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

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

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

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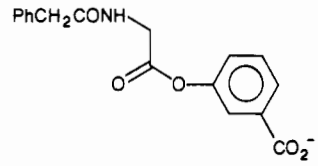

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aminolysis product [(phenylacetyl)glycyl-D-phenylalanine]. The enzyme also catalyzes a competing hydrolysis reaction which produces H, (phenylacetyl)glycine, and P (*m*-hydroxybenzoate), the latter in common with the aminolysis reaction. In the absence of an amine, the enzyme catalyzes the hydrolysis alone (Pratt & Govardhan, 1984; Govardhan & Pratt, 1987).

Scheme I has also been proposed, again to accommodate kinetic data, to apply to the hydrolysis and aminolysis (transpeptidase reaction) of specific peptide substrates catalyzed by a variety of DD-peptidases which have been isolated from *Streptomyces* species (Frere et al., 1973; Ghuysen et al., 1979; Nguyen-Disteche et al., 1986). The latter reaction is of course essential for the cross-linking of bacterial cell walls, and inhibition of the DD-peptidases responsible is the primary goal of β-lactam antibiotics. Since no confirmatory proof of Scheme I has yet been offered and there have been indications that it might be incorrect, or at least incomplete (Pazhanisamy et al., 1989), we have taken the opportunity provided by its appearance in connection with β-lactamases to examine the problem in more detail.

An essential requirement of the kinetic mechanism of Scheme I is that the amine forms a binary complex, EA, with free enzyme. In the case of the P99 β-lactamase-catalyzed aminolysis of **1** by D-phenylalanine, the steady-state kinetics closely define the stability and lability of that complex. The dissociation constant K_1 ($=k_{-1}/k_1$) must be <2 mM at pH 7.5 and <1 mM at pH 9.0. Values for the rate constants have also been determined (Pazhanisamy et al., 1989). The steady-state kinetics require that, at concentrations of **1** above ca. 5 mM, formation of EA be rate determining, i.e., that $k_3[D]/K_2 > k_{-1}$. These quantitative properties of EA provide the basis of the tests for Scheme I given in this paper. A definitive conclusion, that Scheme I cannot be correct, was achieved by application of a double-label isotope-trapping technique. This method appears to be very effective for rapid reactions where incomplete mixing and significant turnover subsequent to the pulse but prior to the quench are problems that need careful attention.

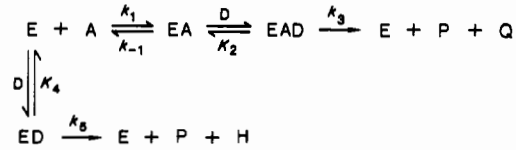
Experimental Procedures

Materials

The *Enterobacter cloacae* P99 β-lactamase and its depsipeptide substrate **1** were obtained as described in the accompanying paper (Pazhanisamy et al., 1989). Amino acids and buffer materials were obtained from commercial sources. Cephalothin was a gift of Eli Lilly and Co. Aldrich supplied phenylboronic acid and (3-aminophenyl)boronic acid. Phenylmethanesulfonyl fluoride was purchased from Sigma Chemical Co. ^3H_2O (1 Ci/mL) and [U-^{14}C]-L-phenylalanine (405 Ci/mol) were obtained from ICN Biomedicals, Inc. [1-^{13}C]-D-Phenylalanine was purchased from MSD Isotopes, Canada. The assay enzymes, D-amino acid oxidase and catalase, were from Sigma.

(3-Dansylamidophenyl)boronic acid was prepared from reaction of (3-aminophenyl)boronic acid (3.25 mmol) and dansyl chloride (Sigma) (3.25 mmol) for 5 h in 50% aqueous acetone containing sodium bicarbonate (5 mmol) at 25 °C.

Scheme I



The product was isolated by evaporation, purified by recrystallization from hot water, and characterized by ^{1}H NMR, absorption, and fluorescence spectra (Burnett et al., 1980).

Attempts To Detect Binding of D-*Phenylalanine to P99 β-Lactamase*

(*1*) *Direct Methods.* (*a*) *Circular Dichroism Spectroscopy.* The CD spectra of the P99 β-lactamase (25 μM) in 0.1 M carbonate buffer (pH 9.0) were obtained in the presence and absence of D-phenylalanine (1–5 mM). The instrument used was a Varian AVIV 60DS CD spectrophotometer (Yale University Instrument Center, New Haven, CT).

