Characterization of a β -lactamase produced in *Mycobacterium fortuitum* D316

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A β -lactamase from *Mycobacterium fortuitum* D316 was purified and some physico-chemical properties and substrate profile determined. On the basis of its N-terminal sequence and of its sensitivity to β -iodopenicillanate inactivation, the enzyme appeared to be a class A β -lactamase, but its substrate profile was quite unexpected, since nine cephalosporins were among the eleven best substrates. The enzyme also hydrolysed ureidopenicillins and some so-called ' β -lactamase-stable' cephalosporins.

INTRODUCTION

Several species of pathogenic mycobacteria inactivate penicillins and cephalosporins by producing specific β -lactamases (Kasic, 1979), which contribute to the resistance exhibited by mycobacteria towards β -lactam antibiotics with, however, the exceptions of cephamycins, imipenem and the clavulanic acid-amoxycillin combination (Cynamom & Palmer, 1982, 1983).

The enzymes are usually described as cell-bound and apparently constitutively expressed. The few available data indicate that both penicillins and cephalosporins are hydrolysed and that isoxazolympenicillins behave as inhibitors.

A recent review (Bush, 1989) on the classification of mycobacterial β -lactamases reports some data on their molecular properties and substrate specificity, but nothing is known about their primary or tertiary structures. The present study was undertaken to purify and characterize the β -lactamase produced by a mutant strain D316 of *Mycobacterium fortuitum* A.T.C.C. 19542.

A preliminary account of this work was presented at the 19th FEBS Meeting held in Rome, Italy, on 2–7 July 1989.

MATERIALS AND METHODS

Chemicals and antibiotics

Sephadex G-75, Polybuffer 74 and the Mono P HR 5/20 column were from Pharmacia (Uppsala, Sweden); acrylamide, bisacrylamide and ammonium persulphate were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Ampholines PAG-Plates 3.5–9.5 and M_r standards were from LKB (Bromma, Sweden). All other chemicals used were of the purest available grade.

Benzylpenicillin, ampicillin, methicillin, oxacillin, cephaloridine, cephalosporin C and cephalothin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Carbenicillin, ticarcillin and talampicillin were kindly given by Beecham Pharmaceuticals (Brentford, Middx., U.K.). Other β -lactams were from commercial sources. The chromogenic cephalosporin PADAC was purchased from Calbiochem (La Jolla, CA, U.S.A.); nitrocefin was kindly given by Dr. Hotston (Glaxo Research Group, Greenford, Middx., U.K.). 6 β -Iodopenicillanate (β IP) was a gift from Pfizer Central Research (Sandwich, Kent, U.K.).

Strain and β -lactamase production

Mycobacterium fortuitum A.T.C.C. 19542 was mutated by treatment with N-methyl-N'-nitro-N-nitrosoguanidine and a mutant, called D316, presenting a 12-fold higher β -lactamase content than the parent strain, was isolated and grown as previously described (Fattorini *et al.*, 1989). β -Lactamase was purified as follows: the solution obtained after concentration on CH 2RA Amicon apparatus with an M_r cut-off of 10000 and containing the β -lactamase activity was dialysed against 0.1 M-Tris buffer, pH 7.4, and loaded on to a Sephadex G-75 column.

The active fractions were pooled and dialysed against 25 mM-Bistris buffer, pH 7.0. After centrifugation, the β -lactamase-containing supernatant was subjected to chromatofocusing over the pH range 7.0–4.0 using a Mono P HR 5/20 column and a Perkin-Elmer series 10 h.p.l.c. apparatus.

Protein and β -lactamase activity determination

Protein content was determined as described by Bradford (1976) or by directly measuring the absorbance at 280 nm. The enzyme activity was assayed in 0.1 M-sodium phosphate buffer, pH 7.2, containing 0.3 M-KCl and BSA (0.05 mg/ml) at 30 °C, using cephaloridine or nitrocefin as substrates. Absorbance variations were monitored at 255 or 482 nm respectively with a Perkin-Elmer 550S spectrophotometer.

One unit of enzyme activity was the amount of enzyme which hydrolysed 1 μ mol of cephaloridine/min at 30 °C and at a saturating substrate concentration.

Kinetic parameters for penicillins and cephalosporins

The experiments were performed at 30 °C in 50 mM-sodium phosphate, pH 7.2, containing 0.2 M-KCl and BSA (0.05 mg/ml). The values of K_m and K_{cat} were obtained either by monitoring the complete time courses of the reactions or by using the initial rates of substrate hydrolysis at various concentrations. In the first case the data were collected from an Ultrospec II instrument coupled with an Apple II microcomputer and analysed as described by De Meester *et al.* (1987); the conditions were chosen so that the reaction was virtually complete within 3 min; under these conditions no enzyme inactivation was observed. The initial rates were obtained with the help of a Perkin-Elmer 550S

Abbreviations used: β IP, 6 β -iodopenicillanate; pCMB, p-chloromercuribenzoate.

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instrument and the kinetic parameters were obtained by using the Hanes plot of $[S]/v$ versus $[S]$.

Inactivation by β IP

Samples of enzyme were incubated with β IP at different β IP/enzyme molar ratios for 30 min at 30 °C and the residual activity was measured in the presence of 200 μ M-cefazolin. The samples were subjected to a prolonged incubation or dialysis and the activity was again determined. In separate experiments, 15 μ M- β -lactamase was incubated with 18 μ M- β IP and spectra recorded immediately after the mixing and continuously for 3 h. During those experiments no re-activation of the enzyme was observed.

The rate of inactivation was measured by the reporter-substrate method (De Meester *et al.*, 1987) at 30 °C in the same buffer as that used for the kinetic studies. Enzyme (20 ng in 10 μ l) was added to 465 μ l of 87 μ M-nitrocefin containing β IP concentrations ranging from 0 to 10 μ M. The A_{482} was recorded with a Beckman DU8 spectrophotometer.

Interaction with non- β -lactam compounds

The enzyme activity was determined in the presence of borate, 3-aminophenylboronic acid, EDTA, nitrilotriacetic acid and *p*-hydroxymercuribenzoate (*p*CMB), using cefazolin or nitrocefin as substrates. *p*CMB inactivated the enzyme. That inactivation was studied by incubating the enzyme with various concentrations of *p*CMB, withdrawing samples and determining the residual activity after different periods of time.

Amino acid composition

The purified β -lactamase was freed from Ampholines by means of an AG 501 X8 ion-exchange column. The samples (usually 10 μ g) were hydrolysed in azeotropic HCl for 22 h at 110 °C in a PICO-TAG Work-station. After drying, aliquots of phenyl isothiocyanate were added in each tube and after 30 min at room temperature and evaporation to dryness, amino acid phenyl isothiocyanate derivatives were analysed on a RP-C18 column as described elsewhere (Bidlingmeyer *et al.*, 1984).

Thiol-group determinations were performed as described by Cavallini *et al.* (1966) for total cysteine residues and by the method of Ricci *et al.* (1986) to quantify free thiol groups.

Determination of M_r

The M_r of the enzyme was determined by SDS/PAGE as described by Laemmli (1970). The proteins were stained with AgNO_3 as described by Heukeshoven & Dernick (1985).

Isoelectric focusing

The pI of the enzyme was determined by running 10 μ g of purified enzyme on PAG-Plates (pH 3.5–9.5) using a Multiphor II LKB apparatus as described previously (Oliva *et al.*, 1987).

Sequence determination

Protein sequence analysis was carried out on a 477A pulse-liquid sequencer (Applied Biosystems, Foster City, CA, U.S.A.) with on-line analysis of the amino acid phenylthiohydantoin derivatives on a 120A Analyzer (Applied Biosystems). The automated Edman degradation was pursued for 49 cycles, using 3 nmol of native protein freed from Ampholines over a Centricon 10 microconcentrator (Amicon).

