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Interaction between non-classical β-lactam compounds and the Zn^{2+}-containing G and serine R61 and R39 D-alanyl-D-alanine peptidases

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Streptomyces albus G secretes a Zn^{2+}-containing D-alanyl-D-alanine peptidase. *Streptomyces* R61 and *Actinomadura* R39 secrete D-alanyl-D-alanine-cleaving serine peptidases. The effect of non-classical β-lactam antibiotics on these three model enzymes has been studied. Mecillinam, cefoxitin, quinacillin, quinacillin sulphone, clavulanate and *N*-formimidoylthienamycin have no effect on the Zn^{2+}-containing enzyme. 6-Aminopenicillanic acid slowly inactivates this enzyme and 7-aminocephalosporanic acid behaves as a reversible inhibitor. Cefoxitin and *N*-formimidoylthienamycin are potent anti-bacterial agents; they effectively inactivate the serine R39 enzyme and, to a lesser extent, the serine R61 enzyme. All the other β-lactam compounds tested, including mecillinam, are slow inactivators of these serine enzymes. The intermediates formed between 6-aminopenicillanic acid and the R61 and R39 enzymes are long- and short-lived respectively, whereas those formed between 7-aminocephalosporanic acid and the same R61 and R39 enzymes are short- and long-lived respectively. Breakdown of the short-lived intermediates thus obtained gives rise to several ninhydrin-positive degradation products. The intermediates formed between clavulanate and the serine enzymes are long-lived. With the R39 enzyme, the inactivated complex formed in a first step undergoes subsequent monomolecular rearrangement to give rise to a second species exhibiting a high absorbance at 276 nm.

The water-soluble DD-carboxypeptidases/transpeptidases of *Actinomadura* R39 (the R39 enzyme) and *Streptomyces* R61 (the R61 enzyme) are D-alanyl-D-alanine-cleaving serine peptidases that are highly and moderately sensitive to β-lactam antibiotics respectively (Frère *et al.*, 1973, 1974, 1976*a*, 1980; Ghuysen *et al.*, 1979; Duez *et al.*, 1981*b*). The water-soluble DD-carboxypeptidase of *Streptomyces albus* G (the G enzyme) is a metallo (Zn^{2+}-containing) D-alanyl-D-alanine peptidase that is highly resistant to these compounds (Duez *et al.*, 1978; Frère *et al.*, 1978; Dideberg *et al.*, 1980*b*). Without regard to individual mechanistic properties and penicillin sensitivities, these enzymes react with β-lactam antibiotics according to the reaction:

$$E + I \xrightarrow{K} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + Ps$$

where E = enzyme, I = antibiotic, K = dissociation constant, k_2 and k_3 = first-order rate constants and Ps = degradation product(s) (Ghuysen *et al.*, 1979; Frère *et al.*, 1980). The k_2/K and k_3 terms of the reaction govern the sensitivity of the enzymes toward a given β-lactam compound. A good enzyme inactivator characterizes itself by both a high k_2/K value (thus causing the rapid formation of complex EI*) and a low k_3 value (thus immobilizing the enzyme in the form of complex EI* for a long period of time). Breakdown of complex EI* under non-denaturing conditions results in enzyme regeneration, but the fate of the antibiotic inactivator varies, depending on the specific complex. With the serine enzymes, benzylpenicillin is slowly fragmented into phenylacetylglycine and *N*-formyl-D-penicillamine (Frère *et al.*, 1975*a*). With the Zn^{2+} enzyme, benzylpenicillin is slowly converted into benzylpenicilloate (Duez *et al.*, 1981*a*). The above studies, carried out with 'classical' 6(7)β-acyl substituted penam and 3-cephem antibiotics, have

Abbreviation used: Ac_2, diacetyl.

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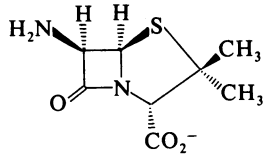
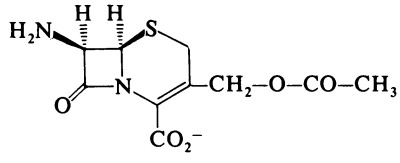
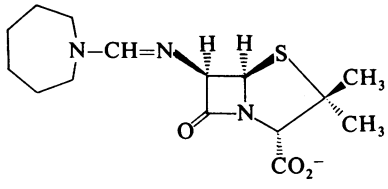


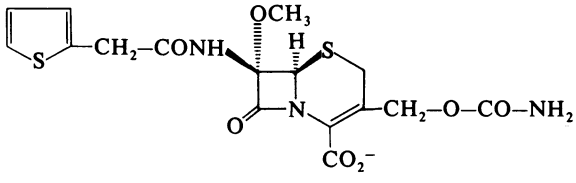
6-Aminopenicillanate



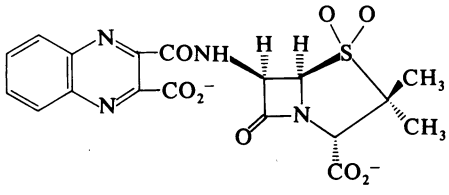
7-Aminocephalosporanate



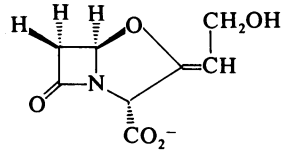
Mecillinam



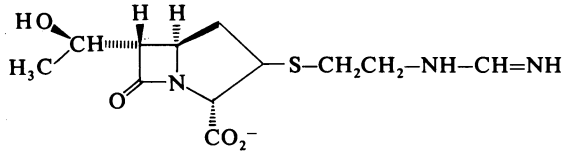
Cefoxitin



Quinacillin sulphone



Clavulanate



N-Formimidoylthienamycin

Fig. 1. *Non-classical β-lactam compounds*

been extended to several 'non-classical' β-lactam compounds. These compounds (Fig. 1) are characterized by the lack of a 6(7)β-acyl side chain on the penam or 3-cephem nucleus (6-aminopenicillanic acid and 7-aminocephalosporanic acid), the occurrence of a 6-amidino side chain on the penam nucleus (mecillinam), the presence of a 6α-methoxy substituent on a 3-cephem nucleus (cefoxitin) and altered fused-ring systems (quinacillin sulphone, the oxapenam clavulanic acid and the carba-2-penem *N*-formimidoylthienamycin).

Material and methods

Enzymes

The R61, R39 and G D-alanyl-D-alanine peptidases were purified to protein homogeneity (Frère *et al.*, 1973, 1974). The β-lactamase I of *Bacillus cereus* (Thatcher, 1975) was purchased from Serva Feinbiochemica, Heidelberg, Germany, and the β-lactamase of *Actinomadura* R39 was prepared as described by Johnson *et al.* (1975).

β-Lactam compounds

[^{14}C]Benzylpenicillin (sp. radioactivity 60Ci/mol), with the radioactive label on the acyl side chain, 6-aminopenicillanic acid and 7-aminocephalosporanic acid were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., Sigma Chemical Co., St Louis, MO, U.S.A., and Aldrich Europe, Beerse, Belgium, respectively. Quinacillin sulphone was a gift from Dr. J. R. Knowles, Harvard University, Cambridge, MA, U.S.A.; quinacillin from Boots Chemical Co., Nottingham, U.K.; mecillinam was from Leo Pharmaceutical Products, Ballerup, Denmark; clavulanic acid from Beecham Pharmaceutical Research Division, Betchworth, Surrey, U.K.; *N*-formimidoylthienamycin and cefoxitin from Merck Institute for Therapeutic Research, Rahway, NJ, U.S.A.

Substrates

$N^{\alpha}N^{\varepsilon}$-$Ac_2$-L-Lys-D-Ala-D-Ala was purchased from UCB Bioproducts, Brussels, Belgium. The ^{14}C-labelled tripeptide was that previously used (Perkins *et al.*, 1973).

Determination of enzyme activity, enzyme inactivation and enzyme recovery

DD-Carboxypeptidase assays with Ac_2-L-Lys-D-Ala-D-Ala as substrate were carried out at 37°C in 50mM-Tris/HCl buffer, pH7.5, containing 5mM-$MgCl_2$ and 50mM-NaCl (the R39 enzyme), in 5mM-sodium phosphate buffer, pH7.0 (the R61 enzyme), and in 10mM-Tris/HCl buffer, pH8.3 containing 3.5mM-$MgCl_2$ (the G enzyme). The released D-alanine was estimated enzymically by using the system D-amino acid oxidase, peroxidase and *o*-dianisidine (Frère *et al.*, 1976*b*). In some cases, [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala was used as substrate and the reaction product [^{14}C]Ac_2-L-Lys-D-Ala was separated from the residual substrate by high-voltage electrophoresis at pH6.5 (Frère *et al.*, 1973).