(*b*) *Fluorescence Spectroscopy.* The fluorescence emission spectrum of D-tryptophan (0.53 mM in 0.1 M carbonate buffer at pH 9.0) produced on irradiation at 310 nm was recorded on a Perkin-Elmer MPF-44 spectrofluorometer in the absence and presence of the P99 β-lactamase (0.07 mM).

(*c*) *Nuclear Magnetic Resonance Spectroscopy.* Chemical shifts and line widths of D-phenylalanine ^{1}H resonances were obtained at 25 °C from a Varian XL-400 spectrometer in the absence and presence of the P99 β-lactamase. Samples in ^2H_2O contained 0.25–5.0 mM D-phenylalanine, 0.10–0.13 mM P99 β-lactamase, and 1 mM each of ethylenediaminetetraacetic acid and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid in either 20 mM MOPS buffer at pH 7.5 or 0.1 M carbonate at pH 9.0. ^{13}C NMR spectra of 1.1 mM [1-^{13}C]-D-phenylalanine were also obtained in the presence and absence of the enzyme under the same conditions as above.

(*d*) *Equilibrium Dialysis.* These experiments were carried out both in classical dialysis cells with a semipermeable cellulose membrane (VWR Scientific, catalog no. 25225-226) and by the batch gel filtration method (Hirose & Kano, 1971; Oberfelder et al., 1984; Na & Timasheff, 1985). The lucite dialysis cells contained two 0.3-mL compartments divided by the membrane. Equal volumes of buffer (20 mM MOPS, pH 7.5, or 0.1 M carbonate, pH 9.0) and buffer containing both enzyme (ca. 0.25 mM) and D-phenylalanine (0.5–1.0 mM) were placed in the cell and equilibrium was established in a thermostated (25 °C) mechanical shaker in 10–15 h. The time required to achieve equilibrium was established by a control experiment with D-phenylalanine alone present. As a further control, an experiment was performed where the D-phenylalanine and enzyme started in different compartments. After equilibrium was established, samples were taken from each side of the membrane, and D-phenylalanine concentrations were determined by the D-amino acid oxidase/peroxidase coupled assay (Putter & Becker, 1983) in 0.1 M Tris buffer at pH 8.7, where the chromophoric reductant whose oxidation was monitored at 460 nm was *o*-dianisidine. The β-lactamase concentration was determined spectrophotometrically (Pazhanisamy et al., 1989). The experiment was repeated four times at pH 7.5 and five times at pH 9.0. The activity of the enzyme at the end of the experiment was also determined to confirm that the enzyme remained functional.

For the gel filtration procedure, a known weight of dry Bio-Rad P-6DG gel (molecular weight exclusion 6000) was swelled in 200 μL of buffer for 4 h. Then D-phenylalanine solution was added by pipet, to a final concentration of about

0.5 mM, and the mixed solution was allowed to stand for 30 min at 25 °C. At this stage, the clear supernatant was removed by syringe and assayed for D-phenylalanine by the D-amino acid oxidase method. This result established the volume available to D-phenylalanine. In a separate experiment both D-phenylalanine and the P99 β-lactamase were added, to final concentrations of about 0.5 mM and 0.3 mM, respectively, and the D-phenylalanine concentration in the supernatant was again determined. The concentration of the enzyme in the supernatant was determined spectrophotometrically.

(*2*) *Indirect (Kinetic) Methods.* (*a*) *Inhibition of Cephalothin Hydrolysis.* The initial rate of cephalothin (74 μM; K_M = 20 μM) hydrolysis in 20 mM MOPS buffer, pH 7.5, 25 °C, was measured spectrophotometrically at 278 nm (Perkin-Elmer Lambda 4B spectrophotometer) in the absence and presence of D-phenylalanine (up to 20 mM).

(*b*) *Protection from Phenylmethanesulfonyl Fluoride Inhibition.* The rate of inactivation of the P99 β-lactamase by phenylmethanesulfonyl fluoride (0.75 mM, added from an acetonitrile stock solution) was determined by following the activity of an enzyme sample by benzylpenicillin (0.5 mM) assay (spectrophotometrically, 232 nm). The concentration of the enzyme was 2.5 μM, and the buffer was 20 mM MOPS, pH 7.5, 25 °C. Pseudo-first-order rate constants of inactivation were determined in the absence and presence of 20 mM D-phenylalanine.

(*c*) *Protection from Inhibition by Arylboronic Acids.* At a concentration of 24.2 μM, phenylboronic acid inhibits cephalothin (73.3 μM) hydrolysis, catalyzed by the P99 β-lactamase (3.2 nM), by some 45%, in 20 mM MOPS buffer containing 0.5 mg/mL bovine serum albumin, pH 7.5, 25 °C. The initial rate of the hydrolysis in the presence of the boronate was also measured with 20 mM D-phenylalanine present.

The inactive binary complex between the P99 β-lactamase and (3-dansylamidophenyl)boronic acid has a dissociation constant of 2.0 ± 0.5 μM and enhanced fluorescence at 550 nm with respect to that of the unbound dansyl compound on irradiation at 338 nm. The effect of D-phenylalanine (22 mM) on both the dissociation constant and fluorescence of the complex was examined.

(*3*) *Double-Label Isotope-Trapping Experiment.* (*a*) *Synthesis of [2-^{3}H]-DL-Phenylalanine.* The procedure of Tabushi et al. (1987) for the racemization of phenylglycine was adapted for this synthesis. D-Phenylalanine (20.5 mg), pyridoxal hydrochloride (3.0 mg), and potassium phosphate ($K_3PO_4 \cdot 12H_2O$, 96 mg) were dissolved in 250 μL of tritiated water (sp act. 1 Ci/mL) in a vacuum reaction tube (Pierce, catalog no. 29550) and heated in an oven at 95 °C for 6.5 h. After cooling, the reaction mixture was freeze-dried, redissolved in H_2O, and freeze-dried again. The residue was taken up into about 1 mL of water and the pH adjusted to about 8. The pyridoxal was then removed by elution of the sample with water through a short ω-aminohexyl-Sepharose 4B (5 mL) column. The radioactive fractions [scintillation counting was carried out in Scintisol (Isolab) (5 mL) solution in a Packard Tri-Carb 460C scintillation counter] were then passed through a 5-mL Dowex 50X4-400 (H^+ form) column, from which, after it had been washed with water, the phenylalanine was eluted with 1 M HCl. Fractions containing radioactivity and the absorption spectrum of phenylalanine were pooled and freeze-dried. The yield of phenylalanine was 14.5 mg (71%) of specific radioactivity of 2.3 Ci/mol.

(*b*) *Synthesis of [U-^{14}C]-DL-Phenylalanine.* The procedure used for the tritiated material was also used here, beginning with 50 μCi of [U-^{14}C]-L-phenylalanine, mixed with 2.0 mg of cold D-phenylalanine, and yielding 1.1 mg of product containing 27.5 μCi (4.13 Ci/mol with respect to total phenylalanine).

The extent of racemization of this sample was determined by the D-amino acid oxidase catalyzed oxidation of D-phenylalanine, followed by separation of the product. The sample for assay (ca. 0.4 μCi) was diluted with cold D-phenylalanine (1 mM) in 1 mL of 0.01 M Tris buffer, pH 8.7. The assay was set up in duplicate. The reaction mixtures consisted of 50 μL of sample, 0.4 mL of the same Tris buffer, 10 μL of D-amino acid oxidase suspension (ca. 0.5 unit), and 25 μL of catalase suspension (32 500 units). This was incubated at 25 °C while four further 10-μL additions of fresh D-amino acid oxidase were made at 5–8-h intervals. The latter, with one (25 μL) further addition of catalase, were added to ensure complete oxidation of the D-phenylalanine. The product of the oxidation (phenylpyruvate) was separated from the unreacted phenylalanine by passage of the reaction mixture through a 2-mL Dowex 50X4-400 (H^+ form) column. The total eluate from the column after a 30-mL water wash was evaporated and the residue taken up into 4 mL of water with 25 μL of 0.1 M NaOH. After this solution was freeze-dried into a scintillation vial, 50 μL of water and 5 mL of Scintisol were added, and the amount of radioactivity was determined. The above assay was repeated three times, and the radioactive phenylalanine was thereby found to be 31.8 ± 2.6% D-phenylalanine. Thus, the specific radioactivity of the D-phenylalanine used in the isotope-trapping experiment was 3.08 Ci/mol. Small amounts of (labeled) L-phenylalanine still present were inconsequential since L-phenylalanine is not a substrate at pH 7.5 (Pazhanisamy et al., 1989). The ^{14}C-labeled phenylalanine was dissolved in 1.5 mL of 20 mM MOPS buffer, pH 7.5, along with 4.57 mg of cold D-phenylalanine in order to make a 20 mM D-phenylalanine stock solution labeled with ^{14}C. Dilutions of this solution were made for the experiment, as needed.

