Vol. 43, No. 3, October 1997 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
Pages 557-562

CHARACTERIZATION OF A BETA-LACTAMASE FROM *Mycobacterium smegmatis* SN_2*

[1]Dhiman Basu, [1]D.V. Narayankumar[§], [2]Josef Van Beeumen and [1]Joyoti Basu[**]

[1]Department of Chemistry, Bose Institute, 93/1 APC Road, Calcutta 700 009, India and [2]Department of Biochemistry, Physiology and Microbiology, Laboratory of Protein Biochemistry and Protein Engineering, Rijksuniversiteit- Gent, Ledeganckstraat 35, 9000 Gent, Belgium

§Present address : Centre for Cellular & Molecular Biology, Hyderabad, India

Received June 24, 1997
Received after revision July 29, 1997

Summary : Beta-lactamases have been reported to be largely responsible for beta-lactam resistance in *Mycobacteria*. We report the characterization of a cell-associated beta-lactamase from *Mycobacterium smegmatis*. The enzyme hydrolyzed the "beta-lactamase-stable" oximinocephalosporins. Nitrocefin was the best substrate. 6-Beta-iodopenicillanate, clavulanate and sulbactam were effective inhibitors, whereas the K_i value for aztreonam was high. From its substrate and inhibitor profile, the enzyme appeared to be a cephalosporinase of group 2e.

Keywords : Beta-lactamase, *Mycobacterium smegmatis*, cephalosporinase

Introduction

Drug resistance is a major problem in the therapy of mycobacterial diseases (1,2), making it necessary to develop newer chemotherapeutic agents and reevaluate the potential of existing ones. Resistance to beta-lactam antibiotics has been attributed largely to the intrinsic low permeability of the cell wall(3) and the production of beta-lactamase (4). The availability of beta-lactamase inhibitors has generated renewed interest in the potential of beta-lactams in the treatment of mycobacterial diseases. Combinations of beta-lactam antibiotics with beta-lactamase inhibitors have been shown to be effective against *M. tuberculosis* both *in vivo* (5) and *in vitro* (6). In order to develop effective beta-lactamase inhibitors and to identify useful beta-lactam antibiotics, detailed knowledge of the beta-lactamases of *Mycobacteria* is essential. An extracellular beta-lactamase of *M. fortuitum* has been

*A portion of this work appeared as an abstract in the proceedings of the 15th International Congress of Biochemistry held in Jerusalem in 1991.

**Author to whom correspondence should be addressed. Fax: 91 33 3506790

1039-9712/97/030557-06$05.00/0
Copyright © 1997 by Academic Press Australia.
All rights of reproduction in any form reserved.

characterized (7) and the gene for a beta-lactamase of M. tuberculosis has been sequenced (8). In this communication, we present the characterization of a cell-associated beta-lactamase of M. smegmatis.

Materials and Methods

Antibiotics and chemicals. Benzylpenicillin, ampicillin, cephaloridine, cefazolin, cephalothin, cefamandole, cefotaxime, ceftazidime, cefuroxime, cefoperazone, cefacetrile, carbenicillin, cefsulodin, cefaclor, cephalosporin C and moxalactam were from Sigma. The following were gifts: ticarcillin, oxacillin, cloxacillin and clavulanic acid (Smith Kline Beecham Pharmaceuticals); aztreonam (Bristol-Myers Squibb); nitrocefin (Glaxo); 6-beta-iodopenicillanate and sulbactam (Pfizer Central Research). Imipenem and flomoxef were products of Merck Sharp and Dohme and Shionogi Pharmaceutical, Japan, respectively. Q-Sepharose Fast Flow, Polybuffer 74 and Mono P HR-5/20 were from Pharmacia, Sweden. Molecular weight markers, phenylmethyl sulfonyl fluoride (PMSF) and DNase were from Sigma. Beef extract and peptone were products of Difco.

Purification of the beta-lactamase. M. smegmatis SN_2 was grown to the early stationary phase in nutrient broth [1% beef extract, 1% peptone and 0.5% NaCl] at 37°C. Cells were harvested, suspended in 50 mM Tris-HCl, pH 7.4 supplemented with 10 mM $MