. These data indicate that in the case of **1**−**3** the presence of methanol not only leads to the methanolysis product, as described above, but also accelerates the rate of disappearance of substrate. These observations are most simply interpreted in terms of Scheme 1 to mean that $k_2 > k_3$ and thus $k_{cat} = k_3$ for **1**−**3** and, conversely, that $k_{cat} = k_2$ for **4**. Previous observations (Murphy & Pratt, 1991) would then suggest that the latter conclusion also applies to **5**.

The noteworthy feature of the above is that the data of Figure 1A (closed circles) regarding compound **1** and the conclusion drawn from it differ from those of an earlier publication from this laboratory where the data were interpreted to mean that methanol afforded no rate acceleration and thus $k_{cat} = k_2$. It is obviously important to reconcile these differences. The essential difference between the earlier and the present experiments is that the former were conducted in phosphate buffer while the latter employed MOPS. Although β-lactamase-catalyzed methanolysis does occur in phosphate buffer to an extent little different from that in MOPS (Table 2), the extent of the rate acceleration is much less (Figure 1A, open circles), to such an extent that it was overlooked in the previous work [cf. Figure 1 in the earlier paper (Govardhan & Pratt, 1987)]. The cause of this phenomenon appears to reside largely with the fact that phosphate behaves as a competitive inhibitor of the solvolysis of **1**. Further, the inhibitory power of phosphate increases in methanol-containing solutions, presumably because of their smaller dielectric constants. Experiments indicated (data not shown) that the competitive inhibition constants of phosphate at pH 7.5 in the absence of methanol and in 3.0 M methanol were 15.3 mM and 5.8 mM, respectively. The effect of this tighter binding of phosphate in solutions of increasing methanol content is, therefore, as observed (Figure 1A, open circles), to reduce the increase in rate produced by methanolysis of E-D (Scheme 2). The competitive presence of phosphate in the early studies is presumably also responsible for the higher K_m value of **1** in phosphate buffer (Table 1).

Scheme 2



The data to this point could also be accommodated by the more complex Scheme 3 where kinetically significant binding of methanol to ED occurs. The reason that Scheme 3 needs to be considered lies in its similarity to previously proposed aminolysis schemes (Pazhanisamy & Pratt, 1991) where the

Table 2: Rate Constant and Product Ratios of Depsipeptide Methanolysis and Hydrolysis

	substrate				
	1[a]	**1**[b]	**2**[a]	**3**[a]	**4**[a]
[H]/[M][c]	0.92 ± 0.05	0.81 ± 0.04	1.06 ± 0.17	−[d]	0.93 ± 0.12
k_4/k_3 (product analysis)	27.7 ± 1.5	31.5 ± 1.6	24.0 ± 3.8	−[d]	27.4 ± 3.5
k_4/k_3 (rate measurements)	28.1 ± 1.2	36.7 ± 6.0[e]	26.6 ± 4.7	25.2 ± 3.0	−[f]
k_2/k_3 (rate measurements)	>10	>2	1.3 ± 0.4	>2	0.18[g]

[a] 20 mM MOPS buffer, pH 7.5. [b] 0.1 M phosphate buffer, pH 7.5. [c] Ratio of hydrolysis to methanolysis by product analysis. [d] Product analysis not performed (see text). [e] Corrected for phosphate inhibition (see text). [f] No kinetic effect (see text). [g] Calculated assuming $k_2 = 22.3\ s^{-1}$ and $k_3 = 125\ s^{-1}$ (Table 1).