(*c*) *Instrumentation.* A Durrum D-132 triple-mix apparatus was used as a rapid-quench device. The 1:1 mixing ratio syringes supplied with the instrument were replaced with 1:10 ratio syringes, thus providing an approximately 1:10:28 pulse:chase:quench mixing ratio. The drive, dump, and collect times were set at 170, 100, and 70 ms, respectively, with the gas pressure for the pneumatic drive set at 60 psi. The delay-line loop between mixing jets 1 and 2 was reduced to 5 cm to minimize the number of turnovers prior to the quench. These conditions ensured complete purging of the solution from the previous run (0.8–0.9 mL of dump volume) and sufficient sample size (ca. 0.6 mL of collect volume) for analysis. The flow rate under these conditions, determined from the volume collected over the set collect time, was 8.5 ± 0.5 mL s^{-1}. The reproducibility of the pulse:chase mixing ratio was checked by using [^{3}H]-D-phenylalanine or methyl red solutions in the pulse. A 10 mg/mL bovine serum albumin solution was also used in the pulse to check the performance of the apparatus when a concentrated protein solution was quenched with 0.1 M HCl.

(*d*) *Isotope-Trapping Experiment.* The pulse solution, consisting of the P99 β-lactamase (ca. 0.3 mM) and [U-^{14}C]-D-phenylalanine (1–20 mM) in 20 mM MOPS buffer, pH 7.5, was loaded into the smallest of the three drive syringes. This syringe and the channel leading to it from the reservoir syringe were dried prior to loading to avoid dilution. Also, to minimize enzyme denaturation while loading, the channel was filled with pulse solution before installation of the drive

Table I: Incorporation of [U-^{14}C]Phenylalanine from the Pulse into the Peptide Product, Catalyzed by the P99 β-Lactamase

[Phe] (mM) in pulse (^{14}C labeled)	turnovers before quench	^{14}C in product × 10^{-4} (cpm)[a]			
		total	from pulse	predicted from pulse[b]	fraction saturation
20.0	69 ± 21	2.93 ± 0.26	1.59 ± 0.17	1.24 ± 0.10	1.27 ± 0.17
10.0	90 ± 10	1.38 ± 0.08	0.52 ± 0.03	1.18 ± 0.05	0.44 ± 0.03
2.0	115 ± 16	0.32 ± 0.05	0.12 ± 0.05	1.18 ± 0.04	0.10 ± 0.04
1.0	130 ± 13	0.22 ± 0.03	0.12 ± 0.04	1.12 ± 0.05	0.11 ± 0.04
1.0[c]	312 ± 36	0.37 ± 0.09	0.22 ± 0.05	1.06 ± 0.12	0.21 ± 0.04

[a] Normalized to 1-mL quenched reaction mixture and 1.0 mM enzyme in the pulse. Quoted uncertainties are standard deviations from at least five separate quenched reaction mixtures. [b] If all enzyme were present as a productive enzyme/D-phenylalanine complex in the pulse. [c] Flow rate 2.0 ± 0.5 mL s^{-1} (cf. 8.5 ± 0.5 mL s^{-1} in the other experiments).

