The importance of the negative charge of β -lactam compounds in the interactions with active-site serine DD-peptidases and β -lactamases

Louis VARETTO,* Fabien DE MEESTER,* Didier MONNAIE,* Jacqueline MARCHAND-BRYNAERT,† George DIVE,* Françoise JACOB* and Jean-Marie FRÈRE*†

*Centre for Protein Engineering and Laboratoire d'Enzymologie, Université de Liège, B6 Sart-Tilman, B4000 Liège, Belgium, and †Laboratoire de Chimie Organique, Université Catholique de Louvain, B1348 Louvain-la-Neuve, Belgium

The interaction between various penicillins and cephalosporins the carboxylate group of which at C-3 or C-4 had been esterified or amidated and different penicillin-recognizing enzymes was studied. In general, our findings reinforced the common assumption that an anionic group at that position is necessary for the effective acylation of these enzymes. However, the relative activities of the modified β -lactams as inactivators of the *Streptomyces* R61 DD-peptidase or as substrates of the *Bacillus licheniformis*, *Streptomyces albus* G and *Enterobacter cloacae* β -lactamases did not fit a general scheme in which the intrinsic electronic and geometric properties of the β -lactam compounds would be sufficient to explain their substrate or inactivator properties towards the various types of enzymes investigated.

INTRODUCTION

The understanding of the catalytic mechanisms of β -lactamases and penicillin-sensitive DD-peptidases has recently been improved by the contributions of X-ray crystallography (Herzberg & Moul, 1987; Dideberg *et al.*, 1987; Kelly *et al.*, 1989; Oefner *et al.*, 1990) and site-directed mutagenesis (Gibson *et al.*, 1990; Ellerby *et al.*, 1990; Jacob *et al.*, 1990).

In spite of these advances, we are still far from being able to propose detailed mechanisms that coherently explain the various interactions and account for the specificities of the different classes of enzymes. Taking a different approach, we have undertaken to analyse closely the commonly accepted assumption that an anionic group, such as the carboxylate group at C-3 of penicillins, the C-4 of cephalosporins or the SO_3^- group in monobactams is necessary for effective biological activity and, in consequence, for the interaction with the penicillin-sensitive DD-peptidases (Varetto *et al.*, 1987a,b).

An independent study of Laws & Page (1989) examined the effect of this carboxylate group on the chemical reactivity of penicillins and cephalosporins and on their interaction with a class A β -lactamase and a class B β -lactamase. To obtain a general overview of these phenomena, we decided to extend the studies to the interactions between various compounds with modified carboxylate group and representatives of each of the major families of active-serine penicillin-recognizing enzymes: a penicillin-sensitive DD-peptidase (from *Streptomyces* R61), two class A β -lactamases (from *Bacillus licheniformis* and *Streptomyces albus* G) and a class C β -lactamase (from *Enterobacter cloacae*).

MATERIALS AND METHODS

Enzymes

The *Streptomyces* R61 DD-peptidase was purified as described by Frère *et al.* (1976) and the β -lactamases of *B. licheniformis* 749C, *S. albus* G and *E. cloacae* 908R were purified as described by Matagne *et al.* (1990). Horseradish peroxidase and pig kidney

D-amino acid oxidase were from Boehringer (Mannheim, Germany).

Substrates

$N^{\alpha}N^{\epsilon}$ -Diacetyl-L-lysyl-D-alanyl-D-alanine ($\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$) was from UCB Bioproducts (Braine-l'Alleud, Belgium). Benzylpenicillin was from Rhône-Poulenc (Paris, France), deacetylcephalosporin C and the corresponding lactone were gifts from Dr. J. B. Ward (Glaxo Research Group, Greenford, Middx., U.K.) and cephalosporin C was a gift from Eli Lilly and Co. (Indianapolis, IN, U.S.A.). The methyl ester and the amide of benzylpenicillin were prepared as described by Jaszberenyi & Gunda (1975).

Buffers

Experiments were performed in the following buffers: pH 5.5, 11.5 mM-sodium cacodylate/HCl; pH 6, 13 mM-sodium cacodylate/HCl; pH 6.5, 11.5 mM-sodium phosphate or 11.5 mM-sodium cacodylate/HCl; pH 7, 10 mM-sodium phosphate. The salt contents of the reaction mixtures were adjusted to a conductance of 0.1 mS with the use of a Methrohm E527 conductometer by slightly modifying the buffer concentrations.

Electrophoresis

The charge of the product resulting from the hydrolysis of the lactone by OH^- ions and by the β -lactamases was estimated by paper electrophoresis (60 V/cm) in water/acetic acid/collidine (1000:2.7:9, by vol.), pH 6.5. Deacetylcephalosporin C and its hydrolysis product were used as standards. The compounds were detected by spraying the paper with a ninhydrin solution, which reacted with the free amino group on the side chain.

Measurement of DD-peptidase activity

The enzyme was incubated at 37 °C in 5 mM-sodium phosphate buffer, pH 7.0, in the presence of 2 mM- $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ and the hydrolysis reaction was terminated by heating the sample

Abbreviation used: EREF, enzyme-rate-enhancement factor.

† To whom correspondence should be addressed.

at 100 °C for 1 min. The amount of D-alanine released was measured by the D-amino acid oxidase assay (Frère *et al.*, 1976).

Spectrophotometric measurements

Absorbance measurements were performed with the help of a Beckman DU-8 spectrophotometer linked to an Apple II micro-computer or of a Uvikon 860 spectrophotometer linked to a Copam PC microcomputer.

Fluorescence measurements were made with a Perkin-Elmer MPF-44 spectrofluorimeter.

Determination of the kinetic parameters

The analysis was based on the three-step model

where EC* is the acyl-enzyme and is the same for the interaction between β -lactams and DD-peptidases or β -lactamases.

For β -lactamases, the classical kinetic parameters

$$k_{cat} = k_2 k_3 / (k_2 + k_3)$$

and

$$K_m = k_3 K' / (k_2 + k_3)$$

where $K' = (k_{-1} + k_{+2}) / k_{+1}$, were derived from the analysis of complete time courses (De Meester *et al.*, 1987) or from initial rates by using the Hanes linearization of the Henri-Michaelis equation. Values of k_{cat} / K_m were also obtained directly from the analysis of complete time courses at $[C] \ll K_m$. Conversely, when K_m values were very low, they were determined as K_1 values with nitrocefin as reporter substrate, and k_{cat} values were derived from initial rates at saturating substrate concentrations.

With the DD-peptidase, values of k_2 / K' and of k_3 were computed from the rate of inactivation and the residual activity at the steady state on the basis of eqns. (2) and (3):

$$k_a = k_3 + \frac{k_2[C]}{[C] + K'} = k_3 + k_t \quad (1)$$

where k_a is the apparent first-order rate constant for inactivation. When $[C] \ll K'$, the equation reduces to:

$$k_a = k_3 + \frac{k_2[C]}{K'} \quad (2)$$

The residual activity at the steady state is given by eqn. (3):

$$\frac{A_{ss}}{A_0} = \frac{k_3}{k_3 + k_t} \quad (3)$$

where A_{ss} and A_0 are the enzyme activities respectively at the steady state and at time zero, just after the addition of the β -lactam. The value of k_a could also be obtained by monitoring the fluorescence decrease that accompanies the formation of the EC* covalent adduct (excitation and emission wavelengths were 280 nm and 320 nm respectively).

The rate constants for the spontaneous hydrolysis of the various β -lactams were determined by monitoring the u.v. absorbance of aqueous solutions adjusted to high pH. The reactions were initiated by diluting a 50 μ l sample of concentrated substrate in 450 μ l of 10 mM-buffer adjusted to the appropriate pH.

Because of the low solubility of the amide and methyl ester of benzylpenicillin in water, all dilutions were made from a 25 mM stock solution containing 75 % (v/v) acetonitrile for the former and 50 % (v/v) acetonitrile for the latter compounds. All the experimental mixtures thus contained 3 % or 2 % of acetonitrile per mM derivative.

THEORETICAL CALCULATIONS

All the degrees of freedom of geometry of the 12 compounds (the six compounds of Table 1 and their methyl esters, used as models for the acyl-enzymes) have been fully optimized in the semi-empirical AM 1 framework (Dewar *et al.*, 1985).

The net charges were calculated by Mulliken population analysis of the STO-3G density matrix at the AM 1 geometry. To calculate the electrostatic potential generated around the molecules, the density matrix elements were obtained at the CNDO/2 level (Pople & Beveridge, 1970).

The electrostatic potentials were calculated with a complete CNDO scheme in which the nuclear attraction integrals are approximated by minus the repulsion integrals between *s* orbitals (approximation I $\gamma\alpha h$) (Giessner-Prettre & Pullman, 1972).

The dimensions of the regular three-dimensional grid were defined as the dimensions of the molecules increased by 0.5 nm (5 Å) in each direction. From these data sets, a contouring algorithm was applied joining the points at the level of -41.8 kJ/mol (-10 kcal/mol).

RESULTS

Hydroxide-ion-catalysed hydrolysis

These experiments were performed to obtain an estimation of the sensitivity of the various compounds to the action of nucleophiles. The results are summarized in Table 1.

The fact that the lactone ring remained intact after hydrolysis of the β -lactam ring by OH⁻ ions and the β -lactamases was demonstrated by the electrophoretic mobility of the product. The following mobilities were recorded after correction for electro-endosmosis: deacetylcephalosporin C, 17 cm/h (one negative charge); lactone, 0 cm/h (no charge); hydrolysed deacetylcephalosporin C, 32 cm/h (two negative charges); product of hydrolysis of the β -lactam ring of the lactone by both OH⁻ and the β -lactamases, 17 cm/h (thus, one negative charge).

In the acyl-enzyme formed with the R61 DD-peptidase, the lactone ring was also intact as discussed previously (Varetto *et al.*, 1987a).

Benzylpenicillin methyl ester was also 20-fold more sensitive to CH₃O⁻ ions than benzylpenicillin itself. When attempts were made to measure the rate of reaction of the same nucleophile with the cephalosporins, secondary reactions, concomitant with

Table 1. Second-order rate constants for the hydrolysis of the β -lactams under study by hydroxide ions

Substrate	k_{OH^-} (M ⁻¹ ·s ⁻¹)		
	25 °C	30 °C	37 °C
Benzylpenicillin amide	0.13 ± 0.02*		
Benzylpenicillin methyl ester	2.13 ± 0.2†	2.49‡	
Benzylpenicillin	0.13 ± 0.02*	0.15‡	0.37§
Cephalosporin C	0.13*		
Deacetylcephalosporin C	0.09		
Deacetylcephalosporin C lactone	19		

* pH 11.25 and 12.25 (potassium phosphate). Decrease in A_{230} for amide and benzylpenicillin and A_{260} for cephalosporin C.

† Buffers as above. Increase in A_{310} . Such a chromophore does not appear with benzylpenicillin under the same conditions. As shown by Laws & Page (1989) the chromophore is formed after neighbouring-group participation by the amide group on C-6.

‡ From Laws & Page (1989).

§ From Frère *et al.* (1982).

|| From Varetto *et al.* (1987a). pH 11.25 and 12.25 (potassium phosphate). Decrease in A_{260} .

Table 2. Interaction of the β -lactams with the R61 DD-peptidase

Abbreviation: N.D., not determined.

Substrate	k_2/K ($M^{-1} \cdot s^{-1}$)	k_3 (s^{-1})
Benzylpenicillin*	9800 ± 900	1.4×10^{-4} (37 °C)
Benzylpenicillin methyl ester	$20 \pm 6/21 \pm 3$ §	1.5×10^{-5}
Benzylpenicillin amide	< 0.1	N.D.
Cephalosporin C†	1150 ± 100 (37 °C)	1×10^{-6}
Deacetylcephalosporin C‡	2525 ± 300	2×10^{-5}
Deacetylcephalosporin C lactone‡	180 ± 10	31×10^{-6}

* From Varetto *et al.* (1987b).† From Frère *et al.* (1975).‡ From Varetto *et al.* (1987a).

§ First value: loss of activity. Second value: fluorescence quenching.

the opening of the β -lactam, were observed that did not allow a reliable determination of $k_{CH_3O^-}$.

Interaction with the R61 DD-peptidase

No inactivation was observed after a 1 h incubation of the enzyme in the presence of 400 μM -amide. This indicated a k_2/K' value lower than 0.1 $M^{-1} \cdot s^{-1}$.

The parameters for the methyl ester were determined by monitoring the activity of the enzyme (0.15 μM) when incubated at 25 °C with 0.1–0.5 mM-benzylpenicillin methyl ester. Samples were removed after contact times ranging from 0 to 30 min and assayed for activity. In addition, the value of k_a could be determined by directly monitoring the fluorescence decrease. In this case, 970 μl of 0.5 mM-methyl ester was rapidly mixed with 30 μl of a 80 μM -enzyme solution in the fluorimeter cuvette. Up to 0.5 mM-methyl ester the value of k_a varied with $[C]$ in a linear manner and the individual values of k_2 and K' were not obtained. The results are summarized in Table 2.

Finally, we verified that the ester was not contaminated by a small proportion of benzylpenicillin by showing that the in-

activation of the R61 DD-peptidase occurred at the same rate after the ester solution had been treated with enough *B. licheniformis* β -lactamase to destroy in 3 min a quantity of penicillin corresponding to 10 % of that of the ester.

The interaction between the R61 DD-peptidase and deacetylcephalosporin C and its lactone has been described previously (Varetto *et al.*, 1987a).

Interaction with the class A β -lactamases

The results are depicted in Table 3. The methyl ester and the amide were 10^3 – 10^4 -fold less sensitive to enzymic hydrolysis than the parent compound. On the contrary, with the *B. licheniformis* β -lactamase the lactone was a much better substrate than both deacetylcephalosporin C and cephalosporin C. In fact, the k_{cat}/K_m and the k_{cat} values for the lactone were respectively 5 % and 20 % of those of benzylpenicillin, which is one of the best substrates for this enzyme. However, as shown in the Discussion section, the sensitivity of the lactone to enzymic hydrolysis is probably strongly dependent on the intrinsic instability of the compound. However, the situation was different with the *S. albus* G enzyme, for which the lactone was not a better substrate than cephalosporin C itself.

Interaction with the *E. cloacae* β -lactamase

The results are depicted in Table 4. The amide was 500-fold less sensitive than benzylpenicillin, as observed with the class A β -lactamase. In contrast, the methyl ester was a surprisingly good substrate, the k_{cat}/K_m value being about 15 % of that observed with benzylpenicillin. The difference was mainly due to an increase in K_m . For benzylpenicillin and the methyl ester, the K_m values were too low to allow direct measurements. They were determined as K_i values with nitrocefin as the reporter substrate. In consequence, the kinetic parameters for nitrocefin were also determined at pH 7.

With class C β -lactamases, low K_m values are often due to values of k_3 significantly lower than those of k_2 . In consequence, the k_{cat} values were also measured in the presence of methanol. The results are presented in Table 5. Moreover under these conditions and with the lactone as substrate a new reaction

Table 3. Interaction of β -lactams with the *B. licheniformis* and *S. albus* G class A β -lactamases

	<i>B. licheniformis</i> β -lactamase (at 25 °C)			
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	
			Ratio	First-order
Benzylpenicillin	0.060 ± 0.004	4000 ± 70	$(66 \pm 6) \times 10^6$	—
Benzylpenicillin methyl ester	> 2.5	> 7.5	—	$(2.2 \pm 0.06) \times 10^3$
Benzylpenicillin amide	> 0.3	> 4	—	$(14 \pm 2) \times 10^3$
Cephalosporin C*	0.1 ± 0.005	14 ± 2	$(150 \pm 20) \times 10^3$	—
Deacetylcephalosporin C	0.25 ± 0.04	11.2 ± 1	$(45 \pm 3) \times 10^3$	$(45.6 \pm 3) \times 10^3$
Deacetylcephalosporin C lactone	0.30 ± 0.02	900 ± 60	$(3 \pm 0.4) \times 10^6$	—
	<i>S. albus</i> β -lactamase (at 30 °C)			k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
	K_m (mM)	k_{cat} (s^{-1})		
Benzylpenicillin*	1.00 ± 0.06	2800 ± 200		$(2800 \pm 100) \times 10^3$
Benzylpenicillin methyl ester	—	—		$(0.8 \pm 0.12) \times 10^3$
Cephalosporin C*	4.5 ± 1.0	170 ± 30		$(40 \pm 10) \times 10^3$
Deacetylcephalosporin C	—	—		$(11 \pm 1.6) \times 10^3$
Deacetylcephalosporin C lactone	—	—		$(40 \pm 6) \times 10^3$

* From Matagne *et al.* (1990) at 30 °C.

Table 4. Interaction of β -lactams with the *E. cloacae* β -lactamase

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Benzylpenicillin	0.4 ± 0.03	14 ± 1	32 ± 5
Benzylpenicillin methyl ester	3.2 ± 0.3	16 ± 1.5	5 ± 1
Benzylpenicillin amide*	> 200	> 4	0.07 ± 0.01
Cephalosporin C†	400 ± 20	1100 ± 100	2.7 ± 0.2
Deacetylcephalosporin C	70 ± 12	500 ± 60	7 ± 2
Deacetylcephalosporin C lactone	350 ± 30	700 ± 15	2 ± 0.2
Nitrocefin	28 ± 3	580 ± 35	21 ± 3.5

* From first-order analysis of complete time courses at 230 nm.
† From Matagne *et al.* (1990) at 30 °C.

Fig. 1. Negative electrostatic potential represented as the surface at the level of -41.8 kJ/mol (-10 kcal/mol)

(a) Benzylpenicillin; (b) benzylpenicillin amide; (c) benzylpenicillin methyl ester; (d) cephalosporin C; (e) deacetylcephalosporin C; (f) deacetylcephalosporin C lactone.

product was detected by h.p.l.c. It was tentatively identified as the α -methyl ester of the lactone, since a compound of identical retention time was obtained by incubating the lactone in dry methanol.

RESULTS OF THE THEORETICAL CALCULATIONS

Fig. 1 compares the negative electrostatic potential maps of the various penicillin and cephalosporin derivatives. Major differences can be seen between the penicillins and the cephalosporins that are due to the different orientations of their carboxylate groups when the β -lactam ring is used as a reference.

Table 5. Influence of methanol (4% and 8%, v/v) on the k_{cat} values of the *E. cloacae* 908R β -lactamase

(K_m)₀ and (k_{cat})₀ are the values of the kinetic parameters in the absence of methanol. (k_{cat})₄ and (k_{cat})₈ are the values of k_{cat} in the presence of 4% and 8% methanol respectively.

	(K_m) ₀ (μM)	[C] (μM)	(k_{cat}) ₀ (s^{-1})	(k_{cat}) ₄ (s^{-1})	(k_{cat}) ₈ (s^{-1})
Benzylpenicillin			14 ± 1	21 ± 2	—
Benzylpenicillin methyl ester	3.7	125	23 ± 3	36 ± 2	51 ± 2
Deacetylcephalosporin C	70		500 ± 35	500 ± 40	—
Deacetylcephalosporin C lactone	322	2000	900 ± 43	1002 ± 57	1115 ± 146

Table 6. Values of dihedral angles and of the positive charges on the carbonyl carbon atoms in the compounds studied and their methyl esters

To simplify the Table, the same numbering was used for all compounds. O⁶C²C³C⁴N¹ correspond to O⁸C⁷C⁶C⁵N⁴ for penicillins, and to O⁸C⁸C⁷C⁶N⁵ for cephalosporins. Abbreviation: N.C., not computed.

	Dihedral angle (°)		Positive charge on C ²
	C ² C ³ C ⁴ N ¹	O ⁶ C ² C ³ C ⁴	
(I) Benzylpenicillin	6.23	175.56	0.282
Benzylpenicillin methyl ester	6.12	175.69	0.281
Benzylpenicillin amide	6.41	175.31	0.279
(II) α -Methyl ester of benzylpenicillin	-71.21	121.38	0.306
α -Methyl ester of benzylpenicillin methyl ester	-71.10	120.49	0.306
α -Methyl ester of benzylpenicillin amide	-72.34	119.83	0.305
(I) Cephalosporin C	3.85	178.73	0.295
Deacetylcephalosporin C	4.47	177.48	0.294
Deacetylcephalosporin C lactone	4.08	178.00	0.298
(II) α -Methyl ester of cephalosporin C	-68.54	65.64	0.307
Deacetylcephalosporin C	-68.95	62.39	0.307
Deacetylcephalosporin C lactone	-68.64	59.00	0.308
(II) α -Methyl ester of aztreonam	-64.94	62.91	N.C.

Conversely, no important differences are observed between benzylpenicillin and its ester or amide derivatives or between deacetyl cephalosporin C and its lactone.

We also compared the fully optimized geometries of all the compounds studied with those of their methyl esters chosen as

Fig. 2. Net charges localized on various atoms of the methyl ester of benzylpenicillin (a) and the deacetylcephalosporin C lactone (b)

models for the serine-linked acyl-enzymes. The most remarkable features were the rotations around the C-5–C-6 (77–78°) and C-6–C-7 (55°) bonds in penicillins and around the corresponding C-6–C-7 (72–73°) and C-8–C-9 (113–119°) bonds in cephalosporins (Table 6).

Finally, the net charges on each atom were also computed. An example is given in Fig. 2.

DISCUSSION

The interaction of β -lactam antibiotics with the serine DD-peptidases and β -lactamases involves a nucleophilic attack by the serine hydroxy group on the β -lactam carbonyl carbon. The efficiency of that process is reflected by the value of the bimolecular rate constant, k_2/K' , measured as k_{cat}/K_m for the β -lactamases and on the basis of the rate of inactivation for the DD-peptidases. The value of this acylation rate constant is expected to depend on the intrinsic susceptibility of the β -lactam ring to nucleophilic attack but, with the *Streptomyces* R61 DD-peptidase, this factor is certainly very far from being the most important one (Frère *et al.*, 1982). Indeed, in a series of penams, the exocyclic acylamido side chain exhibited only moderate effects on the sensitivity of the molecules to hydrolysis by OH^- ions, but spectacular ones on the rate of formation of the acyl-enzyme. These results can certainly be related to the fact that nucleophilic catalysis involves very rigid transition states and could be interpreted by assuming that the structure of the side chain is a determinant factor in adequately positioning the β -lactam ring with respect to the serine hydroxy group. However, if the binding of different molecules to the same enzyme active site involves similar geometries, the intrinsic reactivities of these molecules cannot fail to influence the efficiency of the nucleophilic attack. In order to specify the contribution of the recognition by the enzyme, Laws & Page (1989) have proposed the computation of an 'enzyme-rate-enhancement factor' (EREF) obtained by dividing the k_{cat}/K_m value by k_{OH^-} , the second-order rate constant for the hydrolysis of the same molecule by OH^- ions.

k_{OH^-} values

In an attempt to rationalize the k_{OH^-} values, we computed the net positive charge on C-8 of cephalosporins or C-7 of penicillins. Within each family, the differences were small and only became more significant between the penicillins and cephalosporins (Table 6). Thus the increased sensitivities of the ester and the lactone to nucleophilic attack by OH^- ions could not find a

reasonable explanation in these values, which also predict the cephalosporins to be more unstable than the penicillins. However, this failure might not be surprising if one realizes that the electrophilic character of the carbonyl carbon atom in the ground state is probably not the most important determining factor. Indeed, the rate-limiting step in the hydrolysis of penicillins and cephalosporins by OH^- ions is the formation of the tetrahedral intermediate and exhibits Brønsted β_{lg} values of -0.55 and -0.6 respectively (Proctor *et al.*, 1982). It may be inferred that the transition state is late, close to the tetrahedral intermediate and that differences in OH^- -sensitivities between penicillin and cephalosporin derivatives are related to the $\text{p}K_a$ of the leaving group.

