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PURIFICATION AND PROPERTIES OF THE EXOCELLULAR β-LACTAMASE OF *ACTINOMADURA* STRAIN R39

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The exocellular β-lactamase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) of *Actinomadura* R39 consists of one single polypeptide chain of molecular weight about 15200. It exhibits a highly asymmetrical shape, has a low isoelectric point (at pH 5.0) and contains about 9.3% (w/w) of a polydeoxyribonucleotide with which it forms a rather stable complex. Removal of a substantial amount of this deoxyribonucleotide by treatment with DNAase I has no effect on the enzyme activity. The β-lactamase has a wide spectrum of activity. Penicillins and Δ^3-cephalosporins can be either good or poor substrates. Oxacillin, which is a poor substrate of most β-lactamases from Gram-positive bacteria, is a good substrate of the β-lactamase of *Actinomadura* R39. Its best substrate, however, is nitrocefin (k_{cat}/K_m: 2300000 $M^{-1} \cdot s^{-1}$; catalytic centre activity: 210 s^{-1}). The k_{cat}/K_m values observed with some penicillins and Δ^3-cephalosporins are similar to the values of the bimolecular rate constants that govern the formation of the acyl-enzyme intermediates between these antibiotics and the serine D-alanyl-D-alanine peptidase that is also secreted by the same strain *Actinomadura* R39. Such a relationship, however, is not observed with all the β-lactam compounds tested.

The D-alanyl-D-alanine peptidases (carboxypeptidases-transpeptidases) are involved in the last stages of the bacterial wall peptidoglycan biosynthesis; they are sensitive to inactivation by β-lactam antibiotics. Some of the D-alanyl-D-alanine peptidases are serine enzymes [1–3]. The active serine residue effectively attacks the carbonyl carbon of the amide linkage of the β-lactam ring to form a covalent ester-linked penicilloyl (cephalosporoyl)-enzyme intermediate but the reaction flux stops at this abortive level at least for some time, conferring on the antibiotics the property of behaving as mechanism-based enzyme inactivators. The β-lactamases effectively hydrolyse the β-lactam antibiotics into inactive products. With the serine β-lactamases [4–6], the reaction probably also proceeds via a covalent ester-linked penicilloyl(cephalosporoyl)-enzyme intermediate but this intermediate decays very rapidly, generating high turnover numbers. *Actinomadura* R39 concomitantly secretes a D-alanyl-D-alanine peptidase and a β-lactamase during growth. In previous work, the R39 D-alanyl-D-alanine peptidase has been purified to protein homogeneity (mol. wt. 53000) [7] and characterized as a serine enzyme [3]. This paper describes the purification and properties of the exocellular β-lactamase (EC 3.5.2.6) of *Actinomadura* R39.

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Abbreviations; SDS, sodium dodecyl sulfate; TPCK, L-(tosylamido-2-phenyl)ethyl chloromethylketone.

Materials and Methods

Enzyme units. 1 β-lactamase unit transforms 1 μmol benzylpenicillin into benzylpenicilloate per min, at maximal velocity and at 30°C. The assays were performed by using the starch/iodine method or by using as substrate the chromogenic cephalosporin nitrocefin as described by Duez et al. [8]. 1 D-alanyl-D-alanine peptidase unit (carboxypeptidase assay) hydrolyses 1 μequiv. of the D-Ala-D-Ala-OH linkage of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala per min, at maximum velocity and at 37°C. The assays were performed as described by Frère et al. [9].

Growth of Actinomadura R39. *Actinomadura* R39 was grown in 500-liter tanks as described by Frère et al. [7]. Under these conditions, the culture fluid contained 0.04 β-lactamase unit/ml or 0.006 unit/mg protein.