Enzyme inactivation by the β-lactam compounds was carried out at 37°C and, unless otherwise stated, in the same buffers as those used for the estimation of enzyme activity. Samples of the reaction mixtures were removed after increasing times of incubation and assayed for residual enzyme activity. Most often, the apparent first-order rate constant (k_a) for enzyme inactivation was proportional to the concentration of the β-lactam compound, in which case the k_2/K ratio was computed from the slope of the plot k_a versus [β-lactam]. In some cases, deviation from linearity was observed at high [β-lactam] values, thus permitting evaluation of the individual values of the K and k_2 constants from the reciprocal plots $1/k_a$ versus [β-lactam]. Inactivation of the R61 enzyme by clavulanate was also studied at 23°C, by measuring the β-lactam-induced decrease of fluorescence emission as a function of time (Nieto *et al.*, 1973; Frère *et al.*, 1975*b*). Fluorescence emission was measured at 90° to the excitation beam (285nm) with an Aminco–Bowman recording spectrofluorimeter. Finally, inactivation of both the R61 and R39 enzymes by clavulanate was studied at 25°C by recording the u.v. spectra of the solutions between 330 and 250nm after various times of contact. A Cary 17 double-beam recording spectrophotometer was used for this purpose. The complexes EI* formed with the R61 and R39 enzymes had maximal absorbances at 283 and 276nm respectively.

Breakdown of complex EI* (i.e. determination of the k_3 value) was studied as follows. The enzyme was completely inactivated by incubation at 37°C in the presence of a high concentration of β-lactam compound, the excess of the inactivator was either destroyed by β-lactamase action or eliminated by dialysis at 4°C, and the reaction mixture was further maintained at 37°C. DD-Carboxypeptidase assays were performed on samples removed after increasing times of incubation. 6-Aminopenicillanic acid, mecillinam and quinacillin in excess were hydrolysed by the β-lactamase I of *B. cereus* and 7-aminocephalosporanic acid by the β-lactamase of *Actinomadura* R39. In all cases, β-lactamase concentrations were sufficient to bring about immediate destruction of the free β-lactam compound. Cefoxitin, quinacillin sulphone, clavulanate and *N*-formimidoylthienamycin are β-lactamase-resistant but they form highly stable complexes EI* with the serine enzymes so that the extent of enzyme recovery that occurred during dialysis at 4°C was negligible.

T.l.c. and paper electrophoresis

T.l.c. was carried out on silica-gel G-60 plates with butan-1-ol/acetic acid/ethanol/water (10:3:3:4, by vol.) as solvent and paper electrophoreses were carried out on Whatman 3MM paper, in collidine/acetic acid/water (17:7:2500, by vol.) buffer, pH 6.5, and at 60 V/cm, using a model DW Gilson high-voltage electrophorator. Detection was with reagent containing the following: ninhydrin, 1 g; 2,4,6-trimethylpyridine, 29 ml; ethanol, 700 ml; acetic acid, 210 ml. After spraying, the plates or paper strips were heated at 120°C.

Results

None of the β-lactam compounds inactivated the G enzyme (at least at the concentrations indicated in Table 1) except 6-aminopenicillanic acid. The slow inactivation of the G enzyme by 4 mM-6-aminopenicillanic acid indicated a k_2/K value close to or slightly lower than 1×10^{-2} M$^{-1}\cdot$s^{-1}. At this concentration of 6-aminopenicillanic acid, there was no indication of a reversible inhibition.

The interaction between the G enzyme and 7-aminocephalosporanic acid was reversible. The extent of enzyme inactivation was not a function of time and increased as the concentrations of 7-aminocephalosporanic acid increased. The interaction was therefore studied by steady-state kinetics. Incubating the enzyme (0.2 μg in 60 μl of final volume) with [^{14}C]Ac$_2$-L-Lys-D-Ala-D-Ala at concentrations ranging between 0.10 and 0.33 mM and 7-aminocephalosporanic acid at concentrations ranging from 0 to 0.9 mM yielded a K_i value of 0.2 mM. The data were analysed with the computer program previously described (Schilf *et al.*, 1978); the Fisher–Snedecor variable F was close to zero, suggesting that the experimental data did not fit a non-competitive mechanism better than a competitive one.

The R39 enzyme was inactivated to various extents by all the β-lactam compounds tested and the R61 enzyme by all of them except quinacillin (at 5 mM or less) and quinacillin sulphone (at 0.6 mM or less). The k_2/K and k_3 terms of the reactions between the enzymes and the β-lactam compounds were determined on the basis of enzyme activity measurements as described in the Materials and methods section. The results are given in Table 1. The interactions between both R39 and R61 enzymes and the unsubstituted 6-aminopenicillanic acid, the unsubstituted 7-aminocephalosporanic acid and the β-lactamase inactivator clavulanate deserve some comment.

Interactions between 6-aminopenicillanic acid and the R61 enzyme and between 7-aminocephalosporanic acid and the R39 enzyme

With the system 6-aminopenicillanic acid/R61 enzyme, the plots k_a versus [β-lactam] showed deviation from linearity, permitting determination of the individual K (0.8 mM) and k_2 (2×10^{-4} s^{-1}) values. Since formation of complex EI* was very slow, K could also be estimated by steady-state kinetics, using incubation times so short that the amount of complex EI* formed at the end of the

Table 1. *Kinetic constants at 37°C for the interaction*

$$(E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + Ps)$$

between β-lactam compounds (I) and D-alanyl-D-alanine peptidases (E)

All the constant values were determined on the basis of enzyme activity measurements. The ratio k_2/K is the bimolecular rate constant for enzyme inactivation (formation of complex EI*) and k_3 is the first-order rate constant for enzyme recovery (breakdown of complex EI*). The buffers used are those described in the Materials and methods section.

	R61 serine enzyme		R39 serine enzyme		Zn^{2+}-containing G enzyme	
β-Lactam compound	k_2/K (M$^{-1}\cdot$s^{-1})	k_3 (s^{-1})	k_2/K (M$^{-1}\cdot$s^{-1})	k_3 (s^{-1})	k_2/K (M$^{-1}\cdot$s^{-1})	k_3 (s^{-1})
6-Aminopenicillanic acid	0.25†	$<6 \times 10^{-5}$	1200	5.6×10^{-3}	0.01	Not determined
7-Aminocephalosporanic acid	33	4.4×10^{-3}	200	1×10^{-6}	Reversible inhibitor ($K_i = 0.2$ mM)	
Mecillinam	0.22	$<2 \times 10^{-4}$	32	1×10^{-4}	Non-inactivator at 8 mM	
Cefoxitin	1500	5×10^{-5}	7000	$<3 \times 10^{-5}$	Non-inactivator at 10 mM	
Quinacillin	Non-inactivator at 5 mM		400	6×10^{-5}	Non-inactivator at 5 mM	
Quinacillin sulphone	Non-inactivator at 0.6 mM		10	6×10^{-5}	Non-inactivator at 0.6 mM	
Clavulanate	21	5×10^{-6}	32	$<10^{-6}$	Non-inactivator at 50 mM	
N-Formimidoylthienamycin	1000	7×10^{-6}	10000	2×10^{-6}	Not determined	

† $k_2 = 2 \times 10^{-4}$ s^{-1}; $K = 0.8$ mM.

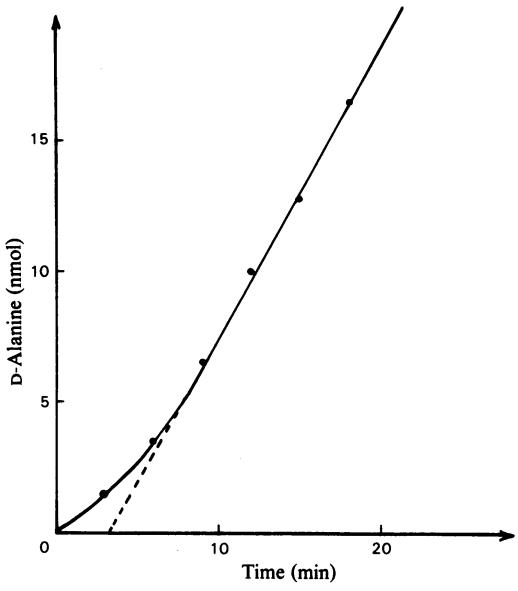


Fig. 2. *Determination of the value of the first-order rate constant k_3 for the degradation of complex EI* formed between 6-aminopenicillanic acid and the serine R39 D-alanyl-D-alanine peptidase*