syringe. The channel leading to the first mixing jet was also filled with pulse solution. The chase solution, 20 mM depsipeptide **1** and 150 mM D-phenylalanine in 20 mM MOPS buffer, pH 7.5, also contained sufficient [2-^{3}H]-D-phenylalanine to give a ca. 1:1 ^{14}C:^{3}H ratio on mixing with the pulse (in a 1:10 volume ratio). The quench syringe was filled with 0.1 M HCl. A 1-mL plastic syringe was used to collect the quenched sample for assay. After each run the sample was transferred into a preweighed vial, a 20–50 μL aliquot was taken for determination of the ^{3}H/^{14}C ratio, and the weight of the remaining sample was obtained by difference. This ^{3}H/^{14}C ratio, since the specific activity of D-phenylalanine, in both pulse and chase, was known, gave the mixing ratio individually for each experiment. The extent of dilution of the pulse solution was also used to calculate the concentration of the enzyme after mixing. The remaining sample was then immediately steam heated for 1 min and then cooled on ice to await further analysis. Control experiments showed no regain of enzyme activity in solutions thus quenched.

The aminolysis product, (phenylacetyl)glycyl-D-phenylalanine, was separated from D-phenylalanine by passage of the sample through a small (5 mL) Dowex 50X4-400 (H^+ form) column. The effectiveness of this procedure was established by control experiments. The total eluate, including 60–80 mL of water wash, was dried for scintillation counting. The ^{3}H/^{14}C ratio channel was used to determine both the ^{3}H and ^{14}C in the sample. Standard ^{3}H/^{14}C D-phenylalanine mixtures were prepared to establish the efficiency of ^{14}C counting in the ^{3}H channel and its invariance over a wide range of ^{3}H/^{14}C ratios. From knowledge of the amount of ^{3}H in the product and the concentration of enzyme after mixing, the number of turnovers between the pulse and the quench could be calculated. After complete mixing, the ratio in which ^{3}H and ^{14}C appear in the product should be that found in the quenched reaction mixture. Thus, the amount of ^{14}C in the product arising from turnovers subsequent to complete mixing could be calculated. This could be subtracted from the total amount of ^{14}C in the product to determine the incorporation of ^{14}C from a β-lactamase/D-phenylalanine complex in the pulse solution.

Results

The spectroscopic methods used in an attempt to detect the P99 β-lactamase/D-phenylalanine complex, viz., fluorescence, ^{1}H and ^{13}C NMR, and CD, gave no hint of the existence of this species under the contions described above. The fluorescence and CD spectra of the enzyme/D-phenylalanine mixtures were identical with the sums of the individual component spectra. The addition of enzyme did not affect either the position or the line width of the ^{1}H and ^{13}C resonances of D-phenylalanine NMR spectra. Nor were protons released or taken up on addition of D-phenylalanine (up to 30 mM) to the P99 β-lactamase (Govardhan, 1986).

D-Phenylalanine did not affect the β-lactamase activity of the enzyme, in the hydrolysis of either benzylpenicillin (Pazhanisamy et al., 1989) or cephalothin. Nor did it affect the rate and position of equilibrium, respectively, of inhibition of the enzyme by phenylmethanesulfonyl fluoride and the arylboronates. The latter compounds probably react with the active site serine hydroxyl group (Bush et al., 1982; Beesley et al., 1983; Crompton et al., 1988). The dansyl fluorescence of the β-lactamase/(3-dansylamidophenyl)boronate complex was also unaffected by the addition of D-phenylalanine to the solution. Compounds known to bind at the active site, β-lactams and **1** for example, did inhibit the binding of the dansyl boronate.

The equilibrium dialysis experiments, at both pH 7.5 and pH 9.0, gave no evidence for association of the P99 β-lactamase with D-phenylalanine. The limits of precision of the experiment, dictated by the enzyme concentrations employed and the assay method, suggested that the dissociation constant of any β-lactamase/D-phenylalanine complex must be greater than 2 mM.