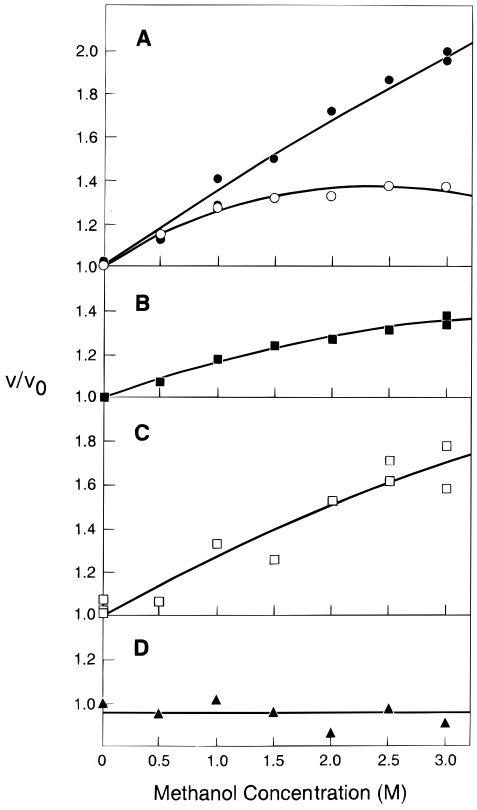


FIGURE 1: Initial rates of solvolysis of **1** (A, solid circles), **2** (B, solid squares), **3** (C, open squares), and **4** (D, solid triangles) in aqueous methanol, catalyzed by the P99 β-lactamase, as a function of methanol concentration. Substrate concentrations were 1.69 mM (**1**), 3.23 mM (**2**), 0.51 mM (**3**), and 9.91 mM (**4**). Also shown (A, open circles) is the result of the same experiment with **1** (4.11 mM) in phosphate rather than MOPS buffer. The rates are given as their ratio to v_0, the solvolysis (hydrolysis) rate in the absence of methanol. The solid lines are calculated from eq 3 as described in the text.

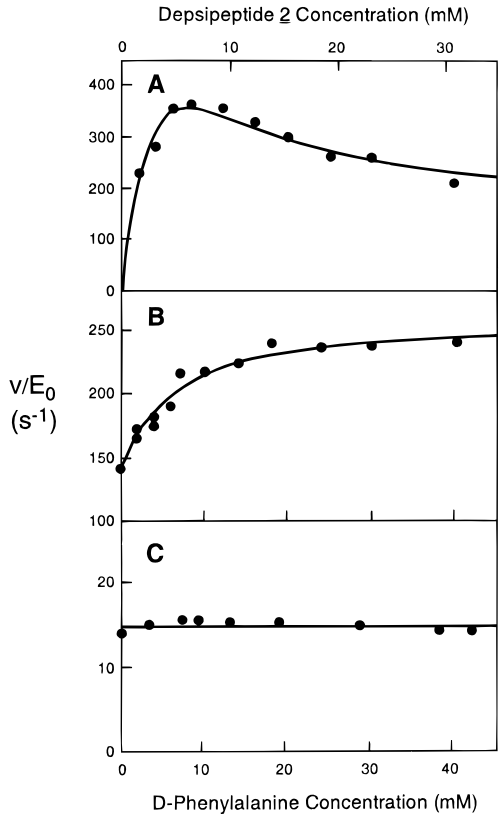
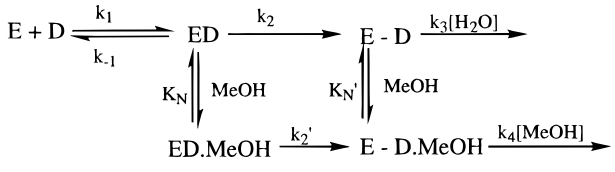


FIGURE 2: Initial rates of total reaction (hydrolysis plus aminolysis) of **2** and **4** in the presence of D-phenylalanine and the P99 β-lactamase. (A) Reaction of varying concentrations of **2** at constant D-phenylalanine concentration (20.1 mM). (B) Reaction of varying concentrations of D-phenylalamine at constant **2** concentration (3.23 mM). (C) Reaction of varying concentrations of D-phenylalanine at constant **4** concentration (7.30 mM). The points are experimental, and the solid lines in panels A and B were calculated from eq 5 as described in the text.

Scheme 3



binding of the (amine) nucleophile to ED seemed required (see discussion below, however). This situation will differ from the simpler combination of Schemes 1 and 2 in that at low substrate concentration the reaction rate would vary with methanol concentration according to:

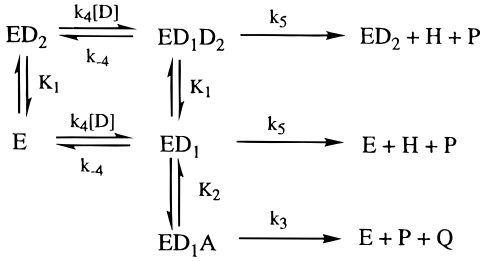
$$v/E_0 = \frac{k_1\{k_2 + (k_2'/K_N)[\mathrm{MeOH}]\}}{k_{-1} + k_2 + (k_2'/K_N)[\mathrm{MeOH}]} \qquad (2)$$

Methanol concentration (0−3 M) dependence of reaction rates was not observed at a substrate concentration of 0.02 mM [the mean value of v/E_0 from 11 runs at different methanol concentrations was $(4.91 \pm 0.21) \times 10^5\ M\ s^{-1}$]. The absence of such dependence would also be observed if, alternatively, the condition $k_2 \gg k_{-1}$ held, and thus v/E_0 under these conditions (i.e., k_{cat}/K_m) would equal k_1, but there is no evidence for this condition at present—certainly k_{cat}/K_m for **1** is far too small to represent a diffusion-controlled reaction. Thus, at present at least, the additional complexity of Scheme 3 is unwarranted for methanolysis.

Schemes 1 and 2 lead to the following equation for the dependence of rate on methanol concentration:

$$v/v_0 = \frac{\alpha\beta(1 + S_0/K_m)}{\alpha\beta + [H_2O]_0(k_2/k_3 + \beta)S_0/K_m} \qquad (3)$$

Scheme 4

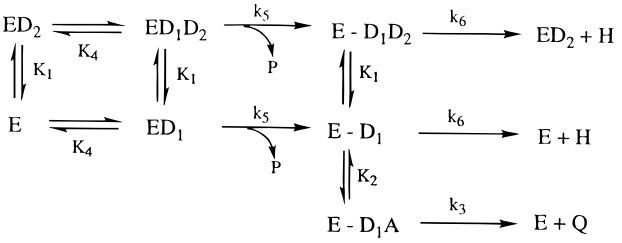


where v is the rate in the presence of methanol, v_0 is the rate in the absence of methanol, $\alpha = (k_2/k_3) + [H_2O]_0$, $\beta = [H_2O] + (k_4/k_3)[MeOH]$, $[H_2O]_0$ is the water concentration in the absence of methanol (55.56 M), and $[H_2O] = 55.56 - 2.25[MeOH]$, where 2.25 is the ratio of molar volumes of methanol and water at 25 °C. The application of eq 3 to the data for compounds **1**−**3**, giving the solid lines in Figure 1, leads to the values of k_4/k_3 also given in Table 2. The same equation can be applied to the data in phosphate buffer (Figure 1A, open circles) on replacement of K_m with K_m (1 + $[P_i]/K_p$), where K_p is the competitive inhibition constant for phosphate (P_i). On the assumption of a K_p value which increased linearly with methanol concentration between the limits of zero and 3 M methanol give above, the solid line of Figure 1A and the k_4/k_3 values shown in Table 2 are obtained. The agreement between all of these values of k_4/k_3 and those obtained from product analysis is clearly very good and strongly supports the simple Scheme 1 where, for **1**−**3**, $k_{cat} = k_3$, and methanol directly competes with water for the acyl-enzyme.

Also available, in principle, from the above analysis are values of k_2/k_3, the ratio of the acylation to deacylation rate constants. The curve fitting indicates values of $k_2/k_3[H_2O]_0$, the ratio of first-order rate constants, to be >10 for **1** and >2 for **3**, and rather smaller, 1.3 ± 0.4, for **2**; the smaller value for **2** is evident in the greater curvature of the data in Figure 1B. This result appears to indicate that acylation of the enzyme by **2** after binding is significantly more difficult than by **1**, contrary to expectations on the basis of their chemical reactivity (see above). Free energies of acylation of the free enzyme, as measured by k_{cat}/K_m, also favor **1** over **2**. These differences presumably reflect the somewhat different geometry of the thiol ester—molecular modeling (Biosym Insight II, version 2.20) suggests that, even with **1** and **2** in a similar conformation, the carboxylate group of **2** would be 0.3−0.4 Å more distant from the ester carbonyl than in **1**. If it is assumed that, for **4**, $k_{cat} = k_2 = 22.3\ s^{-1}$ and $k_3 = 125\ s^{-1}$ (taken from k_{cat} for **1**), then k_2/k_3 for **4** is 0.18. The values of k_2 for **1** relative to those of **4** are reasonable on the chemical grounds described above.

Aminolysis. The P99 β-lactamase also catalyzes the aminolysis of depsipeptides such as **1** by D-amino acids (Pratt & Govardhan, 1984). In order to rationalize the unusual aminolysis kinetics, Scheme 4 has been proposed (Pazhanisamy & Pratt, 1989b). In this diagram, ED_1 represents the noncovalent productive Michaelis complex. Both free enzyme, E, and ED_1 also bind D in a second site, leading to ED_2 and ED_1D_2, respectively. Both ED_1 and ED_1D_2 proceed to hydrolysis products P and H, while the former may also bind amino acid A and proceed to aminolysis products P and Q. The symmetry of rate constants is required to explain the simple hydrolysis kinetics (Pazhanisamy et al., 1989).

Scheme 5



The important features of this reaction scheme are as follows:

1. A second binding site (Site 2) for substrate or substrate analogs is present. The binding of a second substrate is competitive with aminolysis.
2. The amino acid does not bind significantly to the free enzyme (Pazhanisamy & Pratt, 1989a).
3. In the case of the substrate **1**, D-amino acids increased the rate of reaction of the substrate under conditions of partial or complete substrate saturation (Pazhanisamy et al., 1989) and thus must bind to an ES complex prior to the rate-determining step under these conditions. Since acylation was believed to be that step, the amino acid was proposed to bind to the Michaelis complex ED_1 (Scheme 4).

In view of the reinterpretation of the hydrolysis and methanolysis data described above, some reevaluation of the aminolysis reaction is necessary. It seems clear that if *deacylation* of the acyl-enzyme from **1** is rate-determining under saturating conditions, rather than acylation, then D-amino acids, like methanol, must bind to and react with acyl-enzyme. Scheme 5 is indicated. Here acyl-enzyme species E-D_1 and E-D_1D_2 have been added where the former of these can bind amino acid and lead to aminolysis. This scheme accommodates all previous kinetic data (Pazhanisamy et al., 1989; Pazhanisamy & Pratt, 1989a,b) on the aminolysis of **1** as well as does Scheme 4.

The P99 β-lactamase also catalyzes the aminolysis of **2**−**5** as shown, for example, by ^{1}H NMR spectra of reaction mixtures. Steady-state kinetics data on the aminolysis of **2** and of **4** by D-phenylalanine is provided in Figure 2. The results for **2** are qualitatively similar to those for **1** (Govardhan et al., 1989). They can be fitted to Scheme 5 by means of the following equation:

$$v/E_0 = \{k_5k_6K_2(K_1 + [D])[D] + k_3k_5K_1[A][D]\}/ \{k_6K_1K_2K_4 + K_2[K_1(k_5 + k_6) + k_6K_4][D] + (k_5 + k_6)K_2[D]^2 + k_3K_1K_4[A] + K_1(k_3 + k_5)[A][D]\} \quad (4)$$

which, on substituting $k_{cat} = k_5k_6/(k_5 + k_6)$ and $K_m = k_6k_4/(k_5 + k_6)$, can be transformed into

$$v/E_0 = \frac{k_{cat}(K_1 + [D])[D] + k_{cat}K_1B[A][D]}{K_1K_m + (K_1 + K_m)[D] + [D]^2 + K_1K_mB[A] + C[A][D]} \quad (5)$$