The enzyme (3.2 μg) was incubated with 0.33 mM-6-aminopenicillanic acid at 37°C in a total volume of 300 μl of buffer. After 15 min, the reaction mixture was supplemented with 6×10^{-3} units of *B. cereus* β-lactamase I and 500 nmol of Ac_2-L-Lys-D-Ala-D-Ala. Samples (30 μl) were removed after various periods of time, immersed for 1 min in a boiling water bath and the released D-alanine was estimated in each sample. The extrapolation of the linear portion of the graph to [P] = 0 yielded the $1/k_3$ value (Frieden, 1970).

incubation was negligible. Experiments carried out at substrate concentrations ranging between 4.5 and 15 mM and at 6-aminopenicillanic acid concentrations ranging between 0.5 and 2.0 mM yielded a K_i value of about 1–2 mM. Assuming a competitive model, this K_i value was in fair agreement with the K value (0.8 mM) obtained from the reciprocal plot $1/k_a$ versus $1/[\beta$-lactam]. Computer analyses of the data (Schilf *et al.*, 1978) did not indicate a better agreement assuming a non-competitive model. With the system 7-aminocephalosporanic acid/R39 enzyme, a long-lived complex ($k_3 = 1 \times 10^{-6}$ s^{-1}) was formed with moderate efficiency (k_2/K = 200 M$^{-1}\cdot$s^{-1}).

Interactions between 6-aminopenicillanic acid and the R39 enzyme and between 7-aminocephalosporanic acid and the R61 enzyme

Both EI* complexes were short-lived (half-lives 2–3 min). With the system 6-aminopenicillanic acid/R39 enzyme, the k_3 term of the reaction was determined as shown in Fig. 2. In turn, the velocity of formation of complex EI* was estimated by measuring the enzyme activity at the steady state in a mixture containing the enzyme (200 ng in 30 μl, final volume), 1.5 mM-Ac_2-L-Lys-D-Ala-D-Ala and 6-aminopenicillanic acid at concentrations ranging from 3 to 13 μM. The K_i value was 4.5 μM. Since K_i is related to K, k_2 and k_3 by $K_i = k_3 K/(k_2 + k_3)$, it follows that for $k_2 \gg k_3$ (as observed with all the β-lactam compounds tested with the R39 enzyme), $K_i = k_3 K/k_2$. Under this assumption ($k_2 \gg k_3$), the value of k_2/K was estimated to be equal to 1200 M$^{-1}\cdot$s^{-1}.

The very short half-lives of the complexes EI* permitted investigation of the fate of 6-aminopenicillanic acid during interaction with the R39 enzyme, and of 7-aminocephalosporanic acid during interaction with the R61 enzyme. The R39 enzyme {6 nmol in 10 μl of 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer containing 5 mM-$MgCl_2$ and 50 mM-NaCl} and 6-aminopenicillanic acid (6 nmol dissolved in 2 μl of the same buffer) were mixed together and incubated at 37°C. After 10 min, a new addition of 6 nmol of 6-aminopenicillanic acid was made and similar additions were repeated every 10 min during 2 h, so that, eventually, 60 nmol of 6-aminopenicillanic acid was degraded. Similarly, the R61 enzyme (15 nmol in 50 μl of 5 mM-phosphate buffer, pH 7.0) and 7-aminocephalosporanic acid (15 nmol in 5 μl of the same buffer) were mixed together and incubated at 37°C. After 10 min, a new addition of an equivalent amount of 7-aminocephalosporanic acid was made and the same operation was repeated 10 times. Analysis of the two reaction mixtures by ion-exchange chromatography in an automatic amino acid analyser failed to reveal any trace of glycine (the reaction product that should have arisen by fragmentation of the unsubstituted penam and 3-cephem compounds). In turn, analysis of the reaction mixtures by high-voltage paper electrophoresis at pH 6.5 revealed one neutral and four negatively charged ninhydrin-positive compounds. One of these had the same migration as that of the product obtained by degradation of 6-aminopenicillanic acid or 7-aminocephalosporanic acid by β-lactamase action. Finally, the reaction products of the R61 enzyme-treated 7-aminocephalosporanic acid were also analysed by t.l.c. (see the Materials and methods section). Detection with ninhydrin revealed a streak with six dark bands exhibiting R_F values of 0.50, 0.44, 0.31, 0.23, 0.15 and 0.11 respectively. Under the same conditions, glycine gave a red–grey spot with R_F 0.29 and 7-aminocephalosporanic acid formed a streak whose leading edge had R_F 0.36.

