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The importance of the negative charge of β-lactam compounds for the inactivation of the active-site serine DD-peptidase of *Streptomyces* R61

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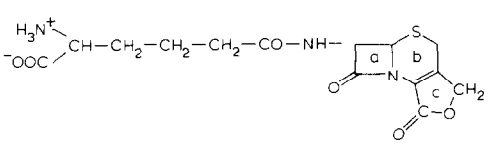
The interaction between the *Streptomyces* R61 penicillin-sensitive DD-peptidase and deacetyl-cephalosporin C or its lactone derivative has been studied at different pH values. The results show the importance of an enzyme group of $pK \cong 9$ which might form an ion pair with the free carboxylate of the former compound. This electrostatic interaction is shown to contribute to the formation of the first, non-covalent enzyme-inactivator complex by a factor of at least 50.

β-Lactam; pH dependence; Enzyme inactivation; Deacetyl-cephalosporin C; DD-peptidase

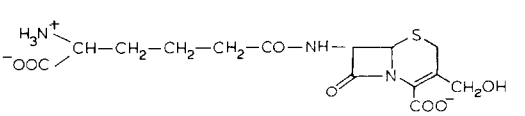
1. INTRODUCTION

The study of the pH dependence of the active-site serine DD-peptidase of *Streptomyces* R61 [1] has revealed that the deprotonation of an enzyme group of $pK \cong 9.3–9.6$ induces a sharp decrease in the rates of enzyme-catalysed hydrolysis of the peptide substrate and inactivation by various β-lactams. The group concerned has been hypothesized to be the side chain of a lysine residue. Indeed, the active-site serine residue of penicillin-binding proteins and β-lactamases is invariably followed by an -X-X-Lys sequence [2–5], suggesting that charge pairing between an enzyme positive group and the C-terminal carboxylate of the substrate or the carboxylate on C_3 of penicillins (or C_4 of cephalosporins) might be important for recognition of substrate and inactivator by the enzyme. Accordingly, esterification of the carboxylate on C_3 of penicillins strongly decreases the antibacterial activity [6]. However, with cephalosporins, lactonization of the C_4 carboxylate with an alcohol function borne by the C_3 substituent yields active compounds. Moreover, the lactone of deacetyl-cephalosporin C (I) is a reasonably good substrate of β-lactamase I from *Bacillus cereus* [7]. In preliminary experiments, inactivation of *Streptomyces* R61 DD-peptidase by I was found to proceed at a non-negligible rate.

(I)



(II)



The present work compares deacetyl-cephalosporin C (II) to the corresponding lactone (I) as inactivators of *Streptomyces* R61 DD-peptidase.

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2. MATERIALS AND METHODS

All experiments were performed at 25°C.

2.1. *Enzymes*

Streptomyces R61 DD-peptidase and *B. licheniformis* β-lactamase were purified as described [8,9].

2.2. *β-Lactam compounds*

Compounds I and II were kind gifts from Dr J.B. Ward (Glaxo Research Group, Greenford, Middlesex, England).

2.3. *Buffers*

The following buffers were used: pH 7.0, 10 mM sodium phosphate; pH 8.0, 7 mM sodium phosphate; pH 9, 12 mM glycine + NaOH; pH 9.5, 15 mM glycine + NaOH; pH 10 and 10.5, 25 mM glycine + NaOH. The ionic strength of all buffers was adjusted to a conductance of 0.1 mS using a Methrom E527 conductometer (c = 11.6 cm^{-1}) either by modifying the buffer concentration or by addition of NaCl in the case of the glycine-NaOH buffer, pH 9.

2.4. *DD-peptidase activity*

Hydrolysis of the synthetic substrate N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine was stopped by heating the sample at 100°C for 1 min and the amount of released D-alanine was measured by the D-amino acid oxidase procedure [10].

2.5. *Model and equations for the interaction with β-lactam compounds* [11]

The results were analysed on the basis of the three-step model

$$E + I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} EI^* \xrightarrow{k_{+3}} E + P$$

where E denotes the enzyme, I the β-lactam, EI* the acyl enzyme and K the dissociation constant of EI. If [I] ≫ [E], the rate of accumulation of EI*, $[EI^*]_t - [EI^*]_{ss}$ (where $[EI^*]_t$ and $[EI^*]_{ss}$ are the concentrations of EI* at time t and at the steady state, respectively) is proportional to e^{-k_a} where $k_a = (k_{+3} + k_f)$ and $k_f = (k_{+2}[I])/(K + [I])$.

2.6. *Determination of the kinetic parameters*

2.6.1. Value of k_{+3}

After complete inactivation of the enzyme, excess β-lactam was eliminated by addition of β-lactamase. The solutions were further incubated and, after increasing time periods, samples were taken to which was added substrate for activity estimation.

2.6.2. Value of k_f

With deacetyl-cephalosporin C, k_{+3} was small (see below) and, in all experiments, $[EI^*]_{ss}$ remained negligible. The value of k_f at low [I] was computed by measuring the enzyme inactivation as a function of time. Faster reactions were monitored by measuring the quenching of the enzyme fluorescence at 320 nm upon formation of the acyl enzyme [11]. These experiments were performed using a Kontron SFM23 spectrofluorometer. Excitation was at 280 nm. A 20 μl aliquot of β-lactam solution was rapidly mixed with 980 μl enzyme solution (5.3 μM) and the exponential decrease in fluorescence was analysed as in [1]. At pH 10.5, the decrease in fluorescence was too small to allow utilization of this method, and the enzyme was too unstable for monitoring the decrease in activity. It was only possible to obtain a rough approximation of k_f by following the decrease in absorbance at 260 nm after adding concentrated enzyme to a 10 μM solution of deacetyl-cephalosporin C. The final enzyme concentration was also 10 μM so that k_f could be computed by using the second-order rate law.

With the lactone, the fluorescence method was used, but k_{+3} was not always negligible when compared to k_a and k_f was obtained from $k_a - k_{+3}$. The instability of the lactone precluded any measurement above pH 10.

The susceptibility of the compounds to nucleophilic attack by OH^- was determined by following the decrease in absorbance of a 150 μM solution in 1 M K_2HPO_4 adjusted to pH 11.25 (for the lactone) or 12.25 (for deacetyl-cephalosporin C) with 10 M KOH [12].

3. RESULTS

3.1. *Values of* k_{+3} (table 1)

Reactivation was markedly more rapid with the

Table 1

pH dependence of k_{+3}

pH	$10^5 \times k_{+3}$ (s^{-1})	
	Deacetyl-cephalosporin C	Lactone
7.0	2 ± 0.4	31 ± 4
8.0	2.4 ± 0.5	53 ± 9
9.0	6.1 ± 0.9	310 ± 50
10.0	12 ± 2	550 ± 80

Deacetyl-cephalosporin C: enzyme (9.4 μM) and β-lactam (625 μM) were incubated at pH 7.0 in a total volume of 170 μl of 1 mM sodium phosphate, pH 7.0. After 15 min, inactivation was complete and 10-μl aliquots were 20-fold diluted in a suitable buffer containing 23 μg β-lactamase. The recovery of activity was measured on 10-μl aliquots over a period of 60 min. Lactone: enzyme (0.44 μM) and the lactone (616 μM) were incubated for 10 min in 0.1 mM sodium phosphate pH 7.0, which resulted in complete inactivation. Samples (50 μl) were then supplemented with 150 μl of suitable buffer containing 5 μg β-lactamase. The reactivation was measured on 10-μl aliquots over a period of 10 (pH 9 and 10) or 30 min (pH 7 and 8)

lactone and, as observed before, the value of k_{+3} sharply increased at high pH [1]. This phenomenon has been shown to be due to direct attack of the acyl enzyme by OH^- [1].

3.2. *Values of* k_f

In a first series of experiments, only one β-lactam concentration was used at each pH (table 2). The decrease in $k_f/[I]$ observed at pH 10 appeared to be significantly larger with deacetyl-cephalosporin C than with the lactone (3.75 ± 0.6 vs 1.7 ± 0.25-fold decrease between pH 8.0 and 10.0). The value of k_f was then measured at various concentrations of β-lactams and plots of $1/k_f$ vs $1/[I]$ allowed the computation of the individual values of k_{+2} and K (fig.1), in the pH range 7–10 for deacetyl-cephalosporin C and at pH 7.0 for the lactone. With this latter compound, experiments were performed at concentrations ranging between 140 and 412 μM. Plots of $1/k_f$ vs $1/[I]$ yielded the following results: $k_{+2}/K = 194 \pm 17\ M^{-1} \cdot s^{-1}$, $k_{+2} = 0.30 \pm 0.18\ s^{-1}$ and $K = 1.5 \pm 0.9$ mM. The large errors in the individual values of k_{+2} and K were due to the fact that the lactone concentrations were too low when compared to K. However, it was not possible to use greater concentrations of the lactone, because the reaction became too rapid and reabsorption of the emitted radiation (in the fluorescence experiments) became too significant. Consequently, it seemed that, for the lactone, the ratio $k_f/[I]$ provided a good approximation of k_{+2}/K at all pH values, since it was very unlikely that K would decrease at high pH values. At pH 10.5, a k_f value of $1.2 \pm 0.3 \times 10^{-3}\ s^{-1}$ was measured with deacetyl-cephalosporin C, indicating a k_2/K value of $120 \pm 30\ M^{-1} \cdot s^{-1}$. The individual values of k_2 and K could not be obtained.

3.3. *Hydrolysis by* OH^-

At pH 12.25 and 30°C, the pseudo-first-order rate constant for the degradation of deacetyl-

Table 2

pH dependence of $k_f/[I]$

pH	Deacetyl-cephalosporin C			Lactone		
	[I] (μM)	k_f (s^{-1})	$k_f/[I]$[a] ($M^{-1} \cdot s^{-1}$)	[I] (μM)	k_f (s^{-1})	$k_f/[I]$ ($M^{-1} \cdot s^{-1}$)
7.0	30	0.035 ± 0.0012	1170 ± 40	412	0.074 ± 0.004	180 ± 10
8.0	30	0.0405 ± 0.0012	1350 ± 40	412	0.082 ± 0.004	200 ± 10
9.0	30	0.0237 ± 0.0012	790 ± 40	412	0.075 ± 0.004	183 ± 10
9.5	50	0.0250 ± 0.002	500 ± 40		N.D.	
10	50	0.018 ± 0.002	360 ± 40	412	0.048 ± 0.004	117 ± 10

[a] The β-lactam concentrations were not much smaller than the K values, so $k_f/[I]$ was not equivalent to k_{+2}/K

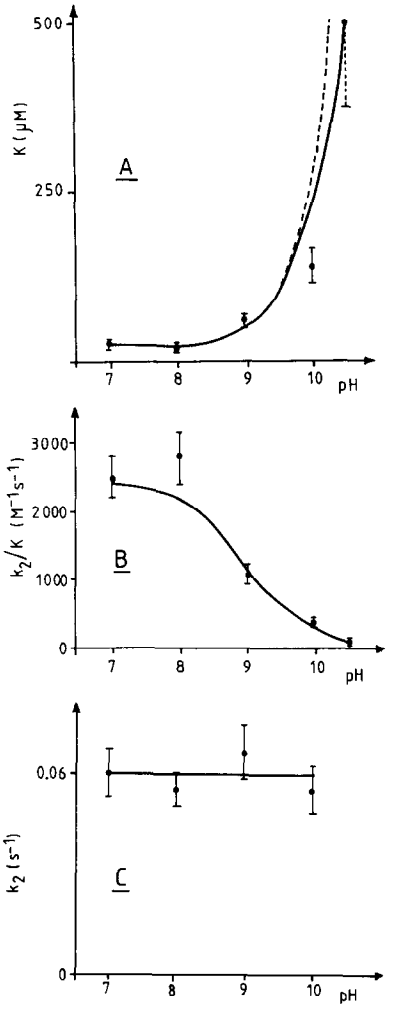
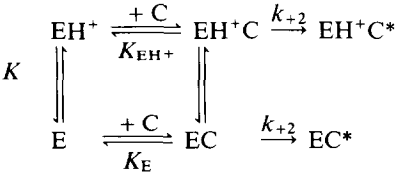


Fig.1. pH dependence of K (A), k_{+2}/K (B) and k_{+2} (C) for deacetyl-cephalosporin C. β-Lactam concentrations varied from 20 to 100 μM (pH 7.0), 30 to 150 μM (pH 8.0), 50 to 150 μM (pH 9.0) and 50 to 500 μM (pH 10). The enzyme concentration was 5.3 μM. At pH 10.5, both enzyme and β-lactam were 10 μM and only the value of k_{+2}/K could be measured. The value of K at that pH was computed by assuming that k_{+2} remained constant (0.06 s^{-1}). The following simple model was used:



The apparent dissociation constant of the non-covalent complex is

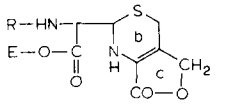
$$K_{app} = (K_{EH+}) \frac{[1 + \frac{K}{[H^+]}]}{[1 + \frac{KK_{EH+}}{K_E[H^+]}]}$$

The solid lines were drawn on the basis of this equation with the following values for the constants: pK = 9.0; K_{EH+} = 25 μM; K_E = 1250 μM. If one assumes that $K_E = \infty$, the dashed curve (A) is obtained. The corresponding curve is not shown in (B), since it is nearly indistinguishable from that displayed.

cephalosporin C was 1.6 ± 0.2 × 10^{-3} s^{-1}, which yielded a value of 0.09 ± 0.01 ± 0.2 $M^{-1} \cdot s^{-1}$ for the second-order rate constant, in good agreement with the values obtained before [12] for similar compounds. The lactone was about 200-fold more unstable: at pH 11.25, the pseudo-first-order rate constant was 34 ± 3 × 10^{-3} s^{-1}, yielding a second-order rate constant of 19 ± 2 $M^{-1} \cdot s^{-1}$.

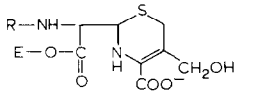
4. DISCUSSION

The relative instability of the acyl enzyme formed upon inactivation of *Streptomyces* R61 DD-peptidase by the lactone seemed to indicate that the lactone c ring remained intact (structure III)



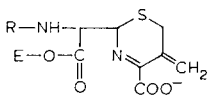
(III)

Indeed, hydrolysis of the lactone would have yielded the same adduct as that obtained with deacetyl-cephalosporin C (IV), since expulsion of the OH group of cephalosporins containing a CH_2OH substituent on C_3 does not seem to occur upon formation of the acyl enzyme [13].



(IV)

Alternatively, rearrangement with expulsion of the leaving group attached to the methylene group on C_3 would have yielded V.



(V)

In the first case, the value of k_{+3} for the lactone would have been the same as that observed with deacetyl-cephalosporin C and, in the second, it should have been even lower [13].

As demonstrated in fig.1, the decrease in $k_f/[I]$ as the pH increased was due to an increased value of K. It could be hypothesized that charge pairing between an enzyme positive group (of $pK \cong 9$) and the cephalosporin carboxylate enhanced the strength of the non-covalent interaction by a large factor, which could be estimated to be close to or greater than 50. Unfortunately, the instability of the enzyme precluded the obtaining of accurate data at pH 10.5 or above. With the lactone and at pH 7.0, the K value was 60 ± 36-fold larger than that observed with deacetyl-cephalosporin C under similar conditions and an increase in pH to 10 had a much less dramatic effect on the k_{+2}/K value. Indeed, the value of the second-order rate constant was 60 ± 10% of that observed at pH 7.0. This slight decrease was probably due to a corresponding increase in K (a larger increase in K would imply a corresponding increase in k_{+2}, a very unlikely phenomenon). The pH dependence of K for the lactone might be due to the presence of a partial negative charge on the carbonyl group of the latter compound.

It is also interesting to note that the first-order rate constants for acylation (k_{+2}) and deacylation (k_{+3}) were greater with the lactone. These observations might be correlated with the fact that the lactone is also a better substrate of β-lactamase I from *B. cereus* [7]. The value of k_{+2} for the lactone was 5 ± 3-fold greater than that for deacetyl-cephalosporin C, while the susceptibility to nucleophilic attack of the former compound was about 200-fold larger than that of the latter. Since both compounds have the same substituent on C_7, the large discrepancy must be attributed to a less productive location of the β-lactam carbonyl near the enzyme active serine, probably due to a poor electrostatic interaction or to steric factors such as a lack of free rotation around the C_3–CH_2O bond.

In conclusion, the present data indicate that charge pairing between the β-lactam carboxylate and an enzyme positive group of $pK \cong 9$ contribute by a factor of at least 50 in the initial, non-covalent interaction between the two reagents.

ACKNOWLEDGEMENTS

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