where $B = k_3/k_6K_2$ and $C = K_1(k_3 + k_5)/K_2(k_5 + k_6)$. Nonlinear regression with three parameters (K_1, B, C) provided the calculated lines shown for **2** in Figure 2 and thus the partition ratio for aminolysis $k_3/k_6K_2 = 1.1 \pm 0.4\ mM^{-1}$. Similar data for **1** (not shown, but see Pazhanisamy et al., 1989) yielded a value for the partition ratio of 1.4 ±

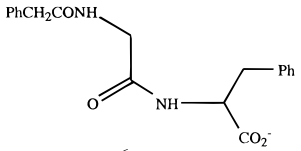
0.4 mM^{-1}. These results are in good agreement and support a common intermediate in aminolysis as provided by Scheme 5. In particular, they do not support a direct aminolysis mechanism where the thiol ester **2** would be expected to react distinctly more rapidly (Bruice & Benkovic, 1966). The inability of a mutant β-lactamase lacking the active site serine hydroxyl group to catalyze depsipeptide aminolysis was also interpreted to support an acyl-enzyme mechanism (Mazzella et al., 1991).

K_1 values of 3.0 mM and 1.8 mM for **1** and **2**, respectively, were also obtained from the curve fitting. The value for **1** is in agreement with the earlier estimate based on interpretation in terms of Scheme 4. The general conclusion at that time was that noncovalent binding in Site 1 (the productive site, filled in ED_1) was stronger than in Site 2. The present view would be that the binding strength in the two sites may well be comparable since, for **1**, $K_4 = K_m(k_5/k_6)$ and, from the methanolysis results, k_5/k_6 is probably >10.

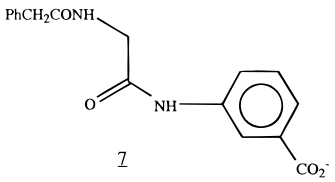
Other questions that arise on consideration of Schemes 4 and 5 are as follows:

1. Does A bind to ED_1? This seems not to be true. The best evidence on this point comes from a consideration of the results from aminolysis of **4** (see below).

2. Does D bind to the Site 2 of E and ED_1, or only to that of E-D_1? Although the hydrolysis and aminolysis data do not now require such binding (the steady-state equations for both hydrolysis and aminolysis generated by Schemes 4 and 5 are identical), there are indications from other experiments that such binding does occur. First, the patterns of inhibition by the peptides **6** and **7**, the latter a very close



6



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structural analog of **1**, appear to require two classes of binding site in both free enzyme and E-D_1 complexes (Pazhanisamy & Pratt, 1989b). Second, measurements of the inhibitory effects of a variety of ligands on the rate of hydrolysis of **1** suggested that Site 2 can be inhabited through all stages of depsipeptide and β-lactam hydrolysis (Dryjanski & Pratt, 1995a,b). Thus, the binding of D to Site 2 on both E and ED_1 still seems likely.

The kinetics of aminolysis of **3** and **4** also fit into the pattern established above. The rates of reaction of **3** at concentrations above K_m increase with D-phenylalanine concentrations in a way similar to that shown in Figure 2 for **2**, in accord with the expectations of rate-determining deacylation at saturation. Also, D-phenylalanine does *not* accelerate the rate of reaction of **4** at such concentrations (Figure 2). This supports the proposal of rate-determining acylation for **4**. ^1H NMR studies confirm, however, that aminolysis of **4** does occur, and to an extent depending on D-phenylalanine concentration. The absence of a rate effect, however, is a strong indication that the amino acid does not bind productively to ED_1.