Table 1 also shows that electron-withdrawing substituents on C-3' and C-4 of cephalosporins contribute to the instability of the β -lactam ring, but the effect is much stronger with the lactone. In this case no expulsion of the potential leaving group on C-3' was observed, in agreement with the results obtained by Laws & Page (1989). The rate differences observed in Table 1 are those expected for purely inductive effects. The β_{lg} values for substituents at C-3' and C-4 have been reported to be respectively -0.06 and -0.5 (Proctor *et al.*, 1982), values that are consistent with a rate-limiting attack by OH^- . The increased instability of the lactone is thus more related to the esterification of the carboxylate group than to the presence of a potential leaving group on C-3'. The relatively small influence of this substituent is in good agreement with the observations made by Mazzella & Pratt (1989), who have shown that, with various enzymes, the departure of this leaving group, when it occurs, is subsequent to and not concerted with the opening of the β -lactam ring.

Enzyme-rate-enhancement factors at pH 7.0

Table 7 shows the influence of the modification of the carboxylate group on the EREF values for the four enzymes studied here. At first sight, the commonly accepted idea that the anionic group is necessary for efficient acylation seems to be confirmed, although a striking exception can be immediately singled out: with the *B. licheniformis* enzyme the EREF of the lactone is nearly the same as that of deacetylcephalosporin C itself, an observation identical with that made by Laws & Page (1989) with the *B. cereus* β -lactamase I, an enzyme closely related to that of *B. licheniformis* (Ambler, 1980).

As seen above, the electrostatic potential maps (Fig. 1) differentiate penicillins from cephalosporins much more than the various derivatives within a given family. Thus the behaviours of the molecules studied, when reacting with the various enzymes, cannot be rationalized on this basis, a conclusion that is reinforced by the fact that the hierarchy of the EREF values varies significantly from enzyme to enzyme, with the result that an explanation found satisfactory in one case cannot be generalized. In consequence, the enzymes are considered separately in the following discussion.

Streptomyces R61 DD-peptidase

The EREF value of the methyl ester is about 1/7000 that of benzylpenicillin and that of the lactone 1/2000 that of deacetylcephalosporin C. Thus esterification of the carboxylate group had similar effects in both families. The k_2 value for the lactone (0.3 s^{-1}) is 5-fold larger than for deacetylcephalosporin C. However, the intrinsic reactivity of the lactone should reflect directly on this value, and, since it is 200 times that of deacetylcephalosporin C, one can conclude that geometric factors decrease the efficiency of the nucleophilic attack on the lactone by about 40-fold. The respective K' values are about 1.5 mM and 0.025 mM for the lactone and deacetylcephalosporin C respectively, and the decrease in the EREF seems to be equally due to

Table 7. Enzyme-rate-enhancement factors at pH 7.0 and 25 °C for the four enzymes under study

A = absolute value; R = relative value (that for benzylpenicillin = 1000).

	EREF							
	<i>Streptomyces</i> R61 DD-peptidase		<i>B. licheniformis</i> β -lactamase		<i>S. albus</i> G β -lactamase (30 °C)		<i>E. cloacae</i> 908R β -lactamase	
	A	R	A	R	A	R	A	R
Benzylpenicillin	7.5×10^4	1000	5×10^8	1000	2.2×10^7	1000	2.5×10^8	1000
Benzylpenicillin methyl ester	10	0.13	1×10^3	0.002	0.4×10^3	0.02	2.3×10^6	10
Benzylpenicillin amide	< 0.8	< 0.01	1×10^5	0.2	—	—	5×10^5	2
Cephalosporin C*	0.88×10^4	120	1.2×10^6	2.3	0.3×10^6	14	2×10^7	80
Deacetylcephalosporin C†	2.8×10^4	260	5×10^5	1	0.12×10^6	6	8×10^7	320
Deacetylcephalosporin C lactone†	10	0.13	1.6×10^5	0.31	0.002×10^6	0.1	1×10^5	0.4

* EREF values at 37 °C (*Streptomyces* R61 DD-peptidase; Frère *et al.*, 1975) and 30 °C (β -lactamases; Matagne *et al.*, 1990).† EREF values for *Streptomyces* R61 DD-peptidase from Varetto *et al.* (1987a).

a lower affinity (K') and to a poorer positioning of the lactone in the active site of the enzyme (k_2 contribution). Accordingly, the value of k_2/K' for deacetylcephalosporin C decreases by about 6-fold between pH 8 and 10, and this is entirely due to an increase in K' (Varetto *et al.*, 1987a). All the results presented and discussed here thus remain in agreement with the simple hypothesis proposed previously that a positively charged group of $pK \approx 9$ in the active site of the enzyme facilitates the binding of the antibiotic by forming an ion-pair with the carboxylate group of the latter molecule.

Identification of this positively charged group remains difficult. The three-dimensional structural data at present available (Kelly *et al.*, 1989) indicate two potential candidates, Lys-65 and His-298, and the former does not seem to be adequately positioned. This leaves only His-298, but this would require the pK of that group to be significantly higher than that of a free imidazolium group.

Class A β -lactamases

With relatively small quantitative differences, our results with the *B. licheniformis* enzyme are similar to those obtained by Laws & Page (1989). In the penicillin family, the EREF of the amide is much smaller than that of benzylpenicillin, and that of the ester is even lower. By contrast, no such differences are found between the EREF values for cephalosporin C, deacetylcephalosporin C and the lactone. Indeed, the lactone is an even better substrate than the free acids, and its lower EREF is only due to its increased intrinsic reactivity. This might be explained by the fact that the two negatively charged cephalosporins are both very poor substrates of the enzymes. The disappearance of the negative charge would not make the situation much worse. Laws & Page (1989) have even suggested that, owing to its *s-trans* conformation, the lactone (A in Fig. 3) might interact with a positively charged substrate, as would the carboxylate group (B in Fig. 3). The low EREF values of the three cephalosporins would then be due to the geometries and electrostatic potentials of the cephem double-ring system, which would induce a poor positioning of the molecules in the enzyme active site, making the nucleophilic attack by the active-serine oxygen atom quite inefficient.

With the ester, an adequate interaction would only be possible with the same *trans* rotamer (C in Fig. 3), steric hindrance impairing the correct positioning of the *cis* rotamer (D in Fig. 3). Since the *cis* rotamer is 16–35 kJ more stable, binding of the ester becomes 600–10⁶-fold more difficult than that of the lactone and

free carboxylate group. The same explanation could account for our results with the *B. licheniformis* enzyme and benzylpenicillin, its ester and its amide. Indeed, one might expect the situation with the amide to be intermediate since, in that case, the two rotamers would be identical (E in Fig. 3) but the steric hindrance of the proton would be much less dramatic than that of the methyl group.

However, even within class A β -lactamases this explanation cannot be generalized. Indeed, with the *S. albus* G enzyme, if the methyl ester remains a much poorer substrate than benzylpenicillin, the lactone is not significantly better than cephalosporin C and deacetylcephalosporin C. It should be underlined that the two *Bacillus* enzymes exhibit very similar primary structures whereas the *S. albus* G β -lactamase appears to be a more distant relative. Thus, even in a clearly homologous group of enzymes, modifications at the level of the substrate carboxylate group can have very different consequences.

E. cloacae β -lactamase

Similarly, the explanation apparently valid for the *Bacillus* enzymes fails even more miserably to explain the behaviour of the class C β -lactamase for which the EREF of the methyl ester is significantly higher than that of the lactone. In a gross manner, the EREF hierarchy for the class C β -lactamase is similar to that for the R61 DD-peptidase. This latter observation might be related to the significant structural similarities between the two enzymes (Joris *et al.*, 1988). The only striking difference is the unexpectedly high k_{cat}/K_m value for the methyl ester of benzylpenicillin, which makes it a relatively good acylating agent for this enzyme, better than some more classical β -lactams such as imipenem and aztreonam (Galleni *et al.*, 1988). It is the only case where the EREF for the ester reaches about 1 % of that observed for benzylpenicillin.

For the cephalosporin C/deacetylcephalosporin C/lactone group a clear parallelism can be established between the DD-peptidase and the 908R β -lactamase. Moreover, with deacetylcephalosporin C, k_2 appears to be smaller than k_3 since the total rate of utilization of the substrate is not increased by the presence of methanol. The values of k_{cat} and K_m thus essentially reflect those of k_2 and K' respectively, i.e. $k_2 = 600 \text{ s}^{-1}$ and $K' = 0.075 \text{ mM}$. With the lactone, methanol increases the reaction rate and k_3 appears to be smaller than k_2 , which also implies that K' is larger than K_m . By analogy with the peptidase, one might assume k_2 to be 5–10-fold that for deacetylcephalosporin C, i.e. about 4000 s^{-1} , which also yields $K' = 2 \text{ mM}$. The ratio of the

Fig. 3. Interaction between a positive charge on the enzyme and the various compounds studied in this work

Key: A, lactone; B, free carboxylate of usual penicillins and cephalosporins; C, methyl ester, *trans*-rotamer; D, methyl ester, *cis*-rotamer; E, amide.

EREF values for deacetylcephalosporin C and the lactone, about 800, not very different from the value of 2000 observed with the DD-peptidase, would thus also be about equally due to a poor recognition ($2\text{ mM}/0.075\text{ mM} = 23$) and to a relatively less efficient k_2 (4000/200 versus 600, taking account of the 200-fold larger intrinsic reactivity of the lactone).

