Steady-State Kinetics of the Binding of β-Lactams and Penicilloates to the Second Binding Site of the *Enterobacter cloacae* P99 β-Lactamase†

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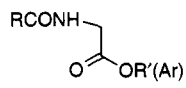
ABSTRACT: Previous research has shown that the class C β-lactamase of *Enterobacter cloacae* P99 is able to catalyze the hydrolysis and aminolysis of acyclic depsipeptides. The steady kinetics of these reactions are complicated by the presence of an additional (depsi)peptide binding site in addition to the active site [Pazhanisamy, S., & Pratt, R. F. (1989) *Biochemistry 28,* 6875−6882]. The present paper presents a steady-state kinetic analysis of the inhibition of depsipeptide hydrolysis by sodium benzylpenicilloate, methyl benzylpenicilloate, 6-aminopenicillanic acid, and 7-aminocephalosporanic acid. The two β-lactams are considerably poorer substrates than the depsipeptide employed, *m*-[[(phenylacetyl)glycyl]oxy]benzoic acid. The aim was to determine the relative affinity of these ligands for the active site and the second site. Three types of experiments were employed: (i) measurements of direct inhibition of depsipeptide hydrolysis, (ii) measurements of the effect of an active-site-directed inhibitor, *m*-(dansylamidophenyl)-boronic acid, on the effectiveness of the ligands as inhibitors, and (iii) measurements of the effect of a preferential second site ligand, *N*-(phenylacetyl)glycyl-D-phenylalanine, on the effectiveness of the ligands as inhibitors. The results suggest that all four ligands preferentially bind to the active site, with weaker binding at the second site. The necessarily weaker binding of a ligand to the second site when the active site is occupied by a transition-state analog inhibitor was analyzed. Perhaps surprisingly, the intact β-lactams appeared to bind more firmly to the alternative site than do the flexible penicilloates. The results also show that ligands can be present in the second site through all stages of depsipeptide and β-lactam hydrolysis. It is likely that the second site (or sites) represents the remnants of substrate binding subsites present on the evolutionary progenitors of β-lactamases, the bacterial DD-peptidases.

β-Lactamases continue to represent an important source of bacterial resistance to β-lactam antibiotics (Neu, 1992). As part of a program of exploration of the chemical properties of the active sites of these enzymes, we have demonstrated that they catalyze not only the hydrolysis of β-lactams but also the hydrolysis and aminolysis of acyclic depsipeptides of general structure **1** (Pratt & Govardhan,



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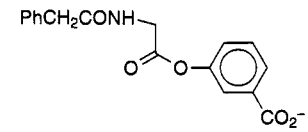
1984; Govardhan & Pratt, 1987). The steady-state kinetics of these depsipeptide reactions are complex, as demonstrated by our detailed study of the hydrolysis and aminolysis of **2** catalyzed by the class C β-lactamase of *Enterobacter cloacae* P99 (Govardhan & Pratt, 1987; Pazhanisamy et al., 1989; Pazhanisamy & Pratt, 1989b). This complexity arises from the presence of a binding site on the enzyme, distinct from the active site, capable of binding acyclic peptides and depsipeptides. Its presence is most directly appreciated by the demonstration that **3**, the product of aminolysis of **2** by D-phenylalanine, is a noncompetitive inhibitor of the hydrolysis of **2**. The specificity of the aminolysis reaction with respect to the structure of the amine acceptor of the acyl group is indicative of an acceptor binding site, although



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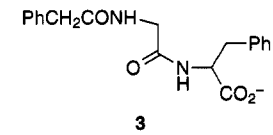

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binding is weak (Pazhanisamy & Pratt, 1989a,b). The aminolysis kinetics indicate a competitive interaction between the second reactant/product binding site and the acceptor site, but the extent of physical overlap between the sites is not known. Thus, there may be one or two small molecule binding sites in addition to the active site where one of them, the acceptor site, must necessarily be directly adjacent to the productive binding site of a depsipeptide substrate.

The existence of an extended binding site in the P99 β-lactamase most likely derives from the past history of β-lactamases and DD-peptidases. Functional and structural studies now strongly support the proposition of Tipper and Strominger (1965) that β-lactamases are the evolutionary descendants of the DD-peptidases that catalyze peptidoglycan cross-linking in the final step of bacterial cell wall biosynthesis. Indeed, it seems likely that the P99 β-lactamase, a typical class C enzyme, is structurally very similar to the *Streptomyces* R61 DD-peptidase (Ghuysen, 1991; Lobkovsky et al., 1993). In view of these considerations, it seems possible, if not likely, that the second binding site detected on the P99 β-lactamase derives from the extended binding site likely present on DD-peptidases to accommodate their polymeric peptidoglycan substrates. Indeed, kinetic studies of the R61 DD-peptidase indicated the productive binding of extended acyl donors and acceptors (Ghuysen et al., 1979;

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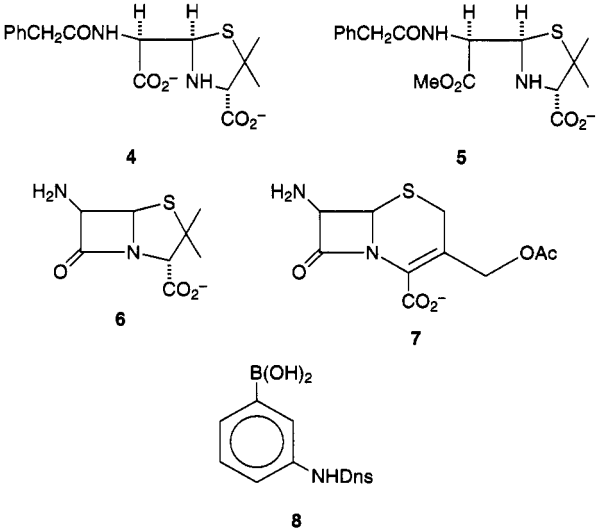
Frère & Joris, 1985). Peptide amines have been shown to inhibit the aminolysis reaction in a way that was best interpreted in terms of a quaternary complex with one acyl donor and two amine acceptors bound to the enzyme (Frère et al., 1973; Perkins et al., 1973; Ghuysen et al., 1974). Initial investigations of the catalystic activity of high molecular weight DD-peptidases, e.g., penicillin-binding protein 1a of *Escherichia coli*, suggested preference for extended disaccharide pentapeptide substrates (Ishino et al., 1980). Further, recent studies of the R61 DD-peptidase with thiol depsipeptide substrates show, by similar steady-state kinetics of aminolysis to those observed with the P99 β-lactamase, the presence of an additional substrate binding site in that enzyme also (Jamin et al., 1993). It seems likely then that the DD-peptidases and therefore, by default, the β-lactamases have a complicated system of binding subsites around the residues encompassing the scissile bond at the active site. No structural analysis of these features has yet been attempted.

We have been interested in these sites as possible targets for extended inhibitor design and, with this in mind, have been exploring the specificity with respect to ligand structure of the second binding site of the P99 β-lactamase. One interesting and important aspect of this inquiry was the question as to whether β-lactams or their hydrolysis products bind at the secondary site. In this paper, we present the results of a steady-state kinetic approach to this question. The experiments involved reaction velocity measurements in the presence of a substrate, the depsipeptide **2**, and the potential ligand, either benzylpenicilloate (**4**), methyl benzylpenicilloate (**5**), 6-aminopenicillanic acid (**6**), or 7-aminocephalosporanic acid (**7**). In order to clarify the mode of binding of these ligands, experiments in the presence of a competitive inhibitor, 3-(dansylamido)phenylboronic acid (**8**), and separately a noncompetitive inhibitor, *N*-(phenylacetyl)-glycyl-D-phenylalanine (**3**) were also performed. The kinetics were complicated by the ability of the substrate to also bind at the secondary site. The results suggest that **4**–**7** all bind to some extent to the secondary site. Peptides are also able to bind there when the active site is occupied by a β-lactam.



EXPERIMENTAL PROCEDURES

Materials. Monosodium benzylpenicilloate (**4**), mp 160–162 °C, was prepared by alkaline hydrolysis of benzylpenicillin (Sigma) and recrystallized from water, as described by Mozingo and Folkers (1949). Material with the same ^{1}H NMR spectrum is obtained as the immediate product from β-lactamase-catalyzed hydrolysis of benzylpenicillin, and thus the isolated alkaline hydrolysis product presumably has the "natural" 5R,6R configuration (**4**). Methyl benzylpenicilloate, also of the 5R,6R configuration (**5**), was prepared by methanolysis of benzylpenicillin as described by Busson et al. (1976); after recrystallization from methanol–diethyl ether (1:9), the product had a mp of 126–127 °C. 6-Aminopenicillanic acid was purchased from Sigma Chemical Co. and used as received. 7-Aminocephalosporanic acid was obtained from Eli Lilly and Co. as a gift. The substrate, *m*-[[(phenylacetyl)glycyl]oxy]benzoic acid (**2**) was prepared as previously described (Govardhan & Pratt, 1987). *m*-(Dansylamidophenyl)boronic acid was available from a previous synthesis in these laboratories (Pazhanisamy & Pratt, 1989b) or purchased from Sigma Chemical Co.; the two preparations had identical inhibitory properties. The peptide **3** was also available from a previous synthesis (Pazhanisamy & Pratt, 1989b).

The β-lactamase of *E. cloacae* P99 was obtained from the Centre for Applied Microbiology and Research (Porton Down, U.K.) and, as previously (Govardhan & Pratt, 1987; Pazhanisamy & Pratt, 1989b), used as supplied.

Kinetic Methods. The rates of hydrolysis of **2** in the presence of **3**–**8** were monitored spectrophotometrically by means of a Perkin-Elmer Lambda 4B spectrophotometer at 300 nm. All reactions were carried out in 20 mM MOPS buffer at pH 7.5 and at 25.0 °C. In all experiments, appropriate volumes of buffered stock solutions of **2**–**8** were mixed in a cuvette with buffer to a total volume of 0.8 mL. After temperature equilibration in the spectrophotometer had been achieved, the reaction was initiated by the addition of a 10-μL aliquot of a stock enzyme solution, giving a final enzyme concentration of ca. 35 nM; the β-lactamase concentrations of stock solutions were determined spectrophotometrically (Pazhanisamy et al., 1989). From the measurements of absorption vs time, initial rates of reaction were determined or, at substrate concentrations, less than 0.05 mM. (K_m = 0.4 mM), pseudo-first order rate constants were determined by nonlinear least-squares curve fitting (Johnson et al., 1976).

Three types of experiments were performed:

(i) Fixed substrate (D) concentration (0.02–10 mM) and variable test ligand (I) concentrations: **4**, 0–38; **5**, 0–20; **6**, 0–15; **7**, 0–2 mM.

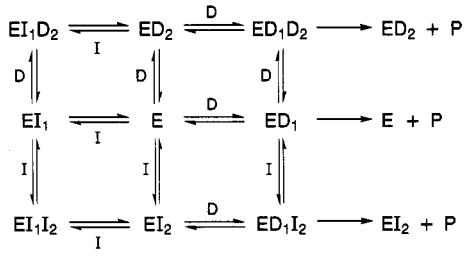
(ii) Fixed D ($\ll K_m$) and competitive inhibitor (J, **8**) (0–10 μM) concentrations and variable test ligand (I) concentrations: **4**, 0–20; **5**, 0–20; **6**, 0–4; **7**, 0–1.4 mM.

(iii) Fixed D ($\ll K_m$) and noncompetitive inhibitor (N, **3**) (0–10 mM) concentrations and variable test ligand (I) concentrations: **4**, 0–20; **5**, 0–12; **6**, 0–4; **7**, 0–1 mM.

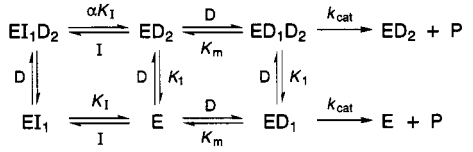
The β-lactams **6** and **7** are of course themselves substrates, but sufficiently poor [k_{cat} values of 0.72 ± 0.04 and 0.46 ± 0.03 s^{-1} and K_m values of 0.29 ± 0.05 and 0.29 ± 0.04 mM were obtained for **6** and **7**, respectively] in contrast to **2** (k_{cat} = 195 s^{-1}, K_m = 0.41 mM) so that little depletion of **6** and **7** would occur during measurements of initial rates of **2** hydrolysis. The rates observed seemed well-behaved and unperturbed by transient phenomena.

Plots of initial velocity, pseudo-first-order rate constants, and certain subsequently derived parameters (p) as a function

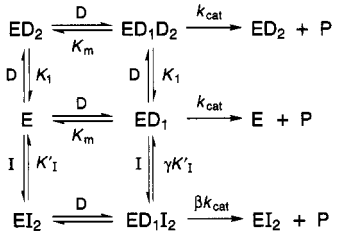
Scheme 1



Scheme 2



Scheme 3



of the variable concentration yielded hyperbolic curves that were fitted by the unweighted nonlinear least-squares procedure referred to above to the general eq 1, where L is the variable ligand and $a-c$ are empirical parameters determined by the curve fitting. In no experiment was a significantly better fit generally obtained, over the concentration range covered, by inclusion of second-order terms in the denominator.

$$v, k_{obs}, p = \frac{a + b[L]}{1 + c[L]} \quad (1)$$

The confidence levels of weak binding were assessed by the likelihood ratio test (Seber & Wild, 1989) applied to fits with and without the weak binding mode.

RESULTS

The general reaction scheme for hydrolysis of **2** in the presence of the *E. cloacae* P99 β-lactamase and its inhibition by the peptide **3** has previously been shown to be that of Scheme 1 (Pazhanisamy & Pratt, 1989b). Two binding sites are required to accommodate the data, one of them the active site (site 1) where hydrolysis of the substrate D to the product P occurs. The peptide **3** was found to be a largely noncompetitive inhibitor, binding considerably more strongly to the second site (site 2) than to the active site, to the extent that the data could be quite well fitted with the assumption of no binding to the latter site. Consequently, as a first approximation to simplify analysis of the present data, it was assumed that **4**–**7** bound preferentially to one of the sites, i.e., that either Scheme 2 or Scheme 3 represented a good approximation. Since in all cases reported here $v \rightarrow 0$ at high [I], irrespective of [D], and the v vs [I] data could be fitted well with a simple hyperbola (see below), it follows that, if Scheme 3 were correct, $\beta \ll 1$ (assumed henceforth to be zero). Under such circumstances, Schemes 2 and 3

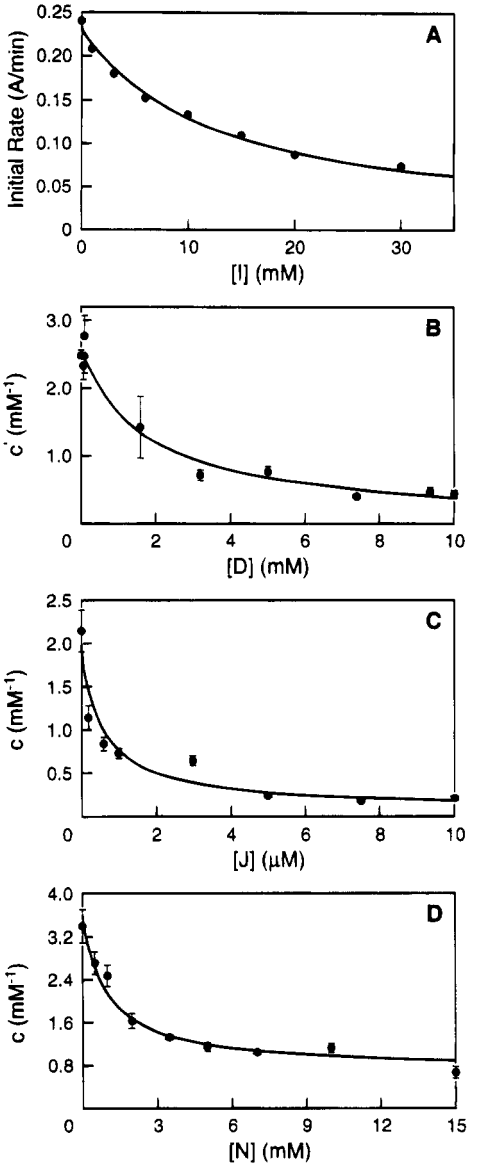


FIGURE 1: Panel A shows the variation of the initial velocity of hydrolysis of **2** catalyzed by the P99 β-lactamase in the presence of increasing concentrations of the inhibitor **4**; the points are experimental, and the line is calculated from eq 1. Also shown for this inhibitor is the variation of the derived parameter c' (eq 4 or 5) with the concentration of **2** (panel B) and the parameter c (eq 8) with the concentrations of **8** (panel C) and **3** (panel D); the points are experimental, the error bars derive from the fits to the primary data (e.g., panel A), and the lines are calculated from best fits to the above-mentioned equations.

