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The pH dependence of the active-site serine DD-peptidase of *Streptomyces* R61

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Titration of the active-site serine DD-peptidase of *Streptomyces* R61 shows that formation of acyl enzyme during hydrolysis of the substrate Ac_2-L-Lys-D-Ala-D-Ala and enzyme inactivation by the β-lactam compounds benzylpenicillin, *N*-acetylampicillin and ampicillin relies on the acidic form of an enzyme's group of p$K \approx 9.5$. It is proposed that protonation of a lysine ε-amino group facilitates initial binding by charge pairing with the free carboxylate of the substrate and the β-lactam molecules. Lowering the pH from 7 to 5 has no effect on the second-order rate constant of enzyme acylation by benzylpenicillin and *N*-acetylampicillin but results in a decreased rate constant of acylation by ampicillin and Ac_2-L-Lys-D-Ala-D-Ala. Protonation of the side-chain amino group of ampicillin and a decreased efficacy of the initial binding of the peptide to the enzyme seem to be responsible for the observed effects. Whatever the molecule bound to the enzyme, there is no sign for the active involvement of an enzyme's histidine residue of pK 6.5–7.0 in the hydrolysis pathway.

Two families of bacterial enzymes specifically interact with β-lactam compounds. The β-lactamases, which hydrolyse the β-lactam ring, can play an important role in resistance. The DD-peptidases, which participate in the last stages of wall peptidoglycan metabolism, are the primary targets of these antibiotics [1, 2]. Many of the β-lactamases (classes A and C) and DD-peptidases characterized so far are serine enzymes and react with β-lactam compounds according to reaction (1).

$$E + D \overset{K}{\rightleftharpoons} E \cdot D \xrightarrow{k_{+2}} E-D^* \xrightarrow{k_{+3}} E + P(s) \qquad (1)$$

where E = enzyme, D = β-lactam, k_{+2} and k_{+3} = first-order rate constants, K = dissociation constant of E · D (Michaelis complex) and E–D* = serine ester-linked acyl enzyme [3]. Most often, the value of k_{+3} is high or very high with the β-lactamases, and so low with the DD-peptidases that a slight stoichiometric excess of β-lactam compound causes long-lasting immobilization of the protein at the level of the acyl enzyme and these latter enzymes behave as penicillin-binding proteins [2–5]. Another major difference is that breakdown of the acyl enzyme formed by reaction between penicillins and DD-peptidases involves either the direct release of the penicilloyl moiety or fragmentation of this moiety, with the formation of *N*-formyl-D-penicillamine and a novel, unstable acylglycylenzyme [6]. The extracellular active-site serine DD-peptidase of *Streptomyces* R61 has been crystallized [7] and its primary structure established [8, 9]. Although this primary structure shows little homology with that of class A β-lactamases, X-ray diffraction studies [10, 11] show striking similarities in the relative positions of secondary structure elements in the R61 DD-peptidase and the *Bacillus cereus* and *Bacillus licheniformis* β-lactamases. Apart from the fact that in all studied DD-peptidases and β-lactamases of classes A and C, the active-site serine is followed by an Xaa-Xaa-Lys sequence (which suggests that this lysine probably plays an important role), little is known concerning the enzymes' functional groups involved in substrate binding and catalysis. Studies of the pH dependence of the kinetic parameters of class A and C β-lactamases [12, 13] indicate the possible involvement of a carboxylate and of a Lys or Tyr residue in the enzymatic mechanisms.

Much less is known about the interaction between the DD-peptidases and their peptide substrates. However, all available data [2, 14] indicate that these enzymes catalyse acyl-transfer reactions from R-D-Ala-D-Ala-terminated carbonyl donors (D) according to a pathway which closely resembles reaction (1)

$$E + D \overset{K}{\rightleftharpoons} E \cdot D \xrightarrow[D\text{-Ala}]{k_{+2}} E-D^* \xrightarrow[HY]{k_{+3}} E + R\text{-D-Ala-Y} \qquad (2)$$

where HY is H_2O or a suitable R-NH_2 peptide or amino acid.

The goal of the present work was to obtain further information on the possible functional groups involved in catalysis and penicillin binding by the *Streptomyces* R61 DD-peptidase. When this study was initiated, it was known [2] that at pH 7.0 and 37°C (unless otherwise stated) (a) the K, k_{+2} and k_{+3} values for the interaction with benzylpenicillin were 13 mM (25°C), 180 s^{-1} (25°C) and 1.4×10^{-4} s^{-1}; (b) the corresponding values for the interaction with ampicillin were 7.2 mM, 0.77 s^{-1} and 1.4×10^{-4} s^{-1}; (c) the K_m and k_{cat} values for the best substrate Ac_2-L-Lys-D-Ala-D-Ala were about 10 mM and 50 s^{-1}, respectively and (d) the activity of the enzyme decreased with increasing ionic strength [15].

MATERIALS AND METHODS

Enzymes

The *Streptomyces* R61 DD-peptidase and the *Bacillus licheniformis* β-lactamase (2000 units/mg protein) were purified as described [16, 17]. Peroxidase and D-amino acid oxidase were from Boehringer, Mannheim, FRG.

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Abbreviation. Ac, acetyl.