Standard proteins and protein determination. Bovine serum albumin (mol. wt. 68000), ovalbumin (mol. wt. 43000), carbonic anhydrase (mol. wt. 29000), chymotrypsinogen (mol. wt. 25700), myoglobin (mol. wt. 17000), lysozyme (mol. wt. 14500) and insulin (mol. wt. 5700) were used as standard proteins. The proteins were estimated by measuring the A_{280} of the solutions, and for more accurate estimation, by total hydrolysis with 6 M HCl followed by reaction of the free amino groups with dinitrofluorobenzene, as described by Duez et al. [10].

Amino acid analyses. Samples were hydrolysed at 110°C for 24 h under vacuum in 1 ml azeotropic HCl. Cysteine was estimated as cysteic acid after performic oxidation [11]. Tryptophan was determined after hydrolysis by mercaptoethanesulfonic acid (96 h at 105°C).

Spectra. Ultraviolet spectra were recorded manually by using a Zeiss MIVQIII spectrophotometer. Fluorescence emission was measured at 90° to the excitation beam with an Aminco-Bowman recording spectrofluorimeter. For the fluorescence excitation spectra, the emission wavelength was fixed at 355 nm and for the fluorescence emission, the excitation wavelength at 285 nm. The protein solutions were in 50 mM sodium phosphate buffer, pH 7.0.

Radioactivity measurements. [^{14}C]Cefoxitin (2.7 mCi/mmol with the ^{14}C on the carbonyl group of the phenylacetyl lateral chain; a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, NJ) and a Packard Tri-Carb 2425 liquid-scintillation spectrometer were used.

Semi-preparative polyacrylamide gel electrophoresis at pH 8.4 under non-denaturing conditions. The enzyme samples to be purified (0.5 mg protein) were made fluorescent by addition of 10 μl of a solution of fluorescamine (1 mg/ml in dry acetone) [12] thus permitting visualization of the protein bands during electrophoresis by illumination with ultraviolet light. Because of the very high mobility of the β-lactamase at pH 8.4, reaction with fluorescamine had virtually no effect on its electrophoretic migration. The electrophoreses were carried out on cylindrical gels (1.0 × 13 cm; containing 7.5% acrylamide and 0.2% *N,N'*-methylenebisacrylamide). Following a preelectrophoresis of the gels for 3 h at 12 mA/gel (without preelectrophoresis, the β-lactamase, as well as the D-alanyl-D-alanine peptidase and pigmented materials in non-completely purified preparations, migrated with the buffer discontinuity), the fluorescent samples were submitted to electrophoresis for 5 h, at 4°C and 12 mA/gel. Other conditions were those described in Ref. 13.

Analytical polyacrylamide gel electrophoresis at pH 8.4 under non-denaturing conditions. Samples of the purified β-lactamase (20 μg protein) were analysed by gel electrophoresis under the same conditions as those described above except that 0.5 × 7.5 cm cylindrical gels were used and that both preelectrophoresis and subsequent electrophoresis of the samples were of 2 h at 4 mA/gel. After electrophoresis, one gel was stained with Coomassie blue and scanned at 265 nm and a second one was sliced into 2-mm thick discs. The discs were eluted with 50 mM sodium phosphate buffer, pH 7.0, and the eluates assayed for β-lactamase activity.

Gel electrofocusing, pH 3–10. Samples of the purified β-lactamase (20 μg protein) were analysed by electrofocusing under the conditions described by Wrigley [14]. Cylindrical gels (0.5 × 7.5 cm) containing 7.5% acrylamide and 0.2% *N,N'*-methylenebisacrylamide were used. One gel was stained with Coomassie blue and two others were sliced into 2-mm thick discs. In one case, the discs were eluted with 1 ml water to determine the shape

of the pH gradient. In the second case, the discs were eluted with 50 mM sodium phosphate buffer, pH 7.0, and the eluates assayed for β-lactamase activity.

Analytical polyacrylamide gel electrophoresis at pH 8.5 in the presence of 0.1% SDS. The electrophoreses were carried out on 15-μg protein samples under the conditions described by Duez et al. [10]. The gels were stained with Coomassie blue.

Equilibrium sedimentations and diffusion coefficient. The equilibrium sedimentations were performed in 50 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl, at 20408 rev./min for 18 h, at 22°C and at an initial protein concentration of 2 mg/ml. For other conditions, see Ref. 13. The initial solute concentrations were determined by a complementary run with the double-sector capillary-type synthetic boundary cell. These latter data were also used for the determination of the diffusion coefficient. The apparent molecular weight ($M_{r,app}$) was calculated as described by O'Donnell and Woods [15], assuming a $\bar{v}$ value of 0.75 cm^3/g.

Non-radioactive β-lactam compounds. Benzylpenicillin was purchased from Rhône-Poulenc, Paris, France or from UCB, Brussels, Belgium and 6-aminopenicillanic acid from Sigma Chemical Co. Ltd., St Louis, MO, U.S.A. Phenoxymethylpenicillin was a gift from Professor H. Vanderhaeghe, Katholieke Universiteit Leuven, Belgium; cefoxitin was from Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey; ampicillin and oxacillin were from Bristol Benelux S.A., Brussels; nitrocefin (cephalosporin 87/312) was from Glaxo Chemical Co., U.K.; carbenicillin, cloxacillin, methicillin were from Beecham Research Laboratories, Brentford, U.K.; cephalosporin C, cephaloglycine, cephalexin and cephalothin were from Eli Lilly and Co., Indianapolis, U.S.A.

Estimation of deoxyribose. Total sugars were determined with the phenol-sulfuric acid technique of Dubois et al. [16] and deoxyribose with the perchloric acid-diphenylamine technique [17].

Results

Purification of the β-lactamase

The β-lactamase had been partially purified in a previous work by Johnson et al. [18]. The procedure was modified as follows.

Step 1. The DEAE-cellulose with both β-lactamase and D-alanyl-D-alanine peptidase bound to it (640 g, corresponding to 60 l culture filtrate) were treated three times with 1 liter 0.1 M Tris-HCl buffer, pH 7.5, containing 0.25 M NaCl and 5 mM $MgCl_2$, thus extracting at least 75% of the D-alanyl-D-alanine peptidase and only minor amounts of the β-lactamase. In turn, the β-lactamase enzyme was eluted with 50 mM sodium phosphate buffer, pH 7.0, containing 1 M NaCl, and then precipitated with acetone as described in Ref. 18.

Step 2 was the same as step 2 described in Ref. 18. It involved chromatography on DEAE-cellulose followed by filtration on Sephadex G-100.

Step 3. Following a chromatography on DEAE-Sephacel (3 × 15 cm) performed under the same conditions as those described for the DEAE-cellulose chromatography in step 2, the pooled active fractions (concentrated to 5 mg protein/ml, dialysed against 25 mM sodium phosphate, pH 7.0, and made fluorescent by addition of fluo-

TABLE I

PURIFICATION OF THE β-LACTAMASE

Purification step	Total β-lactamase units	D-alanyl-D-Ala peptidase units	Total protein (mg)	Specific activity β-lactamase (units · mg^{-1})	Yield (%)	Purification factor
Culture supernatant	2248	690	$356 \cdot 10^3$	$6 \cdot 10^{-3}$	100	1
1	1230	41	$1.9 \cdot 10^3$	0.64	55	100
2	907	1	267	3.4	40	540
3	337	0	1.31 [a]	253	15	40000

[a] The protein concentration in this final preparation was measured by determining the number of amino groups available for dinitrophenylation after total hydrolysis of the protein by 6 M HCl at 110°C.

rescamine) were submitted to a semi-preparative polyacrylamide gel electrophoresis at pH 8.4, as described in Materials and Methods. After elution with 50 mM sodium phosphate buffer, pH 7.0, the pooled active samples (from 60 gels run under identical conditions) were concentrated by ultrafiltration and finally filtered in the same phosphate buffer on a 100-ml (1.2 × 90 cm) Sephadex G-75 column. The main protein peak thus obtained had a constant β-lactamase activity of 253 units/mg protein. Table I summarizes the purification procedure. The preparation previously obtained by Johnson et al. [18] had a specific activity of 198 units/mg protein.

Electrophoretic properties and molecular weight of the purified β-lactamase

Analytical gel electrophoresis at pH 8.4 under non-denaturing conditions revealed one single protein band (migration: 5.5 cm towards the anode) and gel electrofocusing revealed one single protein band with a p*I* at pH 5. In both cases, the protein band corresponded exactly to the area of β-lactamase activity.

Gel electrophoresis in the presence of SDS revealed one major protein band at a position indicating an apparent molecular weight of about 15000. Minor bands of apparent molecular weights of 30000, 45000 and 60000 (i.e., integral multiples of 15000) were also detected. However, their relative intensities depended on the temperature and the detergent concentration used during the treatment of the enzyme sample prior to the electrophoresis, suggesting that they were due to technical artefacts.

The plot of $M_{r,app}$ versus x_n (where x_n is the distance from the rotation axis, as obtained by equilibrium sedimentation; Fig. 1) indicated a constant apparent molecular weight of 15200 for x_n values ranging from 69.10 to 70.5 mm. A $D_{20,w}$ value of $8.70 \cdot 10^{-7}$ cm^2/s was calculated. On the basis that spherical, non-hydrated 15200-M_r protein has a diffusion constant of $12.8 \cdot 10^{-7}$ cm^2/s, the β-lactamase thus appeared to be strongly asymmetrical with a frictional ratio f/f_0 of 1.47. This probably explains that by filtration on a Sephadex G-100 fine column, the β-lactamase was eluted with a K_D value of 0.3, a value which by reference to those of standard proteins would indicate an apparent molecular weight of 29000. (It is known that an elongated molecule has a K_D value smaller than that of a globular molecule of similar molecular weight.)

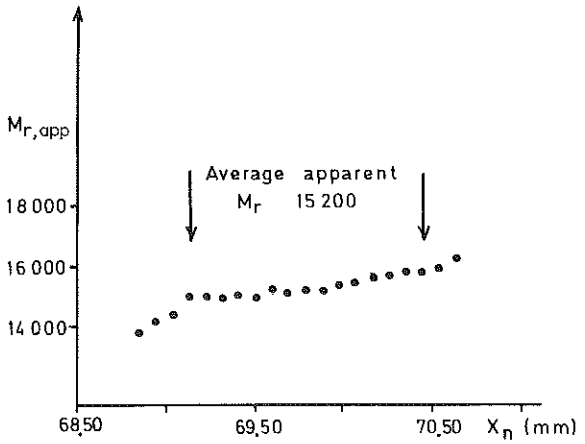

Fig. 1. Equilibrium sedimentation of the purified β-lactamase. For conditions, see the Materials and Methods section.

The molecular weight of the β-lactamase was also estimated by binding experiments using [^{14}C]cefoxitin (a β-lactam antibiotic known to be a poor inactivator of this enzyme [19]). In these experiments, the β-lactamase (90 μg protein in 200 μl, final volume, of 50 mM phosphate buffer, pH 7.0) was incubated with 2.5 mM [^{14}C]cefoxitin for 30 min at 30°C, thus inhibiting the enzyme activity by 75% through formation of an inactive complex of high stability (half-life: 4 h at 30°C). After dialysis to remove the unbound [^{14}C]cefoxitin, estimation of the protein concentration, residual enzyme activity (25%) and radioactivity led to the conclusion that 15700 g inactivated β-lactamase had bound 1 mol cefoxitin.

Absorption and fluorescence spectra

The purified β-lactamase exhibited an unusual ultraviolet spectrum (Fig. 2A) with a maximum at 257 nm and $A^{1\%}_{1cm}$ values of 21 and 63 at 280 and 257 nm, respectively. The fluorescence emission spectrum, however (Fig. 2B), did not present any unexpected property, with the maximum at 355 nm, a value close to that reported for free tryptophan in an aqueous environment [20]. In turn, the fluorescence excitation spectrum was similar to that of most proteins (Fig. 2A) but was clearly

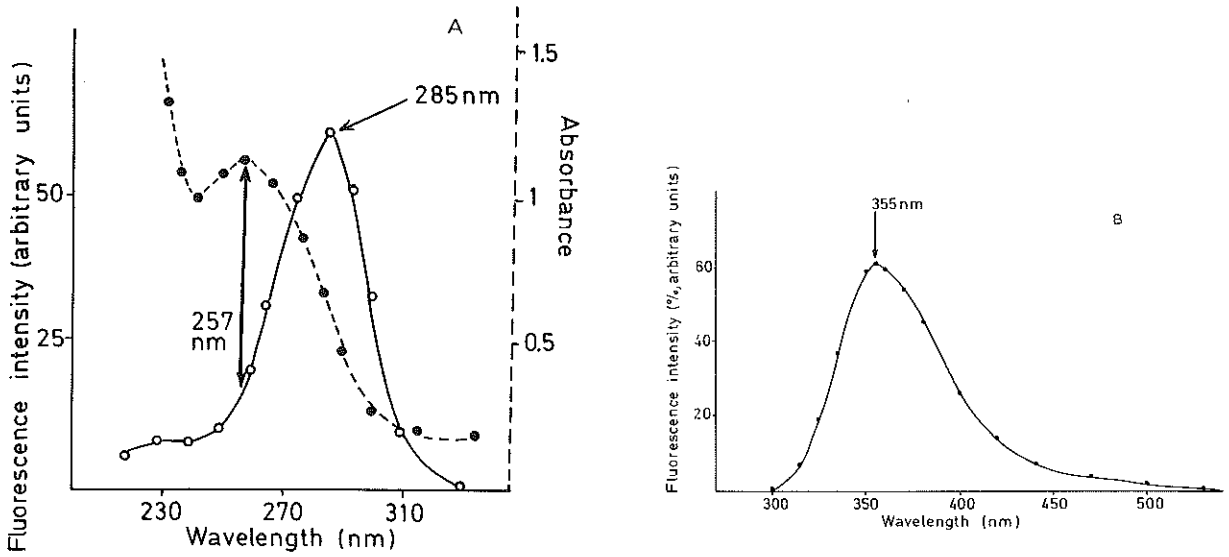

Fig. 2. Absorption and fluorescence spectra of the purified β-lactamase. A. Fluorescence excitation (continuous line, open symbols) and absorption (interrupted line, filled symbols) spectra. B. Fluorescence emission. Protien concentration, 0.35 mg/ml. For conditions, see the Materials and Methods section.

different from the absorption spectrum, thus indicating that tryptophan residues were not the main chromophores responsible for the shape and intensity of the absorption spectrum between 250 and 290 nm.

Presence of a deoxyribonucleotide

The phenolsulfuric acid test (performed on 45 μg β-lactamase) indicated the presence of about 1.6 μg sugar (i.e. 3.6%, w/w). In turn, the perchloric acid-diphenylamine test (performed on 30 μg protein and using calf thymus DNA as standard) indicated the presence of 2.8 μg deoxyribonucleotides (i.e. 9.3%, w/w), a value which translated to 3.4% (w/w) deoxyribose. The good agreement observed between the two tests suggested that deoxyribose was the only sugar present.

The β-lactamase (0.5 mg) was treated with 10 μg of DNAase I (from Boehringer, Mannheim. F.R.G.), at 37°C and in 0.5 ml 0.1 M sodium acetate buffer, pH 5.0, containing 5 mM $MgCl_2$. This treatment produced a hyperchromic effect of 10% after 60 min. (RNAase A from bovine pancreas had no effect). Filtration of the DNAase I-treated β-lactamase through a 50-ml Sephadex G-100 column yielded two fractions. A first fraction, exhibiting the same specific β-lactamase activity as the untreated enzyme, was eluted at a K_D value of 0.3. This fraction no longer had a maximum at 257 nm, but still had an A_{260}/A_{280} ratio larger than 1 (Fig. 3A). A second fraction devoid of enzymatic activity was eluted at a K_D value of