As previously demonstrated (Pazhanisamy et al, 1989), the ratio of the extent of aminolysis to hydrolysis, [Q]/[H], as a function of depsipeptide concentration is a very useful indicator of reaction pathways such as those of Schemes 4 and 5, where D and A compete for an ED complex, and should be so particularly in cases such as with the substrate **4** where the kinetic manifestation of this phenomenon is not present. According to Scheme 5 and eq 4, [Q]/[H] is given by:

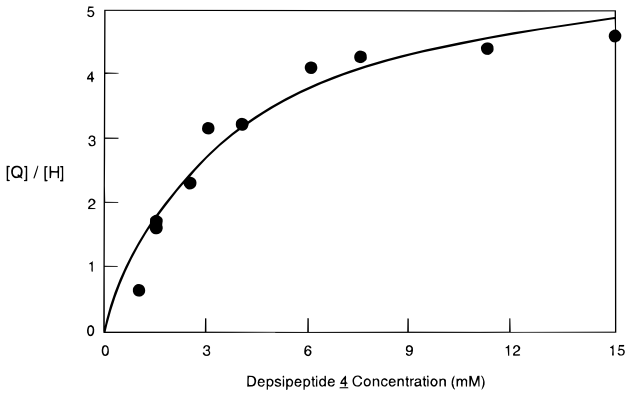


FIGURE 3: The initial ratio of aminolysis to hydrolysis products as a function of depsipeptide concentration for reaction of **4** in D-phenylalanine solutions in the presence of the P99 β-lactamase. D-Phenylalanine concentrations were maintained at twice the concentration of **4**. The points are experimental and the solid line is calculated from eq 6 as described in the text.

$$([Q]/[H]) = \frac{k_3 f}{k_6 K_2}\left(\frac{K_1[D]}{K_1 + [D]}\right) \quad (6)$$

where $f = [A]/[D]$, the ratio of initial concentrations of A and D, which was kept constant in the experiment performed. The results of the relevant experiment are shown in Figure 3. Nonlinear regression to this data gave values for the partition ratio k_3/k_6K_2 of 0.83 ± 0.15 mM^{-1}, in excellent agreement with the values obtained from kinetic data for **1** and **2** (see above), and $K_1 = 3.7 \pm 0.9$ mM. The latter value, interpreted as the dissociation constant of D from E-D_1D_2 to give E-D_1, is very similar to that of K_4 (3.3 mM; taken to be equal to K_m), providing another instance where the binding of a ligand at the two sites is comparable.

The availability of Site 2 in ED_1 to other ligands was further tested by studies of the inhibition of hydrolysis of **4** by the peptide **6**. If acylation were rate-determining to hydrolysis of this depsipeptide at saturation, then the major enzyme−substrate complex under these conditions would be ED_1. The effects of further ligand binding to it would therefore be very likely observed. The data are shown in Figure 4. The simplest analysis, assuming substrate binding at a single site, indicates a predominantly competitive interaction with a weak noncompetitive element. This interpretation, however, ignores past experience and Scheme 5. The evidence of this and previous papers supports Scheme 6 as providing a more accurate representation of the situation. The following equation can be derived as a steady-state rate equation from Scheme 6:

$$v/E_0 = [k_{cat}[D]\{K_1(1 + \beta[I]/\gamma K_i) + [D]\}]/[K_m\{K_1(1 + [I]/K_i) + [D]\} + [D]\{K_1(1 + [I]/\alpha K_i) + [D]\} + K_m[I]\{K_1(1 + [I]/K_i) + [D]\}/\gamma K_i] \quad (7)$$

Reasonable approximations to K_1, K_m, k_{cat}, K_i, and γ are already available: the first three of these from data presented above and the latter two from previous studies of the inhibition of hydrolysis of **1** by **6** (Pazhanisamy & Pratt,