Enzymes. D-Alanyl-D-alanine carboxypeptidase or DD-peptidase (EC 3.4.16.–); β-lactamase (EC 3.5.2.6).

Peptide substrate and inhibitor

Ac_2-L-Lys-D-Ala-D-Ala was from UCB Bioproducts (Braine-l'Alleud, Belgium). [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala and [^{14}C]Ac_2-L-Lys-D-Ala-D-lactate (with the label in the acetyl groups; specific radioactivity 50 Ci/mol) were prepared by reacting L-Lys-D-Ala-D-Ala or L-Lys-D-Ala-D-lactate (from UCB Bioproducts) with [^{14}C]acetic anhydride (from Amersham International, UK) as described [18].

β-Lactam compounds

Benzylpenicillin was from Rhône Poulenc, France, and ampicillin (Pentrexyl®) from Bristol Benelux, Brussels. [^{14}C]Benzylpenicillin (50 Ci/mol) was from Amersham International, UK. Benzylpenicillin amide was a gift from Prof. H. Vanderhaeghe (Rega Instituut, KUL, Leuven, Belgium). *N*-Acetyl ampicillin, *N*-[^{14}C]acetylampicillin and *N*-acetyl α-phenylglycylglycine were synthesized as described in the Appendix.

Buffers

The following buffers were used: pH 5, 10 mM sodium cacodylate/HCl; pH 6, 13 mM sodium cacodylate + HCl or 13 mM sodium phosphate; pH 7, 10 mM sodium phosphate; pH 8, 7 mM sodium phosphate or 25 mM Tris/HCl; pH 9, 6 mM potassium borate or 12 mM glycine + NaOH; pH 10, 2.5 mM borate or 25 mM glycine + NaOH. The ionic strength of all the reaction mixtures was adjusted to a conductance of 0.1 mS using a Methrohm E527 conductometer either by modifying the buffer concentrations or, in the case of the glycine buffers, by adding NaCl. Moreover, in the experiments involving varying substrate concentrations, the ionic strength was adjusted by NaCl addition to that obtained at the highest substrate concentration.

Thin-layer chromatography

Polygram Sil-G plates (Macherey Nagel, Düren, FRG) were used. Solvent A was $CHCl_3$/methanol/acetic acid (88/10/2; v/v/v).

Paper electrophoreses

These were performed at pH 6.5 on Whatman 3MM paper using a Gilson high-voltage electrophorator model DW (60 V/cm). The buffer was collidine/acetic acid/water (9/2.7/1000; v/v/v). Mobilities (in cm/h towards the anode) of the various standard compounds were as follows: benzylpenicillin, 16; benzylpenicilloate, 25; phenylacetylglycine, 22; phenylacetylglycylglycine, 18; *N*-acetylampicillin, 14; penicilloate of *N*-acetylampicillin, 24; *N*-acetyl α-phenylglycylglycine, 17.

Radioactive measurements

Radioactive areas on paper strips were located with a Packard 2000 Radiochromatogram scanner. The amount of radioactivity was measured with a Packard Tri-carb liquid scintillation spectrometer.

DD-Peptidase activity

The hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala was stopped by heating the solutions in boiling water for 1 min and the amount of released D-Ala measured enzymatically (D-amino acid oxidase procedure) as described [19]. Alternatively, the amount of [^{14}C]Ac_2-L-Lys-D-Ala released from [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala was separated from the residual substrate by paper electrophoresis and estimated as described [15].

Estimation of acyl ([^{14}C]Ac_2-L-Lys-D-alanyl) enzyme

[^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (5.3 mM) or [^{14}C]Ac_2-L-Lys-D-Ala-D-Lac (4 mM) was incubated wih the R61 DD-peptidase (25 μM) for 15 s at 0°C, in buffers of varying pH (final volumes 15 μl). The reaction mixtures were supplemented with 15 μl denaturing buffer (0.125 M Tris/HCl pH 6.8 containing 20% glycerol, 2% sodium dodecyl sulphate, 10% 2-mercaptoethanol and 0.001% bromophenol blue), maintained in boiling water for 30 s and submitted to gel electrophoresis at pH 8.3 in the presence of sodium dodecyl sulphate. Estimation of the amounts of radioactive acyl enzyme was performed by fluorography [20, 21] of the gels using preflashed Kodak X-Omat XR films, and scanning. Controls were samples of the R61 DD-peptidase treated with [^{14}C]benzylpenicillin under conditions where all the enzyme was converted into [^{14}C]benzylpenicilloyl enzyme. For more details, see [14].

Kinetic parameters for the interaction with β-lactam compounds

Value of k_{+3}. After complete inactivation of the enzyme by ampicillin or benzylpenicillin and elimination of the free β-lactam compound by addition of the *B. licheniformis* β-lactamase, recovery of enzyme activity was measured as described [22]. With [^{14}C]benzylpenicillin, the radioactive acyl enzyme was isolated by filtration on Sephadex G-25 in water and incubated at 37°C in buffers of varying pH. Samples were removed at increasing intervals, submitted to high-voltage electrophoresis and the released compounds were estimated by radioactivity measurements. With *N*-acetylampicillin, acyl enzyme was not very stable and the value of k_{+3} was measured by adding β-lactamase and tripeptide substrate to mixtures containing the enzyme and a large excess of antibiotic, and monitoring the release of D-alanine with time [23].