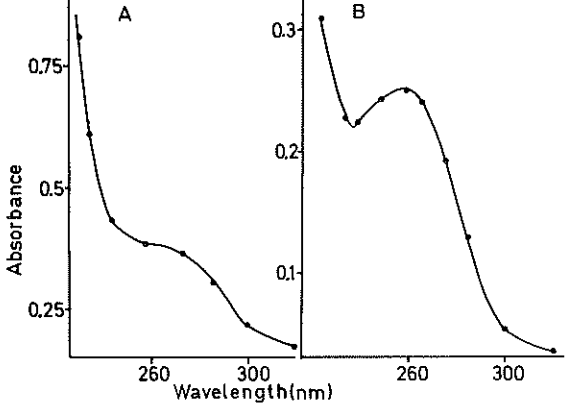

Fig. 3. Absorption spectra of the fractions obtained by Sephadex filtration of the DNAase I-treated β-lactamase. A. Fraction of $K_D = 0.3$. Protein concentration, 0.25 mg/ml. The ratio A_{280}/A_{260} is 0.9. B. Fraction of $K_D = 1.0$. The ratio A_{280}/A_{260} is 0.66.

1.0. This fraction had a spectrum similar to that of a mixture of nucleotides or deoxynucleotides (Fig. 3B).

Amino acid composition

The results are given in Table II. An estimated value of 13850 for the molecular weight of the protein portion of the β-lactamase (assuming that about 9% of the total molecular weight was due to the polydeoxyribonucleotide) was used to estimate the number of amino acid residues.

Sensitivity to proteolytic treatments

The β-lactamase (150 μg; i.e. 10 nmol) was freeze-dried, dissolved in 50 μl 0.2 M ammonium bicarbonate, heated at 100°C for 2 min and incubated for 6 h (total time) with 9 μg TPCK-trypsin (from Worthington, Freehold, NJ, U.S.A.) using 3 μg of the protease at the beginning of the incubation and after 2 and 4 h, respectively. Attempts to obtain a peptide map (under the conditions described in Ref. 21) failed, indicating that most of the protein was trypsin-resistant. The β-lactamase also resisted the action of the *Staphylococcus aureus* protease and chymotrypsin.

TABLE II

AMINO ACID COMPOSITION OF THE EXOCELLULAR β-LACTAMASE OF *ACTINOMADURA* R39

Residue	Number of residues per 13 850 M_r	% (in number)
Lys	2	1.6
His	2	1.6
Arg	7	5.6
Trp	4	3.2
Asx	14	11.2
Thr	8	6.4
Ser	5	4
Glx	18	14.4
Pro	6	4.8
Gly	12	9.6
Ala	13	10.4
1/2 Cys	2	1.6
Val	10	8
Met	2	1.6
Ile	3	2.4
Leu	11	8.8
Tyr	2	1.6
Phe	4	3.2

TABLE III

KINETIC PARAMETERS OF THE EXOCELLULAR β-LACTAMASE AND D-ALANYL-D-Ala PEPTIDASE OF *ACTINOMADURA* R39

Substrate	D-alanyl-D-Ala peptidase [a]	β-Lactamase [b]			*A/B*
	k_2/K ($M^{-1}\cdot s^{-1}$)(*A*) (at 37°C)	K_m (mM) (at 30°C)	*V* (units per mg protein) (at 30°C)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$) (*B*)	
Benzylpenicillin	300000	0.065	253	980000	0.31
Phenoxymethylpenicillin	>70000	0.290	582	505000	>0.14
Ampicillin	280000	0.57	1327	590000	0.48
Carbenicillin	6000	0.26	100	97000	0.06
Methicillin	4000	0.29	70	61000	0.06
Oxacillin	40000	0.54	826	385000	0.10
Cloxacillin	15000	0.42	77	46000	0.32
6-Aminopenicillanic acid	1000	0.072	194	680000	0.0015
Cephalosporin C	200000	1.05	15	3500	57
Cephaloglycine	280000	2.86	548	50000	5.6
Cephalexin	12000	0.91	45	12300	0.98
Cephalothin	>70000	0.12	142	300000	>0.23
Nitrocefin	$\approx 10\cdot 10^6$	0.085	1000	$2.8\cdot 10^6$	≈4

[a] From Ref. 25.
[b] At 30°C and in 50 mM phosphate buffer, pH 7.5.