lead to eqs 2 and 3, respectively. Under constant [D]

$$v/E_0 = \frac{k_{cat}[D]/(K_m + [D])}{1 + \left(\frac{K_m}{K_m + [D]}\right)\left(\frac{1 + [D]/\alpha K_1}{1 + [D]/K_1}\right)\frac{[I]}{K_I}} \quad (2)$$

$$v/E_0 = \frac{k_{cat}[D]/(K_m + [D])}{1 + \left(\frac{K_m}{K_m + [D]}\right)\left(\frac{1 + [D]/\gamma K_m}{1 + [D]/K_1}\right)\frac{[I]}{K'_I}} \quad (3)$$

conditions, eqs 2 and 3 are both of the form of eq 1 (I = L). Plots of v/E_0 *vs* [I] (from experiment i), where I is **4**, fit eq 1 well (for example, see Figure 1A). Thus, a series of c values at various [D] could be obtained. Note that this

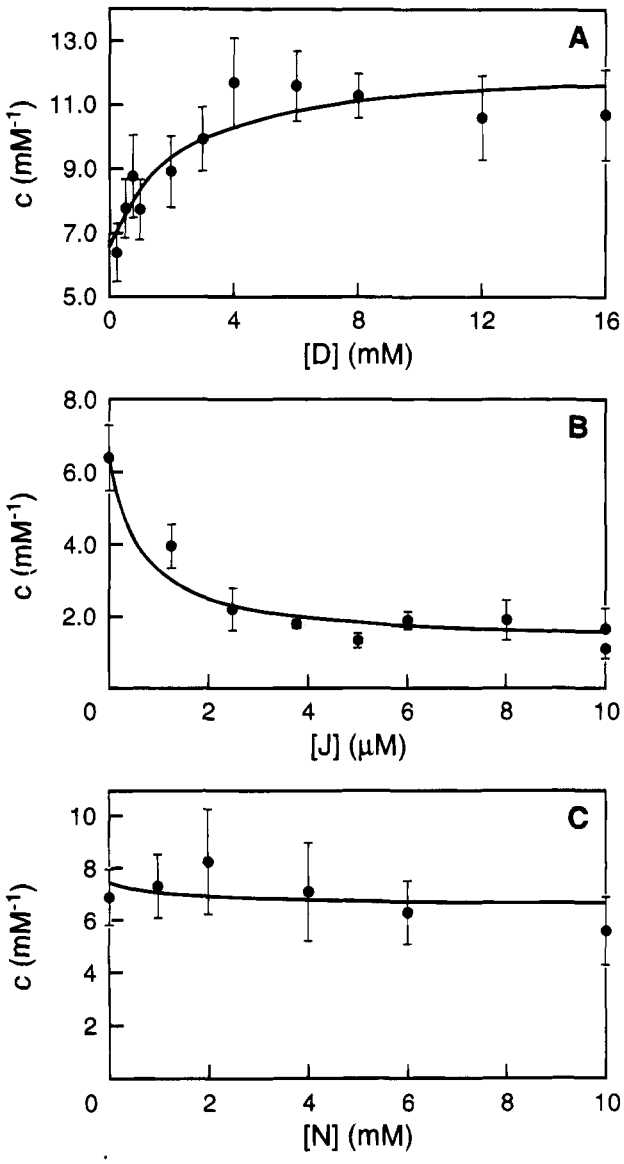
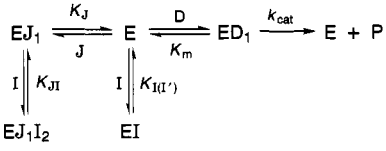


FIGURE 2: Plots of the derived parameter c' or c with concentrations of **2** (panel A), **8** (panel B), and **3** (panel C) as described in the legend to Figure 1 for the inhibitor **6**.

Scheme 4



procedure was preferred to direct fitting of the data to eqs 2 and 3 because the only information required was in the c parameter of the denominator. Direct fitting would propagate uncertainties in the numerator arising from those in E_0—different dilutions of an enzyme stock solution and in some cases different stock solutions were used in a given series of experiments—into c. A plot of c' [$=c(K_m + [D])/K_m$, where K_m was taken to be 0.41 mM)] vs [D] is shown in Figure 1B for **4**. This plot (and the others of Figures 1 and 2 described below) is readily interpreted qualitatively as showing the relative binding strength of I at low variable ligand (here D) concentration, i.e., to E, and at high concentration (here to ED). Figure 2B shows clearly that I binds significantly more strongly to E than to ED. A nonlinear least-squares fit of the data to eq 4, the relationship dictated by Scheme 2 and eq 2, yielded $K_I = 0.39 \pm 0.02$ mM, $K_1 = 1.7 \pm 0.4$ mM, and $\alpha > 8$ (90% confidence).