RESULTS

Purification, M_r and pI determinations

As shown in Table 1 and Fig. 1, the purification procedure was simple and permitted one to obtain a β -lactamase of high purity

Table 1. Purification of the β -lactamase of *Mycobacterium fortuitum* D316

The starting material was obtained from 14 litres of *M. fortuitum* culture.

Step	Total protein (mg)	*Specific activity (units/mg)	Total activity (units)	Purification factor	Yield (%)
Material retained by:					
DC 10LA column	12 500	1.6	20 000	1	100
CH 2RA column	750	23	17 200	14	86
Sephadex G-75	90	142	12 800	89	64
Fast chromatofocusing	5.6	1 300	7 200	807	36

* Cephaloridine was used as substrate; s.d. values for activity determinations $\pm 6\%$.

Fig. 1. SDS/PAGE analysis after different purification steps

A 15% (w/v)-polyacrylamide fixed concentration was used. The proteins were stained with AgNO_3 . The following samples were loaded: A, mixture of M_r standards; B, retained material from concentration on hollow-fibres apparatus (50 μ g); C, active peak from Sephadex G-75 chromatography (50 μ g); D, β -lactamase from fast chromatofocusing (30 μ g). The standard mixture contained the following pure proteins: ovotransferrin (M_r 78 000), BSA (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 30 000), myoglobin (M_r 17 200) and cytochrome *c* (M_r 12 300).

with a reasonable yield. Electrophoretic analysis indicated a homogeneous protein with an M_r of $29\,000 \pm 1\,000$ and a pI of 4.9 ± 0.01 , a result in good agreement with the apparent pI determined in the fast chromatofocusing procedure (Fig. 2).

Amino acid composition

In agreement with its acidic pI, this β -lactamase exhibited a high content of aspartic acid and glutamic acid (Table 2). In separate experiments, two residues of free cysteine were found.

Substrate specificity

The *M. fortuitum* D316 enzyme appeared to have a broad spectrum of specificity. Surprisingly, several cephalosporins exhibited k_{cat} and k_{cat}/K_m values higher than those observed with penicillins, nitrocefin being the best one.

Fig. 2. Elution profile of fast chromatofocusing

A sample of 5 mg of total protein from Sephadex G-75 was loaded on a MONO P HR 5/20 column equilibrated with 25 mM-Bistris (pH 7.2) and eluted with about 60 ml of Polybuffer 74 (diluted 1:10, pH 4.0). Fractions (2 ml) were collected and, after measuring the pH, they were buffered with 0.2 ml of 1 M-phosphate buffer at pH 7.0.

Cefuroxime, cefoperazone, cefotaxime, cefamandole, carbenicillin and ureidopenicillins, usually considered as 'stable' β -lactams, were hydrolysed rather rapidly. 7-(Thien-2-ylacetamido)-3-[2-(4-*NN*-dimethylaminophenylazo)pyridinium methyl]-3-cephem-4-carboxylic acid ('PADAC'), a chromogenic cephalosporin, was not a good substrate, and the progress curves showed biphasic features. The kinetic results are summarized in Table 3 with cephaloridin, both the k_{cat} and k_{cat}/K_m values exhibited rather broad maxima between pH 6.0 and 8.5.

Table 2. Amino acid composition of *M. fortuitum* β -lactamase

The composition is calculated on the basis of an M_r of 30200.

Amino acid	Experimentally obtained	Nearest integer
Asp + Asn	20.0	20
Glu + Gln	31.4	31
Ser	26.3	26
His	3.1	3
Gly	28.4	28
Thr	11.2	11
Arg	21.1	21
Ala	21.1	21
Pro	16.9	17
Tyr	11.6	12
Val	17.9	18
Met	2.1	2
Ile	11.3	11
Leu	23.2	23
Phe	9.7	10
Lys	8.4	8
Trp*	7.2	7
Cys†	1.9	2

* Determined on the basis of A_{280} .

† Results from Cavallini *et al.* (1966) and Ricci *et al.* (1986).

β -Lactamase- β IP interaction

The inactivation of *M. fortuitum* β -lactamase by β IP was complete with an enzyme/inactivator ratio of 1:1. After dialysis

Table 3. Substrate profile of *Mycobacterium fortuitum* D316 β -lactamase

Antibiotic substrate	Wavelength (nm)	$\Delta\epsilon$ ($M^{-1}\cdot cm^{-1}$)	Enzyme (μg)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot M^{-1}$)
Penicillins						
Penicillin G	232	800 ± 100	0.6–2.5	56 ± 8	54 ± 4	1.0×10^6
Ampicillin	235	670 ± 80	0.8–3.0	204 ± 20	58 ± 9	0.3×10^6
Amoxicillin	235	780 ± 100	0.8–3.0	N.D.*	10 ± 2	N.D.
Oxacillin	230	500 ± 100	2.0–5.0	A†	0.25 ± 0.02	N.D.
Carbenicillin	235	830 ± 110	1.0–3.5	104 ± 14	10 ± 2	0.1×10^6
Sulbenicillin	235	740 ± 80	1.0–3.5	N.D.	4.00 ± 0.08	N.D.
Azlocillin	240	1130 ± 140	0.8–3.0	350 ± 40	29 ± 6	0.08×10^6
Mezlocillin	235	1100 ± 140	0.8–3.0	283 ± 36	36 ± 8	0.13×10^6
Piperacillin	235	820 ± 70	0.8–3.0	161 ± 29	33 ± 5	0.2×10^6
Ticarcillin	230	1260 ± 130	0.8–3.0	82 ± 10	13 ± 2	0.16×10^6
Talampicillin	230	3900 ± 230	1.0–3.5	N.D.	0.26 ± 0.02	N.D.
Methicillin	230	900 ± 110	1.0–4.0	A	0.15 ± 0.03	N.D.
Cephalosporins						
Nitrocefin	482	15000 ± 300		115 ± 10	460 ± 30	4.2×10^6
PADAC	570			Biphasic kinetics		
Cephaloridine	255	9000 ± 700	0.07–1.0	91 ± 7	60 ± 4	0.66×10^6
Cefazolin	273	6600 ± 300	0.04–1.0	141 ± 22	165 ± 14	1.20×10^6
Cephalosporin C	262	5500 ± 400	0.08–1.2	138 ± 22	56 ± 6	0.40×10^6
Cefuroxime	273	7500 ± 500	0.4–2.5	35 ± 4	8 ± 0.9	0.20×10^6
Cefonicid	260	5400 ± 250	0.08–1.5	131 ± 20	105 ± 6	0.80×10^6
Cefamandole	260	5900 ± 150	0.08–1.5	201 ± 30	165 ± 12	0.80×10^6
Cefoperazone	260	5700 ± 100	0.4–2.5	123 ± 11	36 ± 4	0.30×10^6
Cefotaxime	264	6700 ± 300	0.4–1.0	25 ± 3 ‡	3 ± 0.2	N.D.
Ceftazole	260	7800 ± 150	0.04–1.0	80 ± 7	187 ± 11	2.30×10^6
Cefacetrile	260	7400 ± 250	0.08–1.2	174 ± 18	210 ± 21	1.20×10^6
Cephalthin	260	6300 ± 400	0.08–1.2	42 ± 4	79 ± 5	1.90×10^6

* N.D. = not determined.

† A = substrate-induced inactivation.

‡ As K_i using 150 μM -nitrocefin as reporter substrate.

or prolonged incubation of these complexes, no re-activation of the enzyme was observed. In separate experiments the spectra of β IP- β -lactamase adducts were recorded (Fig. 3). A new absorbance maximum at 325 nm was found that is characteristic of an enzyme-dihydrothiazine complex; no absorbance peak at 305 nm was observed.

On the basis of these observations it could be concluded that no turnover occurred. The rate of the inactivation was thus studied on the basis of the reaction scheme:

where EC^* is the acyl-enzyme and ECi the irreversibly inactivated

Fig. 3. Absorbance spectrum of 15 μ M- β -lactamase incubated with a stoichiometric amount of β IP read against a solution of 15 μ M-enzyme without inhibitor

enzyme after rearrangement of the β -iodopenicilloyl moiety. The apparent rate constant for the inactivation in the presence of 87 μ M-nitrocefin increased linearly with the β IP concentration up to 9.5 μ M, where an apparent first-order rate constant of 0.05 s was measured. After correction for the protection by the reporter substrate, a value of $9600 \pm 500 \text{ M}^{-1} \cdot \text{s}^{-1}$ was found for k_2/K (average of nine values obtained with β IP concentrations ranging from 2.4 to 10 μ M) (De Meester *et al.*, 1986).

Enzyme interaction with non- β -lactam compounds

EDTA and nitrilotriacetic acid at a concentration of 10 mM did not decrease the enzyme activity after a 10 min preincubation; thus the enzyme most probably did not contain cations as cofactor. Borate and 3-aminophenylboronic acid acted as competitive inhibitors and, with cefazolin or nitrocefin as substrates, K_i values of $1.0 \pm 0.2 \text{ mM}$ and $3.3 \pm 0.3 \text{ mM}$ respectively were obtained.

*p*CMB inactivated the enzyme; the phenomenon was dependent upon the time of preincubation and the inhibitor concentration. Moreover, the K_{obs} values for this inactivation process linearly increased with the *p*CMB concentration up to 0.3 mM, indicating a first-order phenomenon (results not shown). After complete inactivation by *p*CMB, addition of 5,5'-dithiobis(2-nitrobenzoic acid) to the enzyme failed to reveal any free thiol groups, even in the presence of 1% SDS. It was thus likely the *p*CMB reacted with the free cysteine residue of this enzyme. However, sequence data (see below) indicated that the enzyme was quite probably a class-A active-site-serine enzyme

	1				5					10					15					20	
<i>M. fortuitum</i>	A	P	I	D	D	Q	L	[	A	E	L	E	R	R	D	N	V	L	I	G	L
Consensus A	.	a	.	.	k	e	f	.	a	q	l	E	k	q	i	d	a	r	l	G	v

	21				25					30					35					40				
<i>M. fortuitum</i>	Y	A	A	N	[	L	Q	[	S	G	R	R	[	I	T	[	H	R	P	D	E	M	F	A
Consensus A	Y	a	l	d	[	t	q	[	t	g	.	t	[	v	i	a	y	r	a	d	e	r	F	p
Consensus D																								
Consensus C																								

	41		*		45																			
<i>M. fortuitum</i>	M	X	S	T	F	K	.	G	Y	V														
Consensus A	m	a	S	t	f	K	a	l	a															
Consensus D	P	a	S	T	F	K	I	P	.															
Consensus C	l	G	S	v	S	K	T	F	t															

Fig. 4. N-Terminal sequence of the β -lactamase of *M. fortuitum*

The sequence is compared with the consensus sequences of class-A (numbering of Ambler, 1980), class-C (numbering of Galleni *et al.*, 1988) and class-D (numbering of Dale *et al.*, 1985) β -lactamases. In the consensus sequences the capital letters represent residues which are conserved in *all* known members of the class, and the lower-case letters represent residues which are conserved in a majority of known sequences. Dots represent positions where no common residue is present in a majority of the known sequences. For class-A enzymes, a second lower-case letter is given where the residue is also present in some of the proteins. Boxes delimited by a continuous line indicate strict identities with a completely conserved or a 'majority' residue; those delimited by a broken line indicate identities with a 'minority' residue. The solidus (/) in the consensus A sequence indicates a deletion present in most enzymes and which is also found in the *M. fortuitum* β -lactamase.

and, in consequence, binding of the mercuribenzoate moiety resulted in a modification of the enzyme conformation or in steric hindrance if one cysteine residue was near the active site.

N-Terminal sequence

The N-terminal sequence is given in Fig. 4 and compared with the consensus sequences of β -lactamases of classes A, C and D. A high degree of similarity was found with class-A enzymes.

DISCUSSION

The presence of a β -lactamase activity was first recognized in cultures of *Mycobacterium tuberculosis* in the years 1941–1945 (Abraham *et al.*, 1941; Woodruff & Foster, 1945). The production of this enzyme by most mycobacterial species was later reported, with the exception only of the *M. avium* complex (Kasic, 1979). Mycobacteria are usually resistant to the commonly used β -lactam antibiotics, but the role played by the β -lactamase in this phenomenon has not been clearly established (Kasic, 1979; Yabu *et al.*, 1985).

Although the enzymes produced by *M. butyricum* A.T.C.C. 19979 (Choubey & Gopinathan, 1986) and *M. smegmatis* (Kaneda & Yabu, 1983) were purified and some kinetic data were obtained using crude preparations (Kasic & Peacham, 1968), detailed biochemical studies of the mycobacterial enzymes are not available, and it would be useful to elucidate their contribution to resistance and to expand our knowledge of their molecular properties and substrate profiles.

Some of the data reported here are comparable with those obtained by earlier workers (e.g. M_r of about 29000, like the proteins produced by *M. butyricum* A.T.C.C. 19979 and *M. smegmatis*), but quite different from a recent report on the *M. tuberculosis* enzyme, which has been described as a dimeric β -lactamase of M_r 70000 (Wong *et al.*, 1989). The pI is within the 4.6–5.2 range reported for the β -lactamases of these species (Wallace *et al.*, 1985), but differs from those of the *M. smegmatis* A.T.C.C. 607 and *M. phei* N.C.T.C. 527 enzymes (Matthew & Harris, 1976).

The mycobacterial β -lactamases have usually been reported as being cell-bound enzymes (Kasic, 1979), but the D316 strain and the wild-type A.T.C.C. 19542 excreted the enzyme into the medium after 3–6 days of growth (Fattorini *et al.*, 1989).

The rate of the inactivation by β IP and the N-terminal sequence established the *M. fortuitum* β -lactamase as a member of class A. In addition to the SXXK sequence containing the active-site serine residue, the few residues which are totally conserved in the known class-A sequences, namely E37, G45 and F66 [Ambler's (1980) numbering] were found in positions 11, 19 and 39 respectively. It can thus be safely assumed that the amino acid at position 43 is the active-site serine residue. Since the enzyme was inactivated by pCMB, one might also suppose that the unidentified residue in position 42 might be cysteine, as observed in the class-A β -lactamases produced by *Klebsiella pneumoniae* (Joris *et al.*, 1987) and *Citrobacter diversus* (Amicosante *et al.*, 1988).

For a class-A β -lactamase the substrate profile is quite surprising. On the basis of both the k_{cat} and k_{cat}/K_m values, nine out of the eleven best substrates are cephalosporins. Although nitrocefin, the best substrate among those studied here, is often a good substrate for class-A enzymes, several other cephalosporins behaved as better substrates even than benzylpenicillin and ampicillin, the classical best substrates of class-A enzymes. Those data supply a new and striking example of the wide diversity of the catalytic properties of class-A enzymes (Matagne *et al.*, 1990). Another interesting point was the relatively rapid hydrolysis observed with all the ureidopenicillins (piperacillin, mezlocillin and azlocillin) and some so-called ' β -lactamase-

stable cephalosporins' such as cefuroxime, cefoperazone and cefotaxime. With oxacillin and methicillin, substrate-induced inactivation was observed, a rather common phenomenon with class-A enzymes. The same behaviour was observed for the chromogenic cephalosporin PADAC, a situation reminiscent of that reported with the *C. diversus* ULA-27 β -lactamase (Amicosante *et al.*, 1988).

In conclusion, we believe that the catalytic properties of the *M. fortuitum* β -lactamase exhibit some very surprising features for a class-A β -lactamase and that those properties certainly deserve further investigation.

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