Values of the inactivation rate constant. With ampicillin, k_a {i.e. $k_{+2}[D]/([D] + K)$ if $k_{+3} \ll k_a$} was computed by measuring the degree of enzyme inactivation as a function of time. With *N*-acetylampicillin, k_a was not much larger than k_{+3} and it was computed by measuring the residual activity at the steady-state. (For more details, see [5].) The inactivation of the enzyme by benzylpenicillin was monitored by measuring the decrease of protein fluorescence [4] at 320 nm. Fluorescence measurements were performed using a Sigma ZWS-11 stopped-flow mixing unit (Biochem, Munich, FRG) adapted to a Dia-Log optical and detection set-up (Garching Instrument, Düsseldorf, FRG). The signals were analysed by a data acquisition and treatment system for fast, transient optical signals as described [24]. Excitation was at 280 nm. The exponential decrease of fluorescence was analysed on the basis of the linearized equation $\ln [(F_t - F_\infty)/(F_o - F_\infty)] = -k_a t$ where F_o, F_t and F_∞ were the fluorescence intensities at times 0, t and after stabilization of the system was reached. Equal volumes (80 μl) of enzyme solution (4.4 μM) and benzylpenicillin solution (0.67 mM) made in the same buffers (of varying pH) were mixed rapidly. The k_a values were computed by linear regression using 200–400 points at 1-ms intervals.

Curve-fitting

A general multiparametric curve-fitting program was used [25].

RESULTS

Kinetic parameters for the hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala

The effects of pH on K_m and k_{cat} were first determined at substrate concentrations ranging over 2–10 mM, by following the release of D-Ala (i.e. the reaction product of the k_{+2} step) and using Hanes' linearization of the steady-state equation $[D]/v = (K_m + [D])/V$. The ratio k_{cat}/K_m was also determined at a 1 mM (i.e. $\ll K_m$) substrate concentration under which conditions the velocity of the reaction was proportional to [D]. The linearity of product formation with time (5, 10 and 15 min) was verified in all cases. Note that on the basis of reaction (2), k_{cat}/K_m is equivalent to the second-order rate constant k_{+2}/K of enzyme acylation. The experimental values of k_{cat}, K_m and k_{cat}/K_m (i.e. k_{+2}/K) as a function of pH are shown in Fig. 1 A, B and C.

Using [^{14}C]Ac_2-L-Lys-D-Ala-D-lactate at a concentration (4 mM) equivalent to 0.1 K_m [26] and at pH 7, about 1.8% of the enzyme occurred as acyl enzyme at the steady state of the reaction. At this depsipeptide concentration, and if k_{+2} was much larger than k_{+3}, a maximum of 10% of total enzyme would be trapped as acyl enzyme. Using [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala as substrate at a 5.3 mM concentration, no acyl enzyme was detected at the steady state of the reaction whether the pH was 5, 7 or 10.

Inactivation by benzylpenicillin, ampicillin and N*-acetylampicillin*

Preliminary experiments showed that at pH 7, the interaction with *N*-acetylampicillin was characterized by a small k_{+2}/K value (about 8 M^{-1} s^{-1}) and a relatively high k_{+3} value of 5×10^{-4} s^{-1} (half-life 23 min). Four types of experiments were carried out which led to the following observations.

a) The pH dependence of the pseudo-first-order rate constant k_a of acyl-enzyme formation was determined at benzylpenicillin and ampicillin concentrations well below the value of K. Hence $k_a/[D]$ was equivalent to k_{+2}/K. It was assumed that the same condition applied to the interaction with *N*-acetylampicillin. Since with this latter compound the values of k_{+3} was rather high, the rate of formation of the acyl enzyme was estimated by measuring the residual activity after the interaction between the enzyme and the β-lactam had reached the steady state (see [5] and legend of Fig. 2B). The experimental values of $k_a/[D]$ (i.e. k_{+2}/K) as a function of pH are shown in Fig. 2A (for benzylpenicillin), Fig. 2B (for *N*-acetylampicillin) and Fig. 2C (for ampicillin). One should note that benzylpenicillinamide (0.1 mM) did not cause any enzyme inactivation after a 10-min incubation at 37°C, indicating a $k_a/[D]$ value of less than 2 M^{-1} s^{-1}.

b) The pH dependence of the first-order rate constant k_{+3} of acyl enzyme breakdown was examined by following the recovery of the enzyme activity. Fig. 3A, C and D depict the results obtained with benzylpenicillin, *N*-acetylampicillin and ampicillin, respectively.

c) The effects of pH on the nature of the reaction products arising through breakdown of benzylpenicilloyl enzyme are shown in Fig. 3B. Phenylacetylglycine (filled symbols) was produced at a constant rate at pH 5–10 while benzylpenicilloate (open symbols) was produced only at pH > 8. At pH 9 and 10, the experiments were carried out in borate buffers. In glycine-containing buffers, an additional degradation product, presumably, phenylacetylglycylglycine [27], was also formed. The sum of phenylacetylglycine and phenylacetylglycylglycine was equivalent to the amount of phenylacetylglycine alone obtained in borate buffers.