The data from the isotope-trapping experiment at pH 7.5 are shown in Table I. At each D-phenylalanine concentration at least five runs were made, and the numbers reported are the averages and standard deviation of these. The tritium incorporation data (not shown) indicated that about 100 turnovers occurred between complete mixing and the quench. This appears to be an appropriate number in view of the reactant concentrations and reaction time (the latter estimated from the flow rate and channel dimensions to be around 30 ms). Subtraction of the amount of ^{14}C incorporated into the product during these subsequent turnovers gave the amount of ^{14}C incorporated during the pulse (Table I). This may be compared with the incorporation predicted on the basis of the existence of a tight 1:1 complex of the β-lactamase and D-phenylalanine in the pulse, and thus, the apparent fraction of saturation of the enzyme by D-phenylalanine could be calculated. This is shown in Table I and in Figure 1 as a function of D-phenylalanine concentration in the pulse. Also shown in Figure 1 is the theoretical curve for a complex of dissociation constant 1.52 mM (one standard deviation higher than the best estimate, 1.29 mM), and with the dissociation rate reported in the previous paper (Pazhanisamy et al., 1989).

It is clear, on one hand, that the incorporation of D-phenylalanine in the pulse does not follow the pattern expected for a β-lactamase/D-phenylalanine complex with the specified properties. On the other hand, incorporation of ^{14}C has occurred, although to a much smaller extent at low D-phenylalanine concentrations than anticipated for the complex and increasing apparently linearly with the D-phenylalanine concentration of the pulse. At the highest D-phenylalanine concentration employed, the incorporation is greater than 1 mol of D-phenylalanine/mol of enzyme, which is also unanticipated for a putative 1:1 complex. This pattern of ^{14}C incorporation

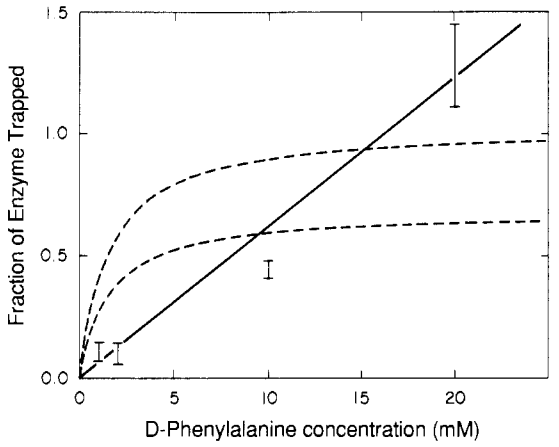


FIGURE 1: Fraction of the P99 β-lactamase apparently associated with [U-^{14}C]-D-phenylalanine, as a function of D-phenylalanine concentration in the pulse, on chasing with [2-^{3}H]-D-phenylalanine and the depsipeptide **1** (20 mM) in the isotope-trapping experiment described in the text. Also shown (--) is the expected result if Scheme I were correct and the previously determined rate and equilibrium constants (Pazhanisamy et al., 1989) obtained (eq 1); the area enclosed by the lines encompasses the standard deviations in k_3/K_2 and k_{-1} which yield the uncertainties in first-order rate constants shown in eq 1.

is probably an artifact stemming from selective ^{14}C incorporation at incomplete stages of mixing. In principle, the isotope-trapping method requires instantaneous mixing, i.e., a mixing time much smaller than the half-life of the complex to be trapped. If this is not completely so, as it is not in this case (the half-life of the complex should be, according to the rate constants derived for Scheme I, around 0.2 ms, which is of the same order as the mixing time), then extra labeling of the product will occur (Rose, 1980). That incomplete mixing, in the time frame of the ensuing reaction, is in fact responsible for the incorporation of [U-^{14}C]-D-phenylalanine into the product shown in Figure 1 is indicated by the results of an experiment where the speed of mixing was decreased by decreasing the flow rate to 2.0 ± 0.5 mL s^{-1} (by lowering the gas pressure on the pneumatic drive ram from 60 to 35 psi). As would be expected, if incomplete mixing were the problem, the number of turnovers between the chase and quench increased, as did that part of the ^{14}C labeling of the product apparently arising from the first turnover, which doubled (Table I).

Discussion

Although the spectroscopic and kinetic methods (methods 1a–c and 2a–c) provided no evidence *for* the existence of a binary P99 β-lactamase/D-phenylalanine, EA, complex of the stability required to accommodate the steady-state kinetics, they could not *prove* that one did not exist, unlikely as it perhaps may seem that the binding of D-phenylalanine to the enzyme would not affect at least one of the diverse properties examined. In view of the importance of the result however—do acyl group acceptors really bind to these enzymes, both β-lactamases and DD-peptidases?—we felt it necessary to go further.