Conclusions

The serine DD-peptidases and β -lactamases form a large family of penicillin-recognizing enzymes (Joris *et al.*, 1988; Kelly *et al.*, 1989; Oefner *et al.*, 1990). However, if some aspects of catalysis of the acylation of some class A β -lactamases can be partly rationalized on the basis of electrostatic interactions, it is not so for another class A enzyme or for the class C β -lactamase and the DD-peptidase. At the present time, no general scheme can be proposed by which the bond geometries and charge distribution of a β -lactam compound would account for its interactions with the various types of enzyme. Our study was mainly concerned with the acylation step of these interactions. It should also be remembered that even larger differences are found for the deacylation rates, which, with benzylpenicillin for instance, vary from $\geq 4000\text{ s}^{-1}$ for class A β -lactamases to $10\text{--}100\text{ s}^{-1}$ for class C lactamases and $10^{-3}\text{--}10^{-6}\text{ s}^{-1}$ for the various DD-peptidases and penicillin-binding proteins.

This work was supported in part by the Fonds de la Recherche Scientifique Médicale (Contract no. 3.4537.88), an Action Concertée with the Belgian Government (Convention 86/91-90), the Fonds de Recherche de la Faculté de Médecine ULq and a Convention Tripartite

between the Région Wallone, SmithKline Beecham (U.K.) and the University of Liège.

REFERENCES

- Ambler, R. P. (1980) *Philos. Trans. R. Soc. London B* **289**, 321–331
- De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frère, J.-M. & Waley, S. G. (1987) *Biochem. Pharmacol.* **36**, 2393–2403
- Dewar, M. J., Zoebisch, E. G., Heuly, E. F. & Stewart, J. P. (1985) *J. Am. Chem. Soc.* **107**, 3902–3909
- Dideberg, O., Charlier, P., Wery, J. P., Dehottay, P., Dusart, J., Erpicum, T., Frère, J.-M. & Ghuysen, J. M. (1987) *Biochem. J.* **245**, 911–913
- Ellerby, L. M., Escobar, A. W., Fink, A. L., Mitchinson, C. & Wells, J. A. (1990) *Biochemistry* **29**, 5797–5806
- Frère, J.-M., Ghuysen, J.-M. & Iwatsubo, M. (1975) *Eur. J. Biochem.* **57**, 343–351
- Frère, J.-M., Leyh-Bouille, M., Ghuysen, J.-M., Nieto, M. & Perkins, H. R. (1976) *Methods Enzymol.* **45**, 610–636
- Frère, J.-M., Kelly, J. A., Klein, D., Ghuysen, J.-M., Claes, P. & Vanderhaeghe, H. (1982) *Biochem. J.* **203**, 223–234
- Galleni, M., Amicosante, G. & Frère, J.-M. (1988) *Biochem. J.* **255**, 123–129
- Gibson, R. M., Christensen, H. & Waley, S. G. (1990) *Biochem. J.* **272**, 613–619
- Giessner-Prettre, C. & Pullman, A. (1972) *Theor. Chim. Acta* **25**, 83–88
- Herzberg, O. & Moulton, J. (1987) *Science* **236**, 694–701
- Jacob, F., Joris, B., Lepage, S., Dusart, J. & Frère, J.-M. (1990) *Biochem. J.* **271**, 399–406
- Jaszberenyi, J. C. & Gunda, T. E. (1975) *Prog. Med. Chem.* **12**, 395–477
- Joris, B., Ghuysen, J. M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frère, J.-M., Kelly, J. A., Boyington, J. C., Moews, P. C. & Knox, J. R. (1988) *Biochem. J.* **250**, 313–324
- Kelly, J. A., Knox, J. R., Zhao, H., Frère, J.-M. & Ghuysen, J.-M. (1989) *J. Mol. Biol.* **209**, 281–295
- Laws, A. P. & Page, M. I. (1989) *J. Chem. Soc. Perkin Trans. 2* **1577–1581**
- Matagne, A., Misselyn-Bauduin, A. M., Joris, B., Erpicum, T., Granier, B. & Frère, J.-M. (1990) *Biochem. J.* **265**, 131–146
- Mazzella, L. J. & Pratt, R. F. (1989) *Biochem. J.* **259**, 255–260
- Oefner, C., Darcy, A., Daly, J. J., Gubernator, K., Charnas, R. L., Heinze, I., Hubschwerlen, C. & Winkler, F. K. (1990) *Nature (London)* **343**, 284–288
- Pople, J. A. & Beveridge, D. L. (1970) *Approximate Molecular Orbital Theory*, McGraw-Hill, New York
- Proctor, P., Gensmantel, N. P. & Page, M. I. (1982) *J. Chem. Soc. Perkin Trans. 2* **1185–1192**
- Varetto, L., Frère, J.-M. & Ghuysen, J.-M. (1987a) *FEBS Lett.* **225**, 218–222
- Varetto, L., Frère, J.-M., Nguyen-Disteche, M., Ghuysen, J.-M. & Houssier, C. (1987b) *Eur. J. Biochem.* **162**, 525–531

Received 20 November 1990/5 April 1991; accepted 17 April 1991