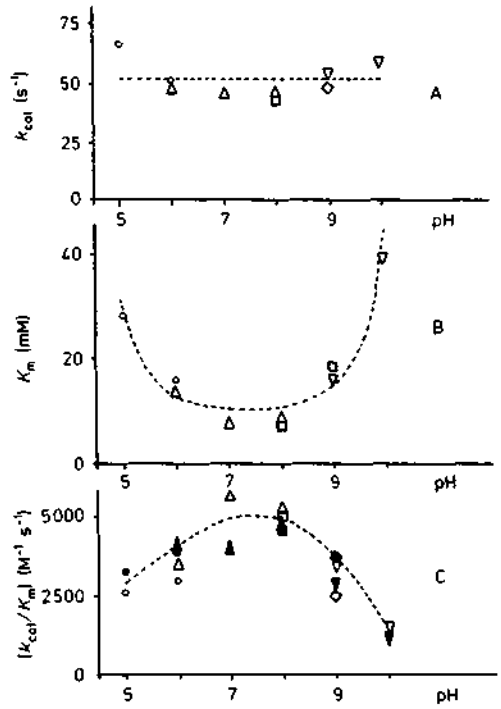

Fig. 1. *pH dependence of* k_{cat} *(A),* K_m *(B) and* k_{cat}/K_m *(C) for the hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala by the active-site serine DD-peptidase of* Streptomyces *R61.* k_{cat} and K_m were obtained from initial velocity measurements using 2, 4, 6, 8 and 10 mM substrate. Filled symbols in (C): the enzyme was incubated with 1 mM substrate for 5, 10 and 15 min. For other conditions, see text. Buffers: (○, ●) cacodylate; (△, ▲) phosphate; (□, ■) Tris/HCl; (◇, ◆) borate; (▽, ▼) glycine/NaOH. Dashed curves were obtained by computer-aided fitting

d) The compounds originating by breakdown of the acyl enzyme formed with *N*-[^{14}C]acetylampicillin were examined by thin-layer chromatography in solvent A (in which system, *N*-acetylampicillin had an R_F value of 0.44). The main compound obtained at pH 10 had an R_F value of 0.05, which is that of the product formed by β-lactamase hydrolysis of *N*-acetylampicillin. The product observed at pH 7.0 had the same R_F (0.22) in solvent A and the same electrophoretic mobility (17 cm/h towards the anode) at pH 6.5 as authentic *N*-acetyl-α-phenylglycylglycine.

DISCUSSION

Benzylpenicillin, ampicillin, *N*-acetylampicillin and Ac_2-L-Lys-D-Ala-D-Ala possess a free carboxylate of p$K \leqq 3$ at one end of the molecule. Ampicillin contains in addition an amino group with a pK value of 7.25 at 25°C [28] or 6.7 at 37°C (as redetermined by titration of the sodium salt of ampicillin).

No acyl enzyme accumulates detectably at the steady state of the reaction with Ac_2-L-Lys-D-Ala-D-Ala used at concentra-

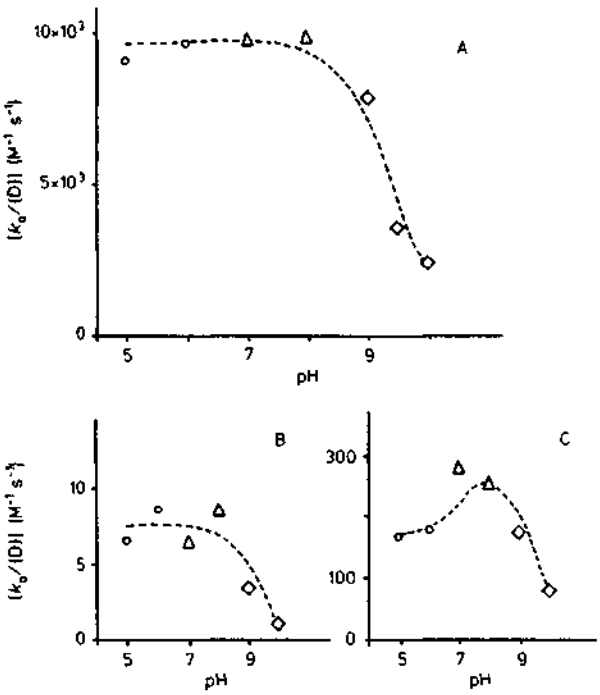

Fig. 2. *pH dependence of the pseudo-first-order rate constant* k_a *of acyl enzyme formation during interaction between the active-site serine* DD*-peptidase and 0.67 mM benzylpenicillin (A; pH 5–9.5), 2 μM benzylpenicillin (A; pH 10), 200 μM* N*-acetylampicillin (B) and 10 μM ampicillin (C).* Dashed curves were obtained by computer-aided fitting. For buffers, see Fig. 1. (A) The values at pH 5–9.5 were obtained at 25°C by fluorescence stopped-flow as explained in the text. The value at pH 10 was obtained by incubating the enzyme (0.6 μM) and benzylpenicillin (2 μM) at the same temperature. Samples (10 μl) were removed at 2-min intervals, and diluted threefold in 5 mM sodium phosphate pH 7.0 containing 2 μg β-lactamase and 60 nmol substrate. The amount of D-alanine produced was estimated after 10 min at 37°C. (B) The enzyme (1.3 μM) was incubated at 37°C with 0.2 mM *N*-acetylampicillin. Samples (10 μl) were removed after 5, 10, 20 and 30 min and supplemented with 20 μl of 2 mM substrate prepared in the corresponding buffer. After 5 min at 37°C, the amount of free D-alanine was estimated. The results indicated that, in all cases, the steady state was reached after less than 20 min of interaction between the enzyme and *N*-acetylampicillin. The value of k_a was estimated using the following equation