The equilibrium dialysis experiments at both pH 7.5 and pH 9.0 gave a lower limit to the value of K_1 (ca. 2 mM), which suggested that an EA complex of the required stability did not exist. Yet the limit provided by these data fell tantalizingly close to the upper limit that would accommodate the kinetic mechanism shown in Scheme I at pH 7.5, also ca. 2 mM. It was felt therefore, also taking into account the possibility of small uncertainties in the dialysis results arising from various artifactual and nonartifactual sources, e.g., adsorption to the surfaces, nonideality of the solutions, etc., that this result, like those above, was not definitive. The situation at pH 9.0 looked clearer since the kinetics there appeared to require a K_1 value of 0.35 mM (Pazhanisamy et al., 1989). However, at pH 9.0 some of the features of the kinetics were different than those at pH 7.5 (Pazhanisamy et al., 1989). Although these differences were not substantial enough to unambiguously indicate the presence of a different or another competing mechanism, they did , along with the differences in amine specificity observed at pH 9.0, suggest that a more detailed examination of the reaction at that pH would be necessary before confident conclusions could be made. In this paper therefore we concentrated more on the reaction at pH 7.5.

The isotope-trapping method, whose application to the detection of noncovalent complexes was largely developed by Rose and co-workers (Rose et al., 1974; Rose, 1980), appeared to be almost ideally suited to this problem. It has an additional advantage over the other methods in that only kinetically competent complexes can be detected. The steady-state kinetics in the present case required that the forward reaction leading to products be faster than dissociation of the binary EA complex at easily achievable depsipeptide, D, concentrations. Under the concentration conditions chosen, the situation was that of eq 1, and thus on addition of sufficient D to preformed EA, most of the EA should be converted to product prior to dissociation to E and A.

$$\mathrm{E} + \mathrm{A} \underset{(1.10 \pm 0.48) \times 10^{3}\ \mathrm{s}^{-1}}{\overset{(8.5 \pm 3.3) \times 10^{5}[\mathrm{A}]\ \mathrm{s}^{-1}}{\rightleftharpoons}} \mathrm{EA} \xrightarrow[\text{(at 20 mM D)}]{(5.9 \pm 0.7) \times 10^{3}\ \mathrm{s}^{-1}} \mathrm{E} + \mathrm{P} + \mathrm{Q} \quad (1)$$

The main difficulty in carrying out the experiment lay with the large rate constants for breakdown of EA, which required that both the mixing of EA with D (the chase) and the quenching of the reaction (to preclude, or at least minimize, production of Q in turnovers subsequent to the trapping of the preformed EA) would have to be done very rapidly. We were able to achieve sufficiently fast mixing with the commercial Durrum D-132 triple-mixing system, where a gas-driven ram drives the contents of two syringes together. After a preset reaction time, determined by a delay loop, the contents of a third syringe, the quench, are equally rapidly mixed with the reaction solution. The estimated mixing time under the conditions employed is estimated by the manufacturer to be ca. 2 ms (99.5% complete). The problem of product arising from additional turnovers prior to the quench was dealt with by (a) use of ^{14}C-labeled A in the initial mixture of E and A to form the putative EA complex, (b) making the delay loop as short as possible, (c) dilution of the ^{14}C-labeled A with a high concentration of cold A in the chase, and (d) use also of ^{3}H-labeled A in the chase. The latter feature was found to be extremely useful in determining the exact mixing ratios for individual runs (which varied slightly) and the exact number of additional turnovers subsequent to complete mixing. From the latter, the amount of EA trapped from the preformed (pulse) complex could be accurately determined. This double-label method should be generally useful for fast reactions when a large number of turnovers between the pulse and the quench is unavoidable. Even with these features, evidence of incorporation of ^{14}C into P from incomplete mixing, a well-known artifact of the method (Rose, 1980), was obtained (presumably from diffusion of D into yet unmixed streams of E with [^{14}C]A). This artifact however was not serious enough to compromise the results of the experiment (see Results).

The data from this experiment (Figure 1) clearly indicate that a productive binary EA complex with the stability and lability required by the steady-state kinetics does not exist at

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).

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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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