$$c' = \frac{(1 + [D]/\alpha K_1)/K_I}{(1 + [D]/K_1)} \quad (4)$$

$$c' = \frac{(1 + [D]/\gamma K_m)/K'_I}{(1 + [D]/K_1)} \quad (5)$$

The same fit to eq 5, from Scheme 3 and eq 3, yielded $K'_I = 0.39 \pm 0.02$ mM, $K_1 = 1.7 \pm 0.4$ mM, and $\gamma > 40$ (90% confidence). The data are thus in accord with the formation of a binary complex between the enzyme and **4** at the concentrations employed. The binding of **4** to ED_1 (Scheme 2) or ED_2 (Scheme 3), however, to form a ternary complex is much weaker. The important point remaining is on whether the binary complex is predominantly EI_1 (Scheme 2) or EI_2 (Scheme 3), i.e. to which site does **4** preferentially bind.

In order to distinguish between these two possibilities, experiments ii and iii, employing ligands that selectively bind to the active site (site 1) and site 2, respectively, were performed. These were carried out at low [D] ([D] $\ll K_m$ and, more importantly, [D] $\ll K_1$, so that the binding of D to site 2 could be neglected). Under these conditions, Scheme 4 was assumed to apply for the active site ligand J (**8**). The binary complex between E and I is designated EI, which could represent EI_1 or EI_2 (or a combination of both). From Scheme 4, eq 6 can be derived, and under the pseudo-first-order conditions of [D] $\ll K_m$, eq 7 gives the expression for the pseudo-first-order rate constant, k_{obs}. Thus, from

$$v/E_0 = \frac{k_{cat}[D]/(K_m + [D])}{1 + \left(\frac{K_m}{K_m + [D]}\right)([I]/K_{I(I')} + [J]/K_J + [I][J]/K_J K_{JI})} \quad (6)$$

$$k_{obs}/E_0 = \frac{k_{cat}/K_m(1 + [J]/K_J)}{1 + \left(\frac{1/K_{I(I')} + [J]/K_J K_{JI}}{1 + [J]/K_J}\right)[I]} \quad (7)$$

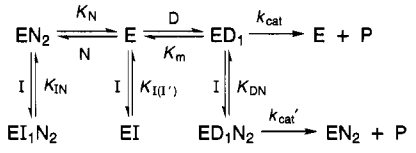
experiments at constant [D] ($\ll K_m$) and [J] and variable [I], c (of eq 1) is given by eq 8. A plot of c vs [J] for such

$$c = (1/K_{I(I')} + [J]/K_J K_{JI})/(1 + [J]/K_J) \quad (8)$$

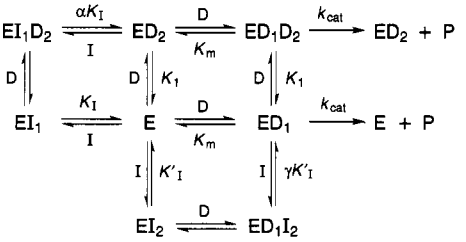
experiments where I is **4** is shown in Figure 1C. A nonlinear least-squares fit of eq 8 to this data for I = **4** (solid line) yielded $K_{I(I')} = 0.53 \pm 0.06$ mM and $K_{JI} = 12 \pm 5$ mM; K_J was fixed at 0.6 μM, the value obtained from experiments with D and J alone. This value of K_J was carefully checked by direct experiment since previous estimates were somewhat larger, 2.0 ± 0.5 μM (Pazhanisamy & Pratt, 1989b). The reason for the difference is not certain but may relate to the difficulty of getting the sparingly soluble **8** into solution. For the present work, **8** was carefully dissolved at pH ca. 9 and then diluted into the lower pH buffer.

Similarly with **3**, N, which prefers site 2, Scheme 5 was applied. This differs from Scheme 4 in the presence of a productive ternary complex of ED_1 with the reference ligand N (Pazhanisamy & Pratt, 1989a), but the equation for c is identical to eq 8 on replacement of J by N and K_{JI} by K_{IN}.

Scheme 5



Scheme 6



The c values obtained are shown in Figure 1D and yielded for **4** via the fitted solid line $K_{I(I')} = 0.28 \pm 0.02$ mM and $K_{IN} = 1.4 \pm 0.3$ mM; K_N was fixed at 1.0 mM, the value obtained from experiments with D and N alone (Pazhanisamy & Pratt, 1989b).

The array of experiments described above was performed also with I = **5**−**7**. In the fits of these data to eqs 2−8, K_I was kept at 1.7 mM, K_J at 0.6 μM, and K_N at 1.0 mM, since in several instances c did not vary significantly with the fixed ligand concentration, and thus the data would not permit the unambiguous determination of all parameters. The parameters yielding the best fits of the data are given in Table 1. The fits for **6**, where c' increases with [D], in contrast to the situation with **4**, is shown in Figure 2; here, the solid lines are those calculated from the parameters of Table 1. The columns of Table 1 headed αK_I and $\gamma K'_I$ represent the alternatives presented by Scheme 2 and eqs 2 and 4 in the former case and by Scheme 3 and eqs 3 and 5 in the latter case. Some confidence in the equations and data treatment was generated by the result that the three experiments yield acceptably similar values of $K_{I(I')}$ for each of the ligands **4**−**7**, as can be seen in Table 1.