$$\frac{[ED^*]_{ss}}{E_o} = \frac{k_a}{k_a + k_{+3}}$$

where k_{+3} was obtained from the data shown on Fig. 3C. (C) The enzyme (0.6 μM) was incubated at 37°C with 10 μM ampicillin, 10-μl samples were removed at increasing intervals and diluted threefold in 5 mM sodium phosphate pH 7.0 containing 20 μg β-lactamase and 60 nmol substrate. The amount of D-alanine was estimated after 10 min

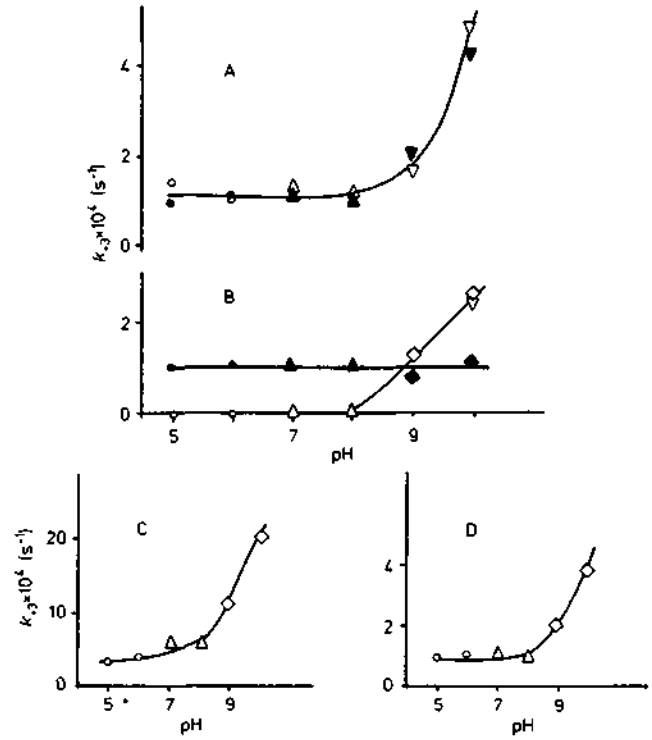

Fig. 3. *pH dependence of the first-order rate constant of enzyme recovery through breakdown of the acyl enzyme formed by reaction between the active-site serine* DD*-peptidase and benzylpenicillin (A), N-acetylampicillin (C) and ampicillin (D). pH dependence of the rate of release of benzylpenicilloate (B; open symbols) and phenylacetylglycine (B; filled symbols) from benzylpenicilloyl enzyme.* For buffers, see Fig. 1. (A, D) The enzyme (51 nM) was completely inactivated by a 10-min incubation at 37°C with 10 μM benzylpenicillin (A) or 100 μM ampicillin (D) in buffers of varying pH. The mixtures were supplemented with 2 μg (A) or 20 μg (D) β-lactamase and maintained at 37°C. After 0, 30, 60 and 90 min, samples (30 μl) were removed and supplemented with 60 nmol substrate. The amount of D-alanine produced was estimated after a 10-min incubation at 37°C. (B) The enzyme (26 nmol) was inactivated by reaction with 100 nmol [^{14}C]benzylpenicillin for 5 min at 37°C in 300 μl of 10 mM sodium phosphate pH 7.0. The buffer salts and the excess of free [^{14}C]benzylpenicillin were eliminated by filtration on Sephadex G-25 in water and the radioactive acyl-enzyme preparation was divided into 100-μl fractions each containing 20 nCi. These fractions were freeze-dried, the residues dissolved in 100 μl buffer of varying pH and the resulting solutions incubated at 37°C. Samples (20 μl) were removed at varying intervals (0, 30, 60 and 90 min) and submitted to high-voltage paper electrophoresis at pH 6.5 for 60 min. The [^{14}C]benzylpenicilloyl enzyme remained at the origin. [^{14}C]Phenylacetylglycine (filled symbols) and [^{14}C]benzylpenicilloate (open symbols) migrated as mentioned in Materials and Methods. (C) The enzyme (200 μM at pH 10, 11 μM in the other cases) was incubated at 37°C with 1.33 mM (at pH 10) or 0.33 mM (in the other cases) *N*-acetylampicillin. After 30 min, inactivation was complete and the solutions were simultaneously supplemented with 20 μg of β-lactamase and 1.2 mM (final concentration) substrate. At varying intervals, 50-μl samples were removed and analysed for their content of D-alanine. The filled symbols in (A) refer to the sum benzylpenicilloate + phenylacetyl glycine whose individual values were measured in B

tions equivalent to $0.2 \times K_m$ at pH 5, $0.65 \times K_m$ at pH 7 and $0.13 \times K_m$ at pH 10. Note also that little variation of the K_m at pH 7.0 was observed between 37° and 0°C (unpublished data). Since 1.8% of total enzyme can be trapped as acyl enzyme during reaction with Ac_2-L-Lys-D-Ala-D-lactate (at 0°C, pH 7 and a concentration equivalent to 0.1 K_m), it is concluded that certainly less than 1% of total enzyme occurs as acyl enzyme during reaction with the peptide. Since $K_m = k_{+3}K/(k_{+2} + k_{+3})$ and as shown elsewhere [5], these levels of enzyme acylation yield k_{+3}/k_{+2} values of 4 (depsipeptide, pH 7) and >22 (peptide, pH 5), >190 (peptide, pH 7) and >14 (peptide, pH 10). Although these data must be taken with caution (since only one substrate concentration has been used), it is evident that the k_{+3}/k_{+2} ratio for the reaction with the peptide is >10 at all pH values and that, consequently, $k_{cat} = k_{+2}$ and $K_m = K$. In agreement with these results, peptide analogues that inhibit the enzyme competitively have K_i values comparable to the K_m for the peptide substrate [29].

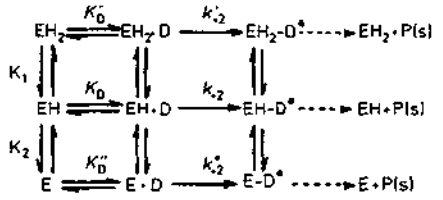

Fig. 4. *General model for the interaction between the* Streptomyces *R61* DD*-peptidase and carbonyl-donor substrate or inactivators.* K_1, K_2, K_D, K'_D and K''_D are dissociation constants. Our discussions assumes that the lower branch is inoperative ($K''_D \gg K_D$) for both substrate and β-lactam, and that the upper branch is only operative for β-lactams (β-lactams: $K'_D = K_D, k'_{+2} = k_{+2}$; substrate: $K'_D \gg K_D$). The deacylation step (k_{+3}) on which no data can be obtained with the substrate, can be studied independently with the β-lactams. In that case, an additional step is found ($EH-D^* \xrightarrow{k_{+4}(OH^-)} EH + P'$. Curve-fitting, when performed with the complete models (i.e. assuming that K'_D and K''_D are not much larger than K_D), does not significantly improve the agreement between the calculated and measured values

The variations of the kinetic parameters shown in Fig. 1 thus reflect the influence of pH on the dissociation constant of the Michaelis complex (K) and of the first-order rate constant (k_{+2}) for the transformation of that complex to acyl enzyme. The effects of pH on the rate of hydrolysis of the acyl enzyme remain unknown.

The value of K_m increases (Fig. 1B) at pH values lower than 7 and higher than 8, expressing a decreased affinity of the free enzyme for the substrate. Assuming that $EH_2 \cdot D$ and $E \cdot D$ (see Fig. 4) are not detectably formed (i.e. K''_D and $K'_D \gg K_D$), the dashed line and curve shown on Fig. 1A and B are obtained and fitting of the data to Eqn (3) gives $k_{+2} = 51.5\ s^{-1}$, $pK_1 = 5.3$, $pK_2 = 9.48$ and $K_D = 10.4$ mM.

$$K_m = K_D\left(1 + \frac{K_2}{[H^+]} + \frac{[H^+]}{K_1}\right). \qquad (3)$$

Fitting the data shown on Fig. 1C (dashed curve) on the basis of Eqn (4) (where $4950\ M^{-1}\ s^{-1}$ is the value of k_{+2}/K_D computed from the results obtained above) yields pK_1 and pK_2 values of 4.92 and 9.47, respectively.

$$\frac{k_{cat}}{K_m} = 4950\left(1 + \frac{K_2}{[H^+]} + \frac{[H^+]}{K_1}\right)^{-1} M^{-1}s^{-1}. \qquad (4)$$

Fitting without prior fixation of the k_{+2}/K_D values has also been attempted but does not significantly decrease the total relative standard deviation and the agreement with the values obtained from analysis of the K_m curve is not so good ($k_{+2}/K_D = 4250\ M^{-1}\ s^{-1}$ and $pK_1 = 4.7$). This suggests that formation of the Michaelis complex with the substrate is possible only with the central form EH and that the observed variations of the k_{cat}/K_m ratio can be accounted for by the sole variation of K_m, i.e. of K.

Eqns (3) and (4) can easily be derived from Eqns (8) and (9) of Kaplan and Laidler [30]; our data correspond to case 4 in Table 1 ($k_2 \ll k_3$) of that article[1], when $(k_{-1} + k_2)/k_2$ is replaced by K_D.