Kinetic parameters

Table III gives the V and K_m values obtained for various penicillins and cephalosporins (at 30°C and in 50 mM phosphate buffer, pH 7.5). The β-lactamase exhibited a wide spectrum of activity. Penicillins and Δ^3-cephalosporins were either good or poor substrates. The best penicillin substrate was benzylpenicillin (k_{cat}/K_m: 980000 $M^{-1} \cdot s^{-1}$) and the best cephalosporin substrate was nitrocefin (k_{cat}/K_m: 2800000 $M^{-1} \cdot s^{-1}$). Remarkably, oxacillin was a fair substrate (k_{cat}/K_m: 385000 $M^{-1} \cdot s^{-1}$) while it is a poor substrate of most β-lactamases from Gram-positive bacteria (with the exception of the *Bacillus cereus* β-lactamase II [22] and the β-lactamase of *Streptomyces cacaoi* [23]).

Comparison between β-lactamase and the serine alanyl-D-alanine peptidase of Actinomadura R39

On the basis of previous works [24,25], the reaction between β-lactam compounds and the serine D-alanyl-D-alanine peptidases (in particular that secreted by *Actinomadura* R39) is known to proceed according to the scheme

$$E + I \overset{K}{\rightleftarrows} EI \overset{k_2}{\rightarrow} EI^* \overset{k_3}{\rightarrow} E + P(s)$$

where E = enzyme; I = antibiotic; K = dissociation constant; EI = Michaelis complex; EI* = serine ester-linked acyl-enzyme intermediate; P(s) = degradation products; k_2 and k_3 = first-order rate constants. Effective enzyme inactivation is caused by a high value for the bimolecular rate constant k_2/K (i.e., a rapid formation of the acyl-enzyme intermediate EI*), and a low k_3 value (i.e., a high stability of the intermediate thus formed). If one assumes that the hydrolysis of a β-lactam antibiotic by the R39 β-lactamase follows the same reaction pathway, then the k_{cat}/K_m value measured with the β-lactamase also expresses the bimolecular rate constant for the formation of the corresponding complex EI*. If one excepts 6-aminopenicillanic acid, which was a very poor inactivator of the D-alanyl-D-alanine peptidase and a very good substrate of the β-lactamase, and

TABLE IV

VALUES OF THE RATIO ((k_2/K) R39 D-ALANYL-D-Ala PEPTIDASE/(k_{cat}/K_m) β-LACTAMASE FOR β-LACTAMASE FROM VARIOUS BACTERIAL STRAINS

When two values are given, they correspond to the lowest and highest values which can be calculated from the different K_m and V values found in the references. *E. coli RTem* values are calculated on the basis of the V values of Richmond [31]; the V values given by Datta and Richmond [33] are 2.5-fold smaller and those given by Fisher and Knowles [6] are 2.4-fold higher.