Some preliminary experiments were also performed with *N*,*N*′-diacetyl-D-alanyl-D-alanine, the preferred small molecule substrate of the *Streptomyces* R61 DD-peptidase (Ghuysen et al., 1979). The peptide proved to be a weak inhibitor of the P99 β-lactamase ($K_I \geq 10$ mM), suggesting weak binding at site 1 at least. It might be noted however that the binding of this compound to the DD-peptidase is also rather weak [K_m, thought to represent K_s, is reported to be 10 mM (Kelly et al., 1986)].

DISCUSSION

A qualitative assessment of the data in the context of previous results (Pazhanisamy & Pratt, 1989a) indicated that they could, initially at least, be discussed within the framework of Scheme 6. In this scheme, D and I represent the depsipeptide substrate (**2**) and inhibitor (**4**−**7**), respectively; P represents the substrate hydrolysis products; and the subscripted numbers indicate occupancy of the active (productive) site (site 1) or the second (nonproductive) site (site 2). The scheme (in accord with the data) also suggests that any ternary complex ED_1I_2 is essentially nonproductive and, at the inhibitor concentrations employed in this work, little ternary complex EI_1I_2 was present in the steady state.

The data relating to experiment i can be interpreted in two alternative ways (Scheme 2 or 3) as indicated in Table 1, involving predominant binding of I either at site 1 or at site 2. Some combination of the two is not excluded by this data; the likely extent of such a combination is discussed further below. Also, taken up again will be the effect of prior D binding (to either site 1 or site 2, depending on which of the above alternatives is correct) on the affinity of the enzyme for **4**−**7**.

The relative affinity of **4**−**7** for the two sites was assessed by means of two further ligands **8** and **3**, which have primary affinity for sites 1 and 2, respectively (Pazhanisamy & Pratt, 1989b). The interactions between the ligands **4**−**7** on one hand and **8** and **3** on the other were determined from separate, pairwise experiments (ii and iii) (Schemes 4 and 5).

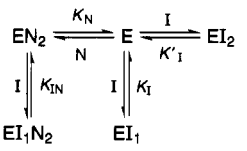
The results of experiment iii (Table 1) indicate that the presence of **3**, preferentially bound in site 2, had only small effect on the affinity of **4**−**7** for the enzyme, i.e., that the association of **4**−**7** with the enzyme could occur with comparable facility irrespective of whether **3** occupied site 2. This result is most readily interpreted of course in terms of the preferential binding of **4**−**7** to site 1, with much smaller, if any, association with site 2. The strongest indication of site 2 binding from this data is presumably in

Table 1: Dissociation Constants for Interaction of Inhibitors **4**−**7** (I) with Various Enzyme Forms

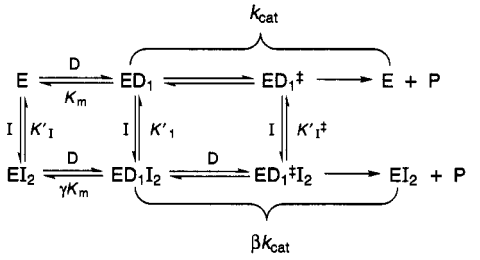
		dissociation constants (mM)[a]				
I	expt	$K_{I(I')}$	αK_I	$\gamma K'_I$	K_{JI}	K_{IN}
4	i	0.39 ± 0.02	>3[e]	>15[e]		
	ii[b]	0.53 ± 0.06			13[f]	
	iii[c]	0.28 ± 0.02				1.4 ± 0.3
5	i[d]	1.4 ± 0.1	3.5 ± 1.0	17.5 ± 5		
	ii[b]	1.5 ± 0.3			8 ± 4[g]	
	iii[c]	1.36 ± 0.2				2.6 ± 0.3
6	i[d]	0.16 ± 0.02	0.082 ± 0.005	0.41 ± 0.03		
	ii[b]	0.15 ± 0.02			0.8 ± 0.2	
	iii[c]	0.13 ± 0.02				0.15 ± 0.02
7	i[d]	0.06 ± 0.01	0.06 ± 0.01	0.29 ± 0.03		
	ii[b]	0.053 ± 0.006			0.31 ± 0.10	
	iii[c]	0.06 ± 0.01				0.06 ± 0.01

[a] K_I and αK_I refer to Scheme 2 (major binding of I to site 1), and their alternatives, K'_I and $\gamma K'_I$ refer to Scheme 3 (major binding of I to site 2); K_{JI} and K_{IN} refer to Schemes 4 and 5, respectively. J represents dansylboronate (**8**), and N is the peptide (**3**). The uncertainties quoted are standard deviations. [b] Calculated with the assumption that K_J (Scheme 4) = 0.6 μM. [c] Calculated with the assumption that K_N (Scheme 5) = 1.0 mM. [d] Calculated with the assumption that K_I (Schemes 2 and 3) = 1.7 mM. [e] >90% confidence. [f] Uncertain limits; >90% confidence that K_{JI} > 3 mM. [g] >90% confidence that K_{JI} > 4 mM.

Scheme 7



Scheme 8



that of **4** where, if it is assumed that K_I (Scheme 7) is 1.4 mM (i.e., assuming no interaction between I in site 1 and N in site 2), a value of K'_I of 0.56 mM can be calculated from the apparent dissociation constant K_I of Table 1 (taking an average value of 0.4 mM for the calculation). The assumption of no interaction between I and N when bound together may well not be a good one however. The binding of **3** to site 2 is weakend 2.8-fold on the binding of D to site 1 for example (Pazhanisamy & Pratt, 1989b). Experiment ii, discussed below, bears further on this point.