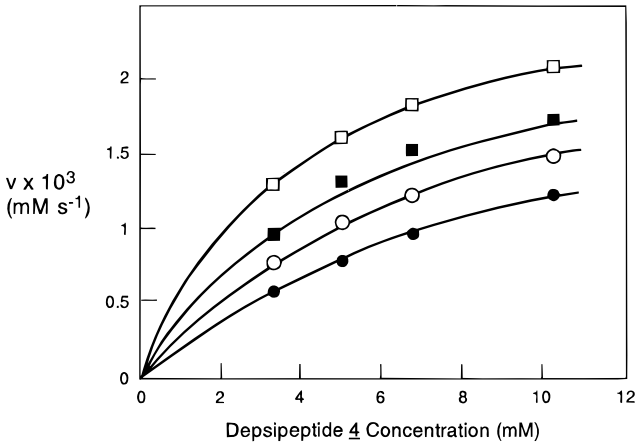
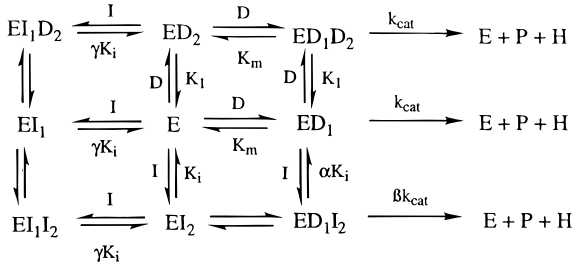


FIGURE 4: Inhibition of the hydrolysis of **4**, catalyzed by the P99 β-lactamase, by the peptide **6**, the latter at concentrations of 0 mM (□), 5.0 mM (■), 10.0 mM (○), and 20.0 mM (●). The points are experimental, and the solid lines were calculated from eq 7 as described in the text.

Scheme 6



1989b). A fitting of the data of Figure 4 to eq 7, with the values of these known parameters fixed [values of 1 mM and 10 were chosen for K_i and γ, respectively (Pazhanisamy & Pratt, 1989b)], yielded values of α and β of 1.05 ± 0.3 and 0.95 ± 0.1, respectively, and the solid lines of Figure 4 to fit the data. Thus, given Scheme 6, significant binding of **6** to the Michaelis complex ED_1 ($\alpha K_i = 1.05$ mM) is indicated. The situation here is similar to that encountered in the hydrolysis of **1** in the presence of **7** (Pazhanisamy & Pratt, 1989b)—again, the more effective binding of the inhibitor to Site 2 than to Site 1 is camouflaged by the reactivity of ED_1I_2.

Concluding Overview. This paper describes the steady-state kinetics of turnover of a series of depsipeptide substrates **1**−**5** by the β-lactamase of *E. cloacae* P99, a typical class C β-lactamase. The results presented provide further support for the previously proposed general reaction path of Scheme 4, now elaborated slightly to Scheme 5, and more firmly establish the nature of the rate-limiting step under conditions of substrate saturation. Contrary to the previous proposal (Govardhan & Pratt, 1987), it is now established that deacylation of the acyl-enzyme is largely rate-determining in the case of the aryl (thio)esters **1**−**3**, while acylation is largely rate-determining for the alkyl (thio)esters **4** and **5**. Alternative nucleophiles such as methanol and D-amino acids can compete with water for the acyl-enzyme, increasing the observed reaction rate of **1**−**3** but not of **4** (Figures 1 and 2).

An intriguing additional feature of the results which deserves brief comment in passing is the marked lack of a leaving group effect on k_{cat}/K_m for the depsipeptides **1**−**3**. From Scheme 1, the chemical essence of which is not changed in Scheme 5, k_{cat}/K_m should reflect, to some extent at least, the nature of the acylation (k_2) transition state which should be leaving group dependent in the ways described above at the beginning of the discussion. Such dependence in the expected direction does appear to be present between the aryl (**1**−**3**) esters as a group and the alkyl (**4**, **5**) esters, but the interpretation of this observed difference (Table 1) in terms of electronic effects only is hazardous because of the pronounced difference in shape of these two classes of leaving groups. Nonetheless, the simplest interpretation of the k_{cat}/K_m data of Table 1 should be in terms of rate-determining formation of the tetrahedral intermediate in the acylation step. The only contrary view is that provided by **3** which appears little more reactive than **1**. This might reflect either an extreme of electrophile participation in the transition state or the adverse steric effect of the nitro group directly adjacent to the carboxylate of the leaving group. The nature of the k_{cat}/K_m transition state is addressed in more detail in the following paper (Adediran et al., 1996).