Antibiotic	β-lactamase						
	S. albus G	*B. cereus* 569/H		*B. licheniformis*		*S. aureus* PCI	*E. coli RTem*
		I	II	749/C	6346/C		
Benzylpenicillin	0.5	0.004	0.71	0.01	0.01	0.0025	0.008
Phenoxymethyl penicillin	>0.14	0.0008				>0.0008	>0.0004–0.002
Ampicillin	0.5	0.002–0.012	1.1			0.0005	0.0008
Carbenicillin	0.13	0.002					0.0006
Methicillin	0.4	0.03	0.005	0.0004	0.0004	5	0.0012
Oxacillin	0.16	0.2	0.05				0.004
Cloxacillin	0.5	0.5	0.002			15	0.0004–0.008
6-Aminopenicillanic acid	0.002	$1 \cdot 10^{-4}$–$2 \cdot 10^{-3}$		0.0002	0.0004	0.01	$4 \cdot 10^{-5}$
Cephalosporin C	26	60–160	0.03–0.20	0.5	0.2	3	0.08
Cephaloglycine	31		0.08				0.05
Cephalexin	10		1				
Cephalothin	>9		0.004–0.02				
Nitrocefin	≈10	0.2					
References	8	22,26–28		29		30	31,32

cephalosporin C which was a poor substrate of the β-lactamase and a very good inactivator of the D-alanyl-D-alanine peptidase, the k_2/K (D-alanyl-D-alanine peptidase)/k_{cat}/K_m (β-lactamase) ratios ranged between 0.06 and 0.48 for the penicillins and between 0.23 and 5.6 for the cephalosporins (Table III). This fair similarity suggested that, in spite of the fact that their molecular weights differ by a factor of more than three (15000 vs. 53000), the β-lactamase and D-alanyl-D-alanine peptidase of *Actinomadura* R39 might be somehow functionally related. This relationship seemed not to be coincidental. Indeed the same k_2/K (R39 D-alanyl-D-alanine peptidase)/k_{cat}/K_m (β-lactamase) ratios were calculated for various β-lactamases other than that of *Actinomadura* R39 (Table IV). With all the β-lactamases used, except that of *Streptomyces albus* G, the ratio values were, most often, not only extremely different from 1 but also extremely variable (depending on the antibiotics).

Discussion

The exocellular β-lactamase of *Actinomadura* R39 was purified to the stage where, as shown by different techniques, the enzyme preparation consisted of one single protein. It still contained, however, 9.3% (w/w) of a polydeoxyribonucleotide, apparently in the form of a rather stable complex. Removal of a substantial amount of the deoxyribonucleotide by treatment with DNAase I had no effect on the enzymatic activity. The R39 β-lactamase exhibited a low molecular weight (15200) and a highly asymmetrical shape. Moreover, it had no strict preference for the penicillins or the cephalosporins and had a relatively low specific activity (253 units/mg protein, to be compared with values higher than 1000 for other β-lactamases). Whether the R39 β-lactamase is a serine enzyme or not remains to be determined. No loss of activity was observed after incubation in the presence of 10 mM ethylenediaminetetraacetate (for 60 min at 25°C or 96 h at 4°C), suggesting that this β-lactamase is not a metalloenzyme.

The 15200-M_r β-lactamase and the 53000-M_r D-alanyl-D-alanine peptidase that are concomitantly secreted by *Actinomadura* R39 have very different molecular weights. In addition, an antiserum prepared agianst the R39 D-alanyl-D-alanine peptidase [34] (which gives rise to a clear, single precipitation line with this peptidase but has no effect on the enzyme activity), neither inactivated nor activated the R39 β-lactamase, had no effect on its thermosensitivity and did not give rise to any precipitation line (not shown in the Results section). In spite of these structural differences, the k_{cat}/K_m values measured for the reactions between the R39 β-lactamase and several penicillins and Δ^3-cephalosporins were similar to the k_2/K values measured for the reactions between the R39 D-alanyl-D-alanine peptidase and the same β-lactam compounds (although not all of them). On this basis, the β-lactamase of *Actinomadura* R39 markedly differred from the other β-lactamases examined (and originating from various Gram-positive and Gram-negative bacteria) except that of *Streptomyces albus* G [8] (mol. wt. 30000–31000). Interestingly, *Streptomyces* sp. and *Actinomadura* sp. are both Actinomycetales. At this stage, however, whether or not an evolutionary relationship exists between β-lactamases and D-alanyl-D-alanine peptidases is unknown and will remain so as long as convincing structural data are not available.

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