[1] It should however be noted that a printing mistake occurred in [30]. Eqn (9) giving $\overline{kc}/\overline{K_m}$ is not obtained as printed by dividing Eqn (7), giving $\overline{kc}$, by Eqn (8), giving $\overline{K_m}$. Instead, Eqn (9) should read

$$\frac{\overline{kc}}{\overline{K_m}} = \frac{k_1k_2}{k_{-1}+k_2}\cdot\frac{1 + \dfrac{aK_a}{[H]} + \dfrac{b[H]}{K_b}}{1 + \dfrac{K_a}{[H]} + \dfrac{[H]}{K_b}}.$$

Table 1. *Values of* pK_2

Method	pK_2
$\frac{k_a}{[D]}$ for benzylpenicillin	9.45
N-acetylampicillin	9.28
ampicillin	9.6
K_m for substrate	9.48
k_{cat}/K_m for substrate	9.47

The rate of enzyme acylation by benzylpenicillin and *N*-acetylampicillin remains unchanged between pH 5 and 8 (fig. 2A and B) and, in that case, $k'_{+2}/K'_D = k_{+2}/K_D$. Curve fitting, performed on the basis of Eqn (5) (dashed curves in Fig. 2A and B) yields pK_2 values of 9.45 (Fig. 2A) and 9.28 (Fig. 2B). The values of k_{+2}/K_D are $9800\ M^{-1}\ s^{-1}$ for benzylpenicillin (at 25°C) and $7.6\ M^{-1}\ s^{-1}$ for *N*-acetylampicillin (at 37°C).

$$\frac{k_a}{[D]} = \frac{k_{+2}}{K_D}\left(1 + \frac{K_2}{[H^+]}\right)^{-1}. \qquad (5)$$

With ampicillin (Fig. 2C) the observed decrease of $k_a/[D]$ at pH 5 and 6 can be attributed to the protonation of ampicillin itself ($D + H^+ \rightleftarrows DH^+$; $pK = 6.7$). Indeed, the dashed curve shown on Fig. 2C is obtained with values of $265\ M^{-1}\ s^{-1}$ for k_{+2}/K_D, $177\ M^{-1}\ s^{-1}$ for k_{+2}, DH^+/K_{DH^+} and 9.6 for pK_2.

Unfortunately, with any of the β-lactam compounds tested, it is not possible to decide whether the observed decrease of k_{+2}/K at high pH is due to a decrease of k_{+2} or to an increase of K. However, since the values of pK_2 derived from the different experiments are strikingly similar (Table 1) and since, as shown above, protonation of an enzyme group of $pK \approx 9.5$ greatly increases the efficacy of the initial binding step of the tripeptide substrate, it is proposed by extension that the same mechanism applies to the interaction with the β-lactam compounds. This more efficient binding might be due to charge pairing between the free carboxylate of the substrate/inactivator and a postively charged group near the enzyme's active site. In support of this view, amidation of the carboxylate group causes, at least, a 5000-fold decrease in the inactivating potency of benzylpenicillin.

Titration of the active-site serine β-lactamases of class A and C [which react with the β-lactam compounds according to reaction (1) but with a k_{+3} of very high value] also reveals an enzyme's group of pK 8.5–10 [12, 13]. Remarkably, the R61 DD-peptidase and these β-lactamases possess a highly conserved lysine residue located at the third position on the carbonyl side of the active-site serine residue (Ser-Xaa-Xaa-Lys). It may thus be hypothesized that the group of pK 8.5–10 detected in all these enzymes is the ε-amino group of that particular lysine residue and that this amino group plays an important role in the formation and/or productiveness of enzyme-ligand associations.

While deprotonation of the group of $pK \approx 9.5$ similarly decreases the affinity of the enzyme for the peptide substrate and penicillins, a major difference between the two classes of compounds is observed at low pH. Whereas interaction with the substrate appears to rely on a group of $pK \approx 5$, in contrast, binding of penicillins remains stable when the pH is lowered from 7 to 5. A group of $pK \approx 5.0$ seems to occur in the active site of serine β-lactamases [12, 13] but the stability of the

DD-peptidase does not allow measurements to be performed at a pH lower than 5. Further experiments are needed to explain coherently the results obtained at pH < 7.

Independent study of the deacylation (k_{+3}) step is only possible with the β-lactam compounds. The value of k_{+3} is low at pH between 5 and 8 but it increases at higher pH. As shown in Fig. 3B, this increase is due to attack of the acyl (benzylpenicilloyl) enzyme by OH^- ions with formation of benzylpenicilloate. The second-order rate constant computed for this reaction is about 4 $M^{-1} s^{-1}$, a value which is not much higher than that observed (0.7 $M^{-1} s^{-1}$) for the hydrolysis of α-methylpenicilloate by OH^- ions [31]. In contrast, the rate of release of phenylacetylglycine is pH-independent between pH 5 and 10 (Fig. 3B). Hence, irrespective of the pH, rupture of the C5–C6 bond of the acyl (benzylpenicilloyl) enzyme and generation of phenylacetylglycyl-enzyme is rate-determining [32]. In some respects, these results are at variance with those reported for the interaction between benzylpenicillin and the membrane-bound DD-peptidase of *Bacillus stearothermophilus* [33]. In this latter case, k_{+3} was maximal at pH 5–6 but experiments were not performed at pH 8. At pH 6.5, phenylacetylglycine was also the only product released.

Finally, it should be stressed that there is no sign of an imidazole group of p$K \approx 7$ as it occurs in the active site of chymotrypsin and related peptidases. Yet, earlier studies suggested that a group of p$K \approx 7.0$ might be involved in the transpeptidation reactions catalysed by the R61 DD-peptidase [15]. Such an enzyme group would play a role neither in the hydrolysis of the peptide substrate nor in the interaction with β-lactam compounds.

APPENDIX

Synthesis of N-*acetyl and* N-[^{14}C]*acetylampicillin*

N-Acetylampicillin was prepared as follows. Ampicillin (100 mg) was dissolved in 0.3 ml acetonitrile by dropwise addition of 1 M NH_4HCO_3 and the solution was supplemented with 0.1 ml acetic anhydride and maintained at 20°C. The disappearance of the free amino group was monitored by spotting 5-µl samples on a filter paper and spraying with a ninhydrin solution. After 20 min, the blue-brown color characteristic of ampicillin failed to appear and the solution was evaporated to dryness. Analysis of the reaction mixture by thin-layer chromatography using solvent A (see below) and detection with bromocresol green (after drying the plates at 120°C for 60 min; acidic compounds appear yellow) revealed one single compound of R_F 0.44. This compound (*N*-acetylampicillin) completely disappeared upon treatment with the β-lactamase and gave rise to one single compound (the corresponding penicilloate) of R_F 0.05.

N-[^{14}C]Acetylampicillin (specific radioactivity 0.82 Ci/mol) was prepared as follows. Ampicillin (4 mg) was dissolved in 55 µl of a mixture of acetonitrile and 1 M NH_4HCO_3 solution (9/1; v/v). The solution was supplemented with 1 µl of 0.5% [^{14}C]acetic anhydride in toluene (Amersham; 120 Ci/mol) and maintained for 2 h at 20°C. Non-radioactive acetic anhydride (20 µl) and acetonitrile (10 µl) were added and after 16 h at 20°C, the reaction mixture was evaporated to dryness and the residue dissolved in 2 ml 50 mM sodium phosphate pH 7.0. The *N*-acetylampicillin concentration (55 mM) was estimated by measuring both the absorbance of the solution at 230 nm ($\varepsilon = 2400\ M^{-1} cm^{-1}$) and the rate with which the preparation inactivated the R61 DD-peptidase (using the non-radioactive *N*-acetylampicillin as control).

Table 2. *NMR characteristics of* N-*acetyl-α-phenylglycylglycine*
The reference for chemical shifts was trimethylsilylpropionate in D_2O and hexamethyl disiloxane in CD_3COCD_3

Solvent	δ	Peak area (number of H)	Interpretation
	ppm		
D_2O + NaOD	7.3	5	phenyl
	3.7	2	methylene of C-terminal glycine
	2.0	3	methyl of acetyl group
CD_3-CO-CD_3	8.5	(2) ?	NH
	7.2	5	phenyl
	5.4	1	methyne of α-phenyl-glycine
	3.8	2	methylene of glycine
	2.0	3	methyl of acetyl

Synthesis of N-*acetyl α-phenylglycylglycine*

a) α-Phenylglycine (5 g, 33 nmol) was dissolved in 100 ml of water by adjusting the pH to 9 with NaOH. Acetic anhydride (10 ml) was added and after 10 min at 20°C, the solution was cooled to 0°C and acidified with HCl. The white crystals were collected, washed with cold water and dried (melting point 190–93°C, yield 4 g).

b) *N*-Acetyl α-phenylglycine was coupled to glycine ethyl ester using dicyclohexylcarbodiimide as described [34], but the procedure for the isolation of the adduct was modified as follows. Urea was eliminated by filtration, the solvent dry-evaporated and the residue redissolved in hot ethylacetate. After cooling and elimination of residual urea, the organic solution was washed successively with a 10% solution of sodium bicarbonate and water, dried over $MgSO_4$ and concentrated until it became slightly cloudy.

c) The ester was crystallized by addition of petroleum ether (40–60°C b.p.) (melting point 222–224°C; yield 0.4 g), dissolved in 30 ml dioxane containing 0.2 g NaOH and saponified during 2 h at 20°C. The solution was cooled to 0°C, acidified with HCl, dry-evaporated, and the residue dissolved in hot acetone. After elimination of the solid (NaCl) and evaporation of the solvent, the residue was dissolved in hot ethylacetate, the solution washed successively with a 7% solution of potassium bicarbonate and water, dried over $MgSO_4$ and concentrated until it became cloudy. Yellowish crystals (melting point 160°C) were obtained by addition of petroleum ether (40–60°C b.p.). They were dissolved in a minimum volume of water containing a stoichiometric amount of NaOH and the solution acidified with HCl. White crystals appeared after several days at 4°C (melting point 182–185°C). They were analysed by thin-layer chromatography in solvent A (one single spot of R_F 0.22–0.24) and by NMR in D_2O + NaOD and CD_3-CO-CD_3 (Table 2). Mass spectra of positive and negative secondary ions were recorded using a quadrupolar extranuclear (model 7-162-8) mass spectrometer [35]. The compound was dissolved in glycerol using camphorsulfonic acid as a promoting agent. *m/z* for positive ions: 251 [MH^+], 233 [MH^+-(H_2O)], 223 [MH^+-(CO)], 176 [MH^+-(NH_2-CH_2-COOH)], 146 [176-(CO)], 106 [C_6H_5-$CH{=}NH_2^+$]; *m/z* for negative ions: 249 [$(M\text{-}H^+)^-$], 204 [$(M\text{-}H^+)^-$-(COOH)], 174 [(?) possibly $(M\text{-}H^+)^-$-(CH_3-CO-NH-OH)], 119 [C_6H_5-CH_2-CO^-].

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