Interaction between clavulanate and the R61 and R39 enzymes

Both R61 and R39 enzymes were slowly inactivated by clavulanate ($k_2/K \simeq 20$–$30\,M^{-1}\cdot s^{-1}$) and the inactivated complexes EI* were highly stable ($k_3 = 5\times10^{-6}\ s^{-1}$ or smaller). When immobilized in the form of complex EI*, the enzymes were no longer able to bind [^{14}C]benzylpenicillin, suggesting that in spite of their structural differences, benzylpenicillin and clavulanate compete for the same site on the enzymes.

Formation of complex EI* with the R61 enzyme was also investigated at 22°C by measuring, as a function of time, the decreased fluorescence emission of the enzyme solution caused by increasing concentrations of clavulanate. The bimolecular rate constant thus obtained ($23\,M^{-1}\cdot s^{-1}$) was virtually identical with that found by measuring the disappearance of enzyme activity. In addition, formation of the complex EI* with both the R61 and R39 enzymes was investigated by u.v. measurements. Solutions of the R61 enzyme (0.45 mg/ml of 5 mM-sodium phosphate buffer, pH 7.0) or the R39 enzyme (0.64 mg/ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-$MgCl_2$ and 50 mM-NaCl) were supplemented with clavulanate (final concentrations: 80 μM in the case of the R61 enzyme and 0.18 mM in the case of the R39 enzyme). The spectra (between 250 and 330 nm) were recorded at $1\,nm\cdot s^{-1}$ immediately after addition of clavulanate to the sample cells and after increasing times of incubation (at 25°C with the R39 enzyme and 21.5°C with the R61 enzyme). The reference cells contained equivalent concentrations of each enzyme dissolved in the corresponding buffers.

Complex EI* formed with the R61 enzyme (in phosphate buffer) had a maximum absorbance at 283 nm and, at this wavelength, the difference ε between the molar absorption coefficient of complex EI* and that of the free enzyme was equal to $15\,600\,M^{-1}\cdot cm^{-1}$. A plot of $\ln[(\Delta A_\infty - \Delta A_t)/\Delta A_\infty]$ versus time (Fig. 3), where ΔA_t and ΔA_∞ are the absorbance differences (between the sample and reference cells) at times t and ∞ respectively, gave rise to a straight line from which a k_2/K value of $17\,M^{-1}\cdot s^{-1}$ could be calculated. This value was similar to those obtained by measuring the disappearance of enzyme activity or the decrease of fluorescence emission. Treatment of complex EI* with 2.5 mM neutral hydroxylamine caused a 50% enzyme recovery after 5 h (apparent pseudo-first-order rate constant $3\times10^{-5}\ s^{-1}$).

When added to the R39 enzyme (in Tris/HCl buffer), clavulanate reacted with the base form (probably) of the buffer, causing the appearance of a compound exhibiting a spectrum similar to that given by complex EI* minus free enzyme. After proper correction of the recorded data (at 276 nm), the plot $\ln[(\Delta A_\infty - \Delta A_t)/\Delta A_\infty]$ versus time was biphasic (Fig. 4) and the linear segment of the curve obtained at prolonged incubation times had a slope of $2.2\times10^{-4}\ s^{-1}$. If one assumes a two-step reaction:

$$E + I \xrightarrow{k_f} EI^* \xrightarrow{k'} EI^{**}$$

the variation of absorbance as a function of time and at a given concentration of clavulanate is given by:

$$\frac{\Delta A_\infty - \Delta A_t}{\Delta A_\infty} = \frac{k_a e^{-k't}(\varepsilon_2 - \varepsilon_1) + e^{-k_a t}(\varepsilon_1 k_a - \varepsilon_2 k')}{\varepsilon_2 (k_a - k')}$$

where $k_a = k_f$[clavulanate] and ε_1 and ε_2 = differences between the molar absorption coefficients of complexes EI* and EI** respectively, and that of the free enzyme. From the absorbance value measured at completion of the reaction, ε_2 was

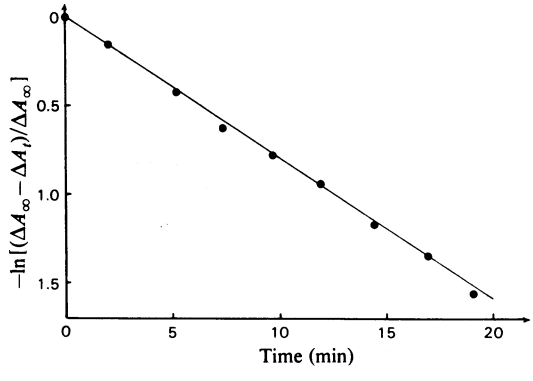


Fig. 3. *Interaction between clavulanate and the serine R61* D-*alanyl*-D-*alanine peptidase as determined by absorbance measurements at 283 nm (and at 25°C)*
For ΔA_∞ and ΔA_t and experimental details, see text.