The binding of I to EJ, measured in experiment ii, is clearly considerably weaker than to E. Since J, the boronate **8**, likely to be a transition state analog (Beesley et al., 1988; Baldwin et al., 1991), is certainly bound in site 1, the implication is clear, in general agreement with that of experiment iii, that the preferential binding of **4**−**7** is to site 1.

Although this conclusion is likely to be correct, it is worth reflecting on one point relevant to the interpretation of experiment ii, the fact, just mentioned, that **8** is a transition-state analog inhibitor. The significance of this with respect to site 2 binding is seen by inspection of Scheme 8. This includes an equilibrium between the transition states $ED_1^‡$ and $ED_1^‡I_2$ which, in reality of course, cannot be established. Nonetheless, on the basis of transition-state theory, a thermodynamic cycle involving the transition states can be employed (Wolfenden, 1975) from which eq 9 follows:

$$K'_I{}^‡ = (\gamma/\beta)K'_I \qquad (9)$$

If $(\gamma/\beta) > 1$, $K'_I{}^‡ > K'_I$, I binds more strongly to E than to $ED_1^‡$, and I is an inhibitor at low [D]. Conversely, if $(\gamma/\beta) < 1$, $K'_I{}^‡$, $< K'_I$, I binds more tightly to $ED_1^‡$ than to E, and I is an activator at low [D]. To take a known example in this system, that of **3**, previous results show it to be a weak inhibitor at low [D] (Pazhanisamy & Pratt, 1989b). In accord with this, experiment yielded $\gamma = 2.8$ and $\beta = 1.7$; thus $(\gamma/\beta) = 1.65$ and hence $K'_I{}^‡ > K'_I$.

A similar analysis should apply when the substrate D is replaced by a transition-state analog $A^‡$. An inhibitor I ($\gamma/\beta > 1$) binding in site 2 will bind more tightly to E than to $EA^‡$, and conversely an activator will bind more tightly to $EA^‡$. For a good transition-state analog, the ratio (γ/β) obtained from studies of the binding of I to E and $EA^‡$ should be the same as that from those of the effects of I on turnover of the substrate D analogous to $A^‡$. This may be a useful criterion of the quality of a transition-state analog and one which could be applied to both noncovalently and covalently (Rahil & Pratt, 1994) bound transition-state analogs. The essential requirement for such a test would be an effector binding site linked to the active site. This is restrictive, but a commonly available possibility is a proton binding site.

With respect to the data of this paper, the implication of the above analysis is that **4**−**7**, all of which are inhibitors at low [D], will bind more weakly to site 2 of EJ_1 than they would to ED_1, because J (**8**) is a transition-state analog. To test this hypothesis further, we performed experiment ii employing **3** (0−10 mM) rather than **4**−**7**. The results (not shown) indicated $K_{JI} \approx 2\ K'_I$, i.e., that **3** bound more tightly to the free enzyme than to the transition-state analog complex EJ, as would be expected of an inhibitor. This result ($\gamma/\beta \approx 2$) is in acceptable agreement with that quoted above for **3** as an inhibitor of D hydrolysis.

Hence the binding of **4**−**7** to site 2, if site 1 were not occupied by a transition-state analog, would be perhaps twice as tight as suggested by K_{JI} of Table 1. Although the binding of these ligands to site 1 would then still be tighter than to site 2, the difference would not be as great as it now appears. If, for example, K_{JI} were 0.4 mM for the binding of **6** to EJ′, where J′ was a ground-state analog, then the binding of **6** to site 2 would not be greatly weaker, ca. 3-fold, than to site 1; the same conclusion would apply to **7**. In fact, if it *were* somewhat tighter, the observation of a small value of β would follow.

The conclusion of substantial, but not absolute, preferences of **4**−**7** for site 1 is supported by the data on the binding of I to ED. Previous results (Pazhanisamy & Pratt, 1989b) showed that the binding of D to Site 1 of E was essentially identically as strong as its binding to ED_2. One might anticipate therefore that if I bound preferentially to site 1, its binding to E would be of comparable strength of that to ED_2. With the exception of **4** (see below), this does seem to be true—K_I is similar in magnitude to αK_I.

On the other hand, the affinity of D for E, binding in site 2, is also very similar to its affinity for ED_1 (Pazhanisamy & Pratt, 1989b). One might therefore anticipate, if I preferably bound to site 2, that the binding of I to E would be comparably strong to that of I with ED_1. This is certainly less true than the alternative above—K'_I is smaller than $\gamma K'_I$ for all **4**−**7**.

Thus, the data seem best interpreted in terms of the preferential binding of **4**−**7** to site 1. Weaker but significant binding to site 2 is also observed, particularly for the β-lactams **6** and **7** as seen most clearly in the K_{JI} values (Table 1). It is now informative to consider these conclusions in terms of the structures of **2**−**8**.

