

Peptidase activity of β -lactamases

Noureddine RHAZI*, Moreno GALLEN†, Michael I. PAGE† and Jean-Marie FRÈRE*¹

*Centre d'Ingénierie des Protéines and Laboratoire d'Enzymologie, Université de Liège, Institut de Chimie B6, Sart Tilman, B-4000 Liège, Belgium, and

†Department of Chemical and Biological Sciences, The University of Huddersfield, Queensgate, Huddersfield HD1 3DH, U.K.

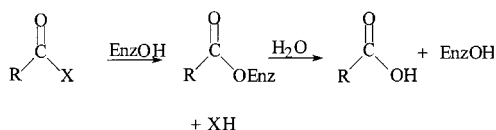
Although β -lactamases have generally been considered as being devoid of peptidase activity, a low but significant hydrolysis of various N-acylated dipeptides was observed with representatives of each class of β -lactamases. The k_{cat}/K_m values were below $0.1 \text{ M}^{-1} \cdot \text{s}^{-1}$, but the enzyme rate enhancement factors were in the range 5000–20 000 for the best substrates. Not unexpectedly, the best 'peptidase' was the class C β -lactamase of *Enterobacter cloacae* P99, but, more surprisingly, the activity was always higher with the phenylacetyl- and benzoyl-D-Ala-D-Ala dipeptides than with the diacetyl- and α -acetyl-L-Lys-D-Ala-D-Ala

tripeptides, which are the preferred substrates of the low-molecular-mass, soluble DD-peptidases. A comparison between the β -lactamases and DD-peptidases showed that it might be as difficult for a DD-peptidase to open the β -lactam ring as it is for the β -lactamases to hydrolyse the peptides, an observation which can be explained by geometric and stereoelectronic considerations.

Key words: catalytic mechanism, kinetics, DD-peptidases.

INTRODUCTION

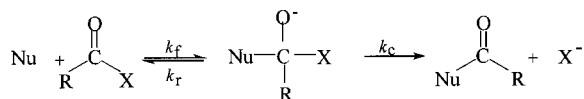
The hydrolysis of many derivatives of carboxylic acids is catalysed by serine enzymes such as peptidases, esterases, lipases and β -lactamases. The basic mechanism involves the formation of an acyl-enzyme intermediate, followed by its hydrolysis (Scheme 1):



Scheme 1

The leaving group XH may be an amine displaced from a peptide, an alcohol from an ester or a thiol from a thiolester (S-ester). These leaving groups have different basicities: thiols (RSH) have the lowest $\text{p}K_a$ values (≈ 9), and may be expelled as the anion, RS^- ; alcohols (ROH) have intermediate $\text{p}K_a$ values (≈ 15), and may be displaced as either the anion (RO^-) or the alcohol; and amines (RNH_2), which are very weak acids ($\text{p}K_a \approx 25$) and always require protonation to aid C–N bond fission.

Displacement at carbonyl centres almost invariably occurs by a two-step process [1,2]. Initial bond formation between the incoming nucleophile and the carbonyl carbon generates a tetrahedral intermediate, before expulsion of the leaving group can take place (Scheme 2):



Scheme 2

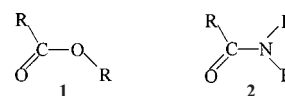
where Nu denotes the nucleophile involved.

Either the formation (k_f) or breakdown (k_c) of the intermediate can be the rate-limiting step; for example, the alkaline hydrolysis

of S-esters ($\text{X} = \text{SR}$) occurs with rate-limiting attack, because the rate of expulsion of RS^- (k_c) is faster than that for the expulsion of OH^- (k_r) (Scheme 2; $\text{Nu} = \text{OH}^-$). Conversely, the alkaline hydrolysis of amides involves rate-limiting breakdown of the tetrahedral intermediate, because it is easier to expel the hydroxide ion (k_r) than the amine anion (k_c), and C–N fission only occurs with protonation of the amine nitrogen.

Proteolytic enzymes therefore require a general acid in the active site to catalyse the hydrolysis of peptides. In principle, these enzymes can catalyse the hydrolysis of other carboxylic acid derivatives without using all of the same catalytic machinery used for the hydrolysis of the relatively unreactive peptides.

Both esters and amides have analogous cyclic forms, i.e. lactones and lactams respectively. However, there are fundamental differences in their geometrical structures. Esters and amides prefer a *trans* (Z) configuration (**1** and **2** respectively):



whereas lactones (**3**) and lactams (**4**) prefer a *cis* (E) arrangement [1–3]:



Enzymes that, as their primary function, hydrolyse peptides are therefore unlikely to have the same relative disposition of catalytic groups in their active sites as those designed for the hydrolysis of lactams.

It has generally been accepted that, although DD-peptidases and β -lactamases can recognize β -lactams, as well as ester and S-ester analogues of the substrates of the former, β -lactamases are

Abbreviations used: Bz, benzoyl; EREF, enzyme rate enhancement factor; S-ester, thiolester.

¹ To whom correspondence should be addressed (e-mail jmfriere@ulg.ac.be).

unable to hydrolyse 'true' peptide bonds [4–6]. We show here that this assumption is not correct, and explore the relative catalytic efficiencies of the two types of enzymes in the hydrolysis of linear peptides, esters and *S*-esters, and in the opening of the β -lactam ring.

MATERIALS AND METHODS

Substrates

DL-Hippurylphenyl lactate was obtained from Sigma (St. Louis, MO, U.S.A.) and the *S*-esters benzoyl (Bz)-Gly-thioglycolic acid and Bz-Gly-D-thiolactic acid were prepared as described previously [7,8]. The benzoylated and phenylacetylated dipeptides were synthesized as follows. The dipeptides Gly-D-Ala, Gly-L-Ala and D-Ala-D-Ala were obtained from Sigma. Peptide (10 mmoles) was dissolved in 40 ml of 500 mM sodium bicarbonate, and one equivalent of phenylacetyl or benzoyl chloride was added dropwise to the solution over a period of 45 min with slow stirring. The reaction mixtures were extracted twice with ethyl acetate, adjusted to pH 1.5 with HCl, and further extracted with ethyl acetate. The latter organic phase was dried over MgSO₄ and the solvent was dry-evaporated. The purity of the compounds was tested by HPLC on an analytical reverse-phase C₁₈ column (5 μ m; Macherey-Nagel, Düren, Germany) and, when necessary, the contaminating benzoic or phenylacetic acid was eliminated by chromatography on a similar preparative column under isocratic conditions [20 % (v/v) acetonitrile/10 mM sodium acetate/acetic acid (pH 3.5)].

β -lactams

Aztreonam and 6- β -iodopenicillanic acid were generously given by Squibb (New Brunswick, NJ, U.S.A.) and Pfizer Central Research (Sandwich, Kent, U.K.) respectively.

Enzymes

The class A TEM-1, NMC-A and *Bacillus licheniformis* 749/C β -lactamases were purified as described, respectively, by Raquet et al. [9], Swarén et al. [10] and Matagne et al. [11]. The class B β -lactamases of *Bacillus cereus* 569/H [12] and *Bacteroides fragilis* [13], the class C β -lactamase from *Enterobacter cloacae* P99 [14], the OXA-2 [15] class D β -lactamase, the *Streptomyces* R61 and K15 DD-peptidases, and the *Actinomadura* R39 DD-peptidase [16] were also purified as described previously.

Incubation conditions

All incubations were performed at 37 °C. β -Lactamases of classes A, C and D were incubated in 50 mM sodium phosphate buffer, pH 7.0. The *B. cereus* and *B. fragilis* metalloenzymes were incubated respectively in either 10 mM cacodylate, pH 6.5, containing 0.1 mM ZnCl₂ and 500 mM NaCl, or 50 mM Tris/HCl, pH 7.5, containing 100 mM NaCl. For the R61 and R39 DD-peptidases, the buffer used was 10 mM sodium phosphate, pH 7.0; for the K15 enzyme, 6 mM Tris/HCl, pH 7.2, was used, containing 5 mM Gly-Gly as an acceptor. The rate of hydrolysis of the peptides by OH[−] ions was measured by incubation in 1 M NaOH, and that of the ester and *S*-ester by incubation in 1 M K₂HPO₄ adjusted to pH 12.0 with concentrated KOH.

The peptides (5 and 10 mM) were incubated for 24, 48 and 72 h with the β -lactamases (10–50 μ g), whereas with the DD-peptidases (0.5–1.0 μ g of R61 and R39, and 5–10 μ g of K15),

shorter periods of time (30, 60 and 90 min) were used for the incubation.

Assay of reaction products

When the reaction was expected to yield D-alanine, the amount of released amino acid was estimated by using the D-amino acid oxidase method [17]. Hydrolysis of the ester and *S*-ester was monitored directly by spectrophotometry at 250 nm [7,8].

RESULTS AND DISCUSSION

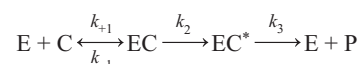
The benzoylated and phenylacetylated dipeptides were poor substrates of all tested β -lactamases (Table 1). The initial rates of the reactions were proportional to the substrate concentrations up to 10 mM, such that only the k_{cat}/K_m values could be derived. The same compounds were also poor substrates for the DD-peptidases, although generally better than for the β -lactamases. Among the latter, the most active enzyme was the *E. cloacae* class C enzyme. To confirm that the observed hydrolysis rates were generated enzymically, the *E. cloacae* and TEM enzymes were first pre-incubated with their respective specific inactivators, aztreonam and 6- β -iodopenicillanic acid, which resulted in residual activities lower than 1.5 (*E. cloacae*) and 8 % (TEM). Moreover, no hydrolysis of Bz-Gly-L-Ala and phenylacetyl-Gly-L-Ala could be detected with any of the studied enzymes over the same periods of time as those used with the D-isomers.

Similarly, the k_{cat}/K_m values were determined with the same enzymes using tripeptide, ester and *S*-ester substrates of the DD-peptidases (Table 2). As expected from previous results, the esters and *S*-esters were much better substrates for the β -lactamases than the similar dipeptides (compare Bz-Gly-thioglycolate with Bz-Gly-D-Ala). However, with the tripeptides as substrates, the difference in reactivity of the DD-peptidases and the β -lactamases is easily discerned: the former enzymes have a preference for the tripeptides by several orders of magnitude over the latter, whereas one of the dipeptides is always among the best substrates of β -lactamases, with the notable exception of the NMC-A enzyme, whose peptide-degrading activity is always very low. The R61 and R39 DD-peptidases clearly have a preference for the acyl-D-Ala-D-Ala dipeptides over acyl-Gly-D-Ala, a trend which is even more marked with the tripeptides [18,22,23]. The other enzymes do not exhibit such a clear specificity although, in several cases, one of the derivatives of Gly-D-Ala is hydrolysed somewhat more efficiently.

Another difference between the DD-peptidases and the active-site serine β -lactamases is that, with the *S*-esters, the former show a moderate (R61) to strong (R39) preference for the D-thiolactate over the thioglycolate leaving group, whereas no such difference is observed with the β -lactamases.

Finally, in a few cases, the rather similar benzoyl and phenylacetyl side chains influence the activity in a somewhat different manner, but, at the present time, the relative behaviour of each specific enzyme is still difficult to rationalize.

The interactions between active-site serine DD-peptidases, β -lactamases and their substrates usually follow a three-step pathway (Scheme 3), involving the transient formation of an acyl-enzyme, EC* [4,5]:



Scheme 3

Table 1 Second-order rate constants k_{cat}/K_m for the hydrolysis of the acylated dipeptides at 37 °C

See the text for appropriate experimental details. S.D. values (not shown) were below 10%. k_{OH^-} is a second-order rate constant for the hydroxide-ion-catalysed hydrolysis. Phac, phenylacetyl.

Enzyme	Substrate ...	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)			
		Bz-Gly-D-Ala	Phac-Gly-D-Ala	Bz-D-Ala-D-Ala	Phac-D-Ala-D-Ala
Class A β -lactamases					
<i>B. licheniformis</i>		1.4×10^{-3}	1.1×10^{-3}	4×10^{-5}	2×10^{-4}
TEM-1		6×10^{-3}	3×10^{-4}	5×10^{-4}	3×10^{-4}
NMC-A		6×10^{-5}	1.4×10^{-4}	1.1×10^{-4}	1.6×10^{-4}
Class B β -lactamases					
<i>B. cereus</i>		2×10^{-3}	1.4×10^{-3}	1.2×10^{-3}	6×10^{-4}
<i>B. fragilis</i>		6.6×10^{-3}	5.5×10^{-3}		
Class C β -lactamase					
<i>E. cloacae</i> P99		1.5×10^{-2}	1.3×10^{-2}	5×10^{-3}	6×10^{-3}
Class D β -lactamase					
OXA-2		1.8×10^{-3}	1.1×10^{-3}	1.8×10^{-3}	1.6×10^{-3}
DD-Peptidases					
S. R61		4.7	5×10^{-2}	52	15
A. R39		4×10^{-2}	0.64	64	175
S. K15		0.22	0.18	7×10^{-2}	1.2
k_{OH^-}		1.2×10^{-6}		2.5×10^{-7}	

Table 2 k_{cat}/K_m values for the hydrolysis of the tripeptide analogues of the transpeptidase substrates, and of an ester and a S-ester substrate

k_{cat}/K_m values were determined for the hydrolysis of the tripeptide analogues of the transpeptidase substrates [N^2 -acetyl-L-Lys-D-Ala-D-Ala (α -Ac-KAA) and N^2 -diacetyl-L-Lys-D-Ala-D-Ala (Ac₂-KAA)], and of ester (Bz-Gly-D-Phla, where Phla denotes phenyl lactate) and S-ester (Bz-Gly-Thg, where Thg denotes thioglycolate) substrates. References in square brackets indicate the sources from which the data are taken; otherwise, the data are drawn from the present study. With the ester substrate, only the D-isomer is hydrolysed. Note that neither Bz-Gly-OCH₂-COOH nor Bz-Gly-OCH(CH₃)-COOH performs better as a substrate. The values in parentheses are those obtained for the D-isomer of Bz-Gly-SCH(CH₃)-COOH, which is the thiolactate ester that is the strict analogue of Bz-Gly-D-Ala [18]. S.D. values (not shown) were < 10%.

Enzyme	Substrate ...	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)			
		Ac ₂ -KAA	α -Ac-KAA	Bz-Gly-D-Phla	Bz-Gly-Thg
Class A β -lactamases					
<i>B. licheniformis</i>		6×10^{-5}	1.2×10^{-4}	80 [7]	4500 [7]
TEM-1		7×10^{-4}	4×10^{-4}	50 [19]	5200 (5700) [20]
NMC-A		1.4×10^{-4}	3×10^{-4}	< 5	250
Class B β -lactamase					
<i>B. cereus</i> II		1.1×10^{-4}	1.5×10^{-5}	55	15
Class C β -lactamase					
<i>E. cloacae</i> P99		4×10^{-5}	9×10^{-4}	20000 [7]	2400 (3600) [19]
Class D β -lactamase					
OXA-2		3×10^{-4}	2×10^{-5}	< 5 [19]	200 (260) [19]
DD-Peptidases					
S. R61		4000 [4]	17 [4]	5200 [19]	100000 (100000) [7]
A. R39		5500 [4]	60000 [4]	6600 [19]	10000 (13000) [7]
S. K15		50 [21]	45 [21]	< 0.2	8 (75) [21]
k_{OH^-}		7×10^{-7}	—	0.26	1.0

The k_{cat}/K_m value corresponds to the second-order rate constant k_2/K' , characteristic of the acylation reaction, where $K' = (k_{-1} + k_2)/k_{+1}$. This allows a comparison to be made with the rate of inactivation of DD-peptidases by β -lactams, for which the same value is determined as an inactivation rate constant. In all cases, the active serine hydroxyl oxygen performs a nucleophilic attack on the carbonyl carbon atom of the scissile bond, resulting in the putative formation of a tetrahedral intermediate, which yields the acyl-enzyme upon expulsion of the leaving group. Electron-withdrawing substituents in the leaving group enhance the rate of nucleophilic attack on the carbonyl carbon, and

facilitate the scission of the bond to the leaving group, but have the opposite effect on its protonation. The rate of the acylation reaction is thus influenced by the nature of the leaving group, as demonstrated by a comparison of the k_{cat}/K_m values of similar peptides, esters and S-esters (see Table 2). To account for the specific contribution of the enzymes when acting on substrates of very different chemical reactivities, Laws and Page have introduced the concept of an enzyme rate enhancement factor (EREF) [24], i.e. the ratio of the k_{cat}/K_m value to k_{OH^-} , the second-order rate constant for the hydrolysis of the substrate by OH⁻ ions. By comparing the EREFs of each enzyme for its various substrates

Table 3 EREF values for the hydrolysis of peptides, an ester and an *S*-ester (a), and for the opening of the β -lactam ring (b), by the various enzymesPhla, phenyl lactate; Thg, thioglycolate; Ac₂-KAA, *N*²*N*⁶-diacetyl-L-Lys-D-Ala-D-Ala. S.D. values of EREFs were < 20%.

(a)

Enzyme	Substrate				
	Bz-Gly-D-Ala (peptide)	Bz-D-Ala-D-Ala (peptide)	Ac ₂ -KAA	Bz-Gly-D-Phla (ester)	Bz-Gly-Thg (S-ester)
<i>β</i> -Lactamases					
TEM	5 000	2 000	1 000	300	4 500
<i>B. cereus</i> II	1 700	5 000	1 500	220	15
P99	12 000	20 000	50	80 000	2 400
OXA-2	1 500	7 000	500	< 5	200
D α -Peptidases					
R61	7 $\times 10^6$	2 $\times 10^8$	6 $\times 10^9$	20 000	100 000
R39	30 000	2.5 $\times 10^8$	4.0 $\times 10^{10}$	25 000	10 000
K15	200 000	300 000	8 $\times 10^7$	< 1	8

(b)

Penicillins and cephalosporins				
Enzyme	Benzylpenicillin	Ampicillin	Cephaloglycin	Cephalosporin C
<i>β</i> -Lactamases				
TEM	6 $\times 10^8$	2.5 $\times 10^8$	300 000	1 $\times 10^6$
<i>B. cereus</i> II	4 $\times 10^6$	6.4 $\times 10^6$	300 000	Not done
P99	2.5 $\times 10^8$	9 $\times 10^6$	3.2 $\times 10^6$	2 $\times 10^7$
OXA-2	Burst	1.5 $\times 10^7$	Burst	Burst
D α -Peptidases				
R61	130 000	800	180	13 000
R39	2.3 $\times 10^6$	500 000	70 000	500 000
K15	1 000	[700]*	[20]*	[6 000]†

* Derived from the IC₅₀ value; †value for cefoxitin, the best β -lactam inactivator of the K15 D α -peptidase [21].

or inactivators, one can thus determine the efficiency of the enzymic process in the scission of the CO–X bonds, where X is N, O or S. This comparison is shown in Table 3, and highlights the following observations. (i) Both β -lactamases and D α -peptidases are reasonably good catalysts for the hydrolysis of all three types of acyclic substrates, i.e. peptides, esters and *S*-esters. The highest EREF values for ester substrates are in the range 10⁴ to 10⁵ for both β -lactamases and peptidases and, importantly, > 10⁴ for peptide substrates of β -lactamases. (ii) The EREFs of the R61 and R39 peptidases with their good peptide substrates are several orders of magnitude higher than with the ester, *S*-ester and β -lactams, the variation of EREFs for different peptides reflecting the importance of molecular recognition. (iii) Conversely, and as expected, the β -lactams behave as the preferred substrates of the β -lactamases (although the EREFs are never larger than 10⁹); however, β -lactamases do show effective peptidase activity, and their EREF values are similar to those for esterase and thiol-esterase activities. (iv) More surprisingly, the EREFs of the β -lactamases with their best peptide substrate(s) are not much lower than those of the peptidases with their best β -lactam inactivators.

It should, however, be remembered that, with β -lactams, both the k_{cat}/K_m values of the lactamases and the k_2/K' values of the peptidases (compare benzylpenicillin and ampicillin with the R61 enzyme and ampicillin and cephaloglycin, which have the same side chain) vary greatly with the exact structure of the studied compound as a result of the varying contributions of the binding interactions between substrate and enzyme. In consequence, the

Table 4 Comparison of the EREF values of the β -lactamases and D α -peptidases with their best peptide and β -lactam substrates/inactivators α -Ac-KAA, *N*²-acetyl-L-Lys-D-Ala-D-Ala; Ac₂-KAA, *N*²*N*⁶-diacetyl-L-Lys-D-Ala-D-Ala. The S. D. values (not shown) of the EREF ratios were < 40%.

Enzyme	Substrates or inactivators	EREF ratios
<i>β</i> -Lactamase		
TEM	Best penicillin/best peptide	
	Benzylpenicillin/Bz-Gly-D-Ala	120 000
P99	Benzylpenicillin/Bz-D-Ala-D-Ala	10 000
OXA-2	Ampicillin/Bz-D-Ala-D-Ala	2 000
<i>B. cereus</i> II	Benzylpenicillin/Bz-D-Ala-D-Ala	800
D α -Peptidase		
	Best peptide/best penicillin	
R61	Ac ₂ -KAA/benzylpenicillin	48 000
R39	α -Ac-KAA/benzylpenicillin	180 000*
K15	Ac ₂ -KAA/cefoxitin	14 000

* This ratio assumes the same k_{OH^-} value as that for Ac₂-KAA. With the latter compound, the EREF ratio is 1.8 $\times 10^4$.

following analysis is restricted to the penicillins and cephalosporins exhibiting the highest EREFs with each enzyme.

The fourth observation is particularly interesting since, up to the present time, it had been assumed that β -lactamases do not significantly catalyse the hydrolysis of 'normal' peptides [4–6]. More strikingly, Table 4 shows a direct comparison of the

peptidase and ' β -lactam-opening' activities of the two types of enzymes performed with the best peptide substrates and the best β -lactam substrates/inactivators of each protein. The results indicate that it is about as difficult for a peptidase to open the β -lactam ring as it is for the β -lactamase to hydrolyse the peptide. Note that the R39 enzyme is generally considered to be a 'highly penicillin-sensitive' peptidase, whereas the R61 and K15 enzymes are of medium and medium-low sensitivity respectively [4,16], and that the behaviour of the *B. cereus* II metalloenzyme cannot sensibly be compared with that of the serine enzymes.