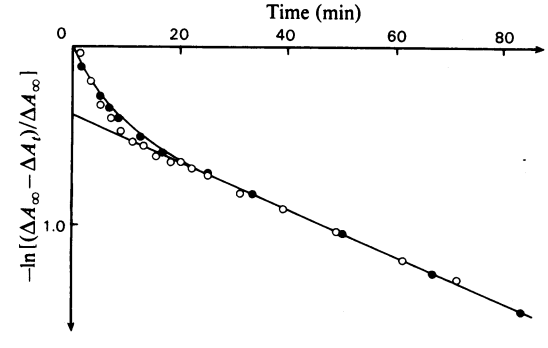


Fig. 4. *Interaction between clavulanate and the serine R39* D-*alanyl*-D-*alanine peptidase as determined by absorbance measurements at 276 nm (and 21.5°C)*
For ΔA_∞ and ΔA_t and experimental details, see the text. The ΔA_∞ and ΔA_t values were corrected as indicated in the text. O, Experimental data; ●, theoretical data.

estimated to be equal to $52000\,M^{-1}\cdot cm^{-1}$. In turn, correction of the first part of the curve (to remove the contribution of the second part) permitted estimation of a pseudo-first-order rate constant of $3.25\times10^{-3}\ s^{-1}$, from which a k_f value of $18\,M^{-1}\cdot s^{-1}$ could be calculated. This value was in good agreement with that obtained by measuring the disappearance of enzyme activity. Finally, when extrapolated to $t = 0$, the straight portion of the curve intersected the ordinate at a value of $-0{\cdot}38$. Since this value corresponds to $\ln[k_a(\varepsilon_2-\varepsilon_1)/(k_a-k')\varepsilon_2]$, an ε_1 value of $18700\,M^{-1}\cdot cm^{-1}$ could be calculated. Fig. 4 shows both the experimental curve and the theoretical one as obtained by using the following values: $\varepsilon_1 = 18700\,M^{-1}\cdot cm^{-1}$; $\varepsilon_2 = 52000\,M^{-1}\cdot cm^{-1}$; $k_a = k_f[\text{clavulanate}] = 3.25\times10^{-3}\ s^{-1}$ and $k' = 2.2\times10^{-4}\ s^{-1}$. Experiments carried out with other concentrations of clavulanate yielded similar values of ε_1, ε_2, k_f and k'. Treatment of complex EI** with neutral 3mM-hydroxylamine did not result in enzyme regeneration. Higher concentrations of hydroxylamine inactivated the R39 enzyme.

Discussion

The metallo (Zn^{2+}) G D-alanyl-D-alanine peptidase is highly resistant to the classical penicillins and Δ^3-cephalosporins. Not surprisingly, this enzyme is not effectively inactivated by any of the unusual β-lactam compounds studied here. 7-Aminocephalosporanic acid behaves as a reversible competitive inhibitor of the enzyme with a K_i value of 0.2mM. A 0.7mM K_i value had been found previously for the interaction between the G enzyme and cephalosporin C, but in this case, the inhibition was found to be non-competitive (Frère *et al.*, 1978). Recent studies (H. Labischinski, G. Barnickel, H. Bradaczek, O. Dideberg, P. Charlier, J.-M. Frère & J.-M. Ghuysen, unpublished work) have shown that crystals of the G enzyme soaked in the mother liquor containing 0.1mM- (or less) cephalosporin C 'crack' very rapidly. Moreover, small-angle X-ray scattering studies suggest that, in solution, cephalosporin C causes aggregation of the enzyme molecules. Like 7-aminocephalosporanic acid, *p*-iodo-β-phenylacetylaminocephalosporanic acid is a competitive inhibitor of the G enzyme; it was successfully diffused in a native enzyme crystal, permitting visual detection of the enzyme binding site (Dideberg *et al.*, 1980*a*).