Evidence has also been provided in this work for the presence and general kinetic significance of the extended active site of this enzyme, where the results for substrates **2** and **4** extend those already available for **1**. It now appears that small substrates, probably including β-lactams (Dryjanski & Pratt, 1995a), and their peptide analogs such as **6** can bind to the enzyme concurrently with a substrate and affect the rate and nature of the reaction. Most striking in this regard is the ability of an additional ligand to control the access of amine nucleophiles to the acyl-enzyme and thus the nature of the reaction product. It seems likely that this effect is indirect rather than by direct competition (Dryjanski & Pratt, 1995b). This conclusion is supported, although not proven, by the observation that competition for Site 2 does not occur between either water or methanol and substrate in the acyl-enzyme derived from **1**. Simple Michaelis−Menten kinetics were observed both in completely aqueous solution and in 2.5 M methanol (data not shown), suggesting that E-D_1 and E-D_1D_2 were equally susceptible to hydrolysis and to methanolysis. The strength of the binding of substrates and their analogs to Site 2 generally appears comparable to that to the active site itself.

It is important to set these results in context with respect to recent developments with related enzymes. There is little comparably detailed work with other β-lactamases yet, but particularly relevant are the extensive studies of Frère and co-workers of the *Streptomyces* R61 DD-peptidase. This enzyme is structurally very similar to the P99 β-lactamase (Ghuysen, 1991; Lobkovsky et al., 1993), and although it does not catalyze the hydrolysis of β-lactams, it does catalyze the hydrolysis and aminolysis of acyclic depsipeptides, thiodepsipeptides, and peptides (Ghuysen et al., 1979; Adam et al., 1990; Jamin et al., 1991). Complicated peptide aminolysis kinetics were long recognized (Ghuysen et al., 1979) and originally interpreted in terms of an ordered reaction sequence with the nucleophile binding first. More recent experiments with depsipeptides have led to the proposal of a new scheme where it was recognized that the kinetic complexity arose from a second substrate binding site (Jamin et al., 1993). The kinetic data could be accommodated most parsimoniously by a scheme which required additional substrate binding to the ternary complex of the acyl-enzyme with amine (i.e., to E-D_1A of Scheme 5) to yield a hydrolytically competent quaternary complex

E-D_1D_2A. Additional binding of D to E, ED_1 or E-D_1 was not required to fit the data but was not excluded (presumably, E-D_1D_2A could also arise through binding of A to E-D_1D_2). A recent report from the same group dealing with the amide aminolysis kinetics of the *Streptomyces* K15 DD-peptidase does include data which have been interpreted in terms of additional substrate binding to a noncovalent enzyme−substrate complex, i.e., of the existence of ED_1D_2 (Grandchamps et al., 1995). The presently available data for the P99 β-lactamases and depsipeptides **1**−**5** do support substrate binding to a second site in E and ED_1, as well as in E-D_1. It does not require the E-D_1D_2A quaternary complex however. The presence of a significant amount of such a complex would lead to nonlinearity in plots of [H]/[Q] *vs* 1/[A], which has not been observed (Figure 4b in Pazhanisamy et al., 1989) and to a bell-shaped curve in Figure 3 of the present paper.

The reasonable conclusion from the above paragraph is that the penicillin-recognizing enzymes generally possess an extended substrate binding site, presumably in order to optimally interact with cell wall peptidoglycan, their polymeric substrate (DD-peptidases), or ancestral substrate (β-lactamases). On investigation of the active sites of these enzymes with small substrates and substrate analogs, evidence of multiple and complicated occupancy of this extended site may be, not surprisingly, observed. The details of the dominant modes of binding and their effect on catalysis will vary depending on the enzyme and the substrates under study. The structural basis for the extended binding in any particular case has not yet been investigated. It is possibile, however, that knowledge of such structure might lead to extended-binding antibiotics.

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