First, it is presumably unsurprising to find that the preferential binding of the β-lactams **6** and **7** is to site 1, the active site, since these compounds are substrates of this enzyme. The inhibition constants of these compounds, K_I, should therefore be equal to the K_m values for their turnover. The K_I value for **6**, 0.15 ± 0.02 mM, is not unreasonably different from the directly determined K_m, 0.29 ± 0.05 mM, but that for **7**, 0.06 ± 0.01 mM, seems distinctly different from its K_m, 0.29 ± 0.04 mM. The reason for these differences are not immediately clear but may well be the same as those responsible for a variety of substrate-specific hysteretic events that are not infrequently observed with β-lactamases (Zyk & Citri, 1968; Citri et al., 1976; Hashizume et al., 1988; Monks & Waley, 1988) although not yet

satisfactorily explained in molecular terms. In the present case, since the common substrate **2** is involved in all experiments, the qualitative interpretation of the results should be unaffected. The deacylation step is most likely rate-determining to the turnover of **6** and **7** at saturation (Knott-Hunziker et al., 1982; Govardhan & Pratt, 1987; Mazzella & Pratt, 1989; Monnaie et al., 1992), and thus K_m^{-1} probably represents binding to the stage of the acyl enzyme. That similar dissociation constants are obtained for **6** and **7** in the presence of **2** (αK_I) and **3** (K_{IN}) indicates that (depsi)-peptide species may bind to site 2 at the acyl enzyme stage of turnover. The presence of these ligands, at least, has little influence on acyl enzyme formation. Jamin et al. (1993) have concluded that depsipeptides may also bind to a second site on acyl enzymes formed on reaction between the same depsipeptide and the *Streptomyces* R61 DD-peptidase. This is likely true for the P99 β-lactamase also (Mazzella et al., 1991).

The penicilloates **4** and **5** both inhibit the P99 β-lactamase, with the dianionic dicarboxylate **4** being somewhat more effective in binding to the free enzyme (lower K_I). This result contrasts strikingly with that observed with the class A β-lactamase I of *Bacillus cereus* where **5** is by far the better inhibitor (Jones et al., 1989). The analogous penilloate, lacking the β-lactam-derived carboxyl group, is also a better inhibitor of this enzyme than is **4** (Kiener & Waley, 1978). Similarly perhaps, substrates of the class A enzyme having additional negative charge adjacent to the β-lactam ring, such as carbenicillin and sulbenicillin, are poorer substrates than their neutral analogs (Hardy et al., 1984). These observations with the class A enzyme have been interpreted in terms of unfavorable electrostatic interaction between the additional carboxylate and a negative charge in the active site, proposed to be Glu 166 by Jones et al. (1989). An alternative explanation would be a hydrophobic environment, although the crystal structures certainly indicate a rather polar active site. The present results with **4** and **5** could be incorporated into the electrostatic model in that class C enzymes do not have a carboxylate group in an analogous position to that of Glu 166 of the class A (Lobkovsky et al., 1993). They *do* have what may be a negatively charged Tyr 150 side chain, but the position of the anion would be different (Oefner et al., 1990; Lobkovsky et al., 1994). Thus, perhaps more under the influence of a local positive charge, e.g. of Lys 67, **4** may be the better inhibitor of the class C P99 β-lactamase. It is interesting that the presence of **2** or **3** in site 2 weakens the binding of **4** more than that of **5**; this could represent unfavorable electrostatic interaction between the negatively charged ligands.

Perhaps the most interesting result is the finding that **4**–**7** bind to site 2 when the active site is occupied by **8**, a transition-state analog (K_{II}). This makes it likely that such binding would also occur when a substrate undergoing turnover occupied the active site. As discussed above, this binding would probably be weakest at the transition state or, equivalently, to a transition-state analog. Certainly **2** and **3**, as also discussed above, seem to be able to occupy site 2 during substrate turnover. Extrapolation of the latter result to the flexible **4** and **5** would not perhaps be surprising, but the suggestion that **6** and **7**, compact β-lactam structures, bind so well to site 2 is certainly interesting particularly in view of the fact that their binding to site 1 involves acyl enzyme formation. Whether any covalent chemistry might be involved in the interaction of **6** and **7** with site 2 is not known at present. It may be significant, in this regard at least, that the ED_1I_2 complexes where I is **6** or **7** have little or no ability to regenerate free enzyme, i.e., β (Scheme 8) $\ll 1$. This is a matter worthy of further investigation. It might be noted here also that Ghuysen and co-workers at one stage favored a β-lactam binding site separate from the active DD-peptidase site of the R61 enzyme, but later analysis showed that the evidence for this was compromised by problems with analysis of the kinetics (Frère & Joris, 1985). The presence of additional binding was not disproved however.

The curiously strong binding of **6** and **7** to site 2 in EJ may reflect the fact that although **8** may be a transition-state analog as far as the boronate moiety is concerned, the remainder of the inhibitor is not substrate-like. It is a matter of some interest in fact why **8** and, to a lesser extent, other aryl boronates (Beesley et al., 1988) are such good inhibitors of class C β-lactamases. It may be that hydrophobic binding of the dansylamidophenyl moiety to the protein specifically enhances the binding of **6** and **7** to site 2.

The situation with respect to D-Ala-D-Ala peptide binding to site 2 was not pursued in this work. It seemed clear that binding of *N,N'*-diacetyl-L-lysyl-D-alanyl-D-alanine was weak at site 1, and if binding at site 2 occurred, it had little linkage with Site 1. Under these circumstances, further exploration of the binding of this and other peptides was postponed until a more direct assay of site 2 binding was developed (Dryjanski & Pratt, 1995).

The present work provides further evidence for the existence of at least one small peptide binding site beyond the active site of the P99 β-lactamase. There is clear evidence that ligand binding to this site can occur in the free enzyme, at the acyl enzyme stage, and that the ligand may be present as the enzyme passes through transition states. It is possible that an extended binding area is involved and the various test ligands **4**–**7** occupy different or overlapping regions of it. Further studies are needed to define its localization with respect to the active site and its existence and role in DD-peptidases.

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