Among the β-lactam compounds studied, cefoxitin (O'Callaghan, 1979) and *N*-formimidoylthienamycin (as well as thienamycin; Cama & Christensen, 1978) are potent anti-bacterial agents. These two compounds are the only ones that effectively inactivate the serine R39 and, to a lesser extent, R61 D-alanyl-D-alanine peptidases. Mecillinam is highly active at least against Gram-negative bacteria. It is unique in that it induces conversion of *Escherichia coli* rods into osmotically stable spherical cells by interacting specifically with one single membrane-bound protein referred to as penicillin-binding protein no. 2 (Spratt, 1977). The possible enzymic function of this particular protein is unknown. Mecilinam is a very poor inactivator of the R61 and R39 serine enzymes.

The interaction between the R61 and the R39 serine enzymes and classical 6(7)β-substituted penicillins (Δ^3-cephalosporins) involves the formation of an acyl-enzyme. The stability of this acyl-enzyme and the fate of the bound metabolite depend on both the enzyme and the particular β-lactam under study (Ghuysen *et al.*, 1979). In this respect, one interesting result brought about by the present study is that the intermediates formed between the unsubstituted 6-aminopenicillanic acid and the serine R61 and R39 enzymes are long- and short-lived respectively, whereas those formed between the unsubstituted 7-aminocephalosporanic acid and the serine R61 and R39 enzymes are short- and long-lived respectively. In addition, breakdown of the short-lived intermediates formed between the R39 enzyme and 6-aminopenicillanic acid or between the R61 enzyme and 7-aminocephalosporanic acid gives rise to several ninhydrin-positive degradation products. None of them is glycine, the product that would have been formed if breakdown of the intermediates had involved fragmentation of the acyl moieties, as it occurs with the benzylpenicilloyl–R61 or –R39 enzyme intermediates (Frère *et al.*, 1975*a*). The present study also permits comparison of the various effects that a given acyl side chain exerts on the k_2/K term of the reaction, depending on both the structure of the β-lactam nucleus and the enzyme under consideration. Hence, on the basis of data published previously for ampicillin and cephaloglycine (Frère *et al.*, 1975*b*; Fuad *et al.*, 1976), the comparable pairs 6-aminopenicillanic acid/ampicillin and 7-aminocephalosporanic acid/cephaloglycine show that, with the R39 enzyme, attachment of a C_6H_5–$CH(NH_2)$–CO β side chain to the penam or 3-cephem nucleus results in 210- and 1250-fold increases in the k_2/K values respectively. With the R61 enzyme, however, the k_2/K value of ampicillin is 400 times that of 6-aminopenicillanic acid, and the k_2/K value of cephaloglycine is 0.64 times that of 7-aminocephalosporanic acid.

Clavulanate has no anti-bacterial effect by itself, but is one of the most potent β-lactamase inactivators known at this time (Fisher *et al.*, 1980). In parallel with this, it is a very weak inactivator of the serine R61 and R39 enzymes. It inactivates the two D-alanyl-D-alanine peptidases with the same very low rate, an observation that may be surprising,

since the R61 enzyme has always been found less sensitive to β-lactam antibiotics than the R39 enzyme.

The reaction between clavulanate and the R61 enzyme causes an increase of $15600\,M^{-1}\cdot cm^{-1}$ in the absorption coefficient of the enzyme at 283 nm. This value is close to that reported by Cartwright & Coulson (1979) for the inactivated complex formed between clavulanate and the β-lactamase of *Staphylococcus aureus*. In the case of the R39 enzyme, a similar inactive complex is formed ($\Delta\varepsilon_{276} = 18700\,M^{-1}\cdot cm^{-1}$), which undergoes a further monomolecular reaction, giving rise to a second complex that has a much higher absorbance ($\Delta\varepsilon_{276} = 52000\,M^{-1}\cdot cm^{-1}$).

Finally, quinacillin (a relatively poor substrate of the TEM β-lactamase) and quinacillin sulphone (a rather good inactivator of the same β-lactamase; Fisher *et al.*, 1980) do not inactivate the serine R61 enzyme (at the concentrations indicated in Table 1) and are poor inactivators of the R39 enzyme. In this respect, it is noteworthy that oxidation of the sulphur atom at position 1 in quinacillin to a sulphone causes a 40-fold decrease in the k_2/K value.

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