The hydrolysis of β -lactams and peptides by OH^- ions involves the rate-limiting formation and breakdown of the tetrahedral intermediates respectively [3]. However, in the enzymic reactions, where the rate of acyl-enzyme formation can be increased 10^7 – 10^{10} -fold, it has not been possible to identify the rate-limiting step in this bimolecular reaction, and the rate of β -lactamase-catalysed hydrolysis of the best substrates might even be diffusion-limited [25,26]. From the above discussion, it would be tempting to conclude that the breakdown of the tetrahedral intermediate is the slowest enzymic step in all cases. Indeed, if the direction of the nucleophilic attack is chosen as a reference, protonation of the leaving group occurs on the *cis* face with the β -lactams, but on the *trans* side with the peptides. It can be assumed that β -lactamases and DD-peptidases have been optimized for these distinct stereochemistries. The similar EREF ratios shown in Table 4 might just reflect, for each enzyme, the difficulty of delivering a proton on the wrong side of the leaving group. The activation energy for the inversion of an amide nitrogen is known to be low in a free compound, but the situation is significantly different in the enzyme active sites, where the leaving group is thought to be rather strongly and specifically anchored [17,27]. Alternatively, it could be proposed that the more rigid structures of penicillins result in a more favourable positioning of the scissile bonds in β -lactamases, whereas the active sites of DD-peptidases are better in accommodating the more flexible peptides. In this respect, it would be interesting to test compounds such as the benzoyl and phenylacetyl derivatives of both Gly-D-Pro and D-Ala-D-Pro with both DD-peptidases and β -lactamases.

For both DD-peptidases and β -lactamases, the relatively low EREF values for the ester and *S*-ester (when compared with the best substrates) can probably be explained by a poorer positioning in the enzymes' catalytic clefts.

Finally, it is also interesting to calculate a minimum value for the rates of hydrolysis of the acyl-enzymes that β -lactamases form with their 'linear' substrates. Table 2 shows the $k_{\text{cat}}/K_{\text{m}}$ values for the ester and *S*-esters, and for all the β -lactamases, the K_{m} values are > 1 mM. The k_{cat} values are thus larger than 5 s^{-1} for the *B. licheniformis* and TEM enzymes, larger than 20 s^{-1} for P99, and larger than 0.2 s^{-1} for OXA-2. These supply minimum values for the hydrolysis of the enzyme-Gly-Bz adducts (k_3). For the P99 enzyme, this rather high value is of particular interest, since the corresponding values, determined with a representative set of β -lactams, range from 10^{-3} to $> 1000 \text{ s}^{-1}$ [28,29]. In particular, with penicillins, the k_3 value is never larger than

20 s^{-1} . With the 'linear' substrates, the deacylation step is thus not particularly impaired when compared with that of the 'normal' substrates of the enzyme. Under our experimental conditions (10 mM peptide substrates) the acylation of the β -lactamases thus remains the severely rate-limiting step.

This work was supported by the Belgian Government (PAI P4/03). We thank Dr. G. Dive for many useful discussions.

REFERENCES

- 1 Page, M. I. (1984) *Acc. Chem. Res.* **17**, 144–151
- 2 Page, M. I. (1991) *Philos. Trans. R. Soc. London Ser. B* **332**, 149–156
- 3 Page, M. I. and Laws, A. P. (1998) *Chem. Commun.* 1609–1617
- 4 Frère, J. M. and Joris, B. (1985) *CRC Crit. Rev. Microbiol.* **11**, 299–396
- 5 Frère, J. M. (1995) *Mol. Microbiol.* **16**, 385–395
- 6 Murphy, B. P. and Pratt, R. F. (1991) *Biochemistry* **30**, 3640–3649
- 7 Adam, M., Dambion, C., Plaitin, B., Christiaens, L. and Frère, J. M. (1990) *Biochem. J.* **270**, 525–529
- 8 Adam, M., Dambion, C., Jamin, M., Zorzi, W., Dusart, V., Galleni, M., El Kharroubi, A., Piras, G., Spratt, B. G., Keck, W. et al. (1991) *Biochem. J.* **279**, 601–604
- 9 Raquet, X., Lamotte-Brasseur, J., Fonze, E., Goussard, S., Courvalin, P. and Frère, J. M. (1994) *J. Mol. Biol.* **244**, 625–639
- 10 Swarén, P., Maveyraud, L., Raquet, X., Cabantous, S., Duez, C., Pédélecq, J. D., Mariotte-Boyer, S., Mourey, L., Labia, R., Nicolas-Chanoine, M. H., Nordmann, P. et al. (1998) *J. Biol. Chem.* **273**, 26714–26721
- 11 Matagne, A., Misselyn-Bauduin, A. M., Joris, B., Erpicum, T., Granier, B. and Frère, J. M. (1990) *Biochem. J.* **265**, 131–146
- 12 Carfi, A., Pares, S., Duée, E., Galleni, M., Duez, C., Frère, J. M. and Dideberg, O. (1995) *EMBO J.* **14**, 4914–4921
- 13 Carfi, A., Duée, E., Paul-Soto, R., Galleni, M., Frère, J. M. and Dideberg, O. (1998) *Acta Crystallogr.* **D54**, 47–57
- 14 Joris, B., De Meester, F., Galleni, M., Reckinger, G., Coyette, J., Frère, J. M. and Van Beeumen, J. (1985) *Biochem. J.* **228**, 241–248
- 15 Ledent, P., Raquet, X., Joris, B., Van Beeumen, J. and Frère, J. M. (1993) *Biochem. J.* **292**, 555–562
- 16 Granier, B., Jamin, M., Adam, M., Galleni, M., Lakaye, B., Zorzi, W., Grandchamps, J., Wilkin, J. M., Fraipont, C., Joris, B. et al. (1994) *Methods Enzymol.* **244**, 249–266
- 17 Frère, J. M., Leyh-Bouille, M., Ghuysen, J. M., Nieto, M. and Perkins, H. R. (1976) *Methods Enzymol.* **45**, 610–636
- 18 Dambion, C., Ledent, P., Zhao, G. H., Jamin, M., Dubus, A., Vanhove, M., Raquet, X., Christiaens, L. and Frère, J. M. (1995) *Lett. Pept. Sci.* **2**, 212–216
- 19 Dambion, C., Zhao, G. H., Jamin, M., Ledent, P., Dubus, A., Vanhove, M., Raquet, X., Christiaens, L. and Frère, J. M. (1995) *Biochem. J.* **309**, 431–436
- 20 Dubus, A., Wilkin, J. M., Raquet, X., Normark, S. and Frère, J. M. (1994) *Biochem. J.* **301**, 485–494
- 21 Grandchamps, J., Nguyen-Distèche, M., Dambion, C., Frère, J. M. and Ghuysen, J. M. (1995) *Biochem. J.* **307**, 335–339
- 22 Leyh-Bouille, M., Coyette, J., Ghuysen, J. M., Idczak, J., Perkins, H. R. and Nieto, M. (1971) *Biochemistry* **10**, 2163–2170
- 23 Leyh-Bouille, M., Nakel, M., Frère, J. M., Johnson, K., Ghuysen, J. M., Nieto, M. and Perkins, H. R. (1972) *Biochemistry* **11**, 1290–1298
- 24 Laws, A. P. and Page, M. I. (1989) *J. Chem. Soc. Perkin Trans. II*, 1577–1581
- 25 Hardy, L. W. and Kirsch, J. F. (1984) *Biochemistry* **23**, 1275–1282
- 26 Christensen, H., Martin, M. T. and Waley, S. G. (1990) *Biochem. J.* **266**, 853–861
- 27 Varetto, L., De Meester, F., Monnaie, D., Marchand-Brynaert, J., Dive, G., Jacob, F. and Frère, J. M. (1991) *Biochem. J.* **278**, 801–807
- 28 Galleni, M. and Frère, J. M. (1988) *Biochem. J.* **255**, 119–122
- 29 Galleni, M., Amicosante, G. and Frère, J. M. (1988) *Biochem. J.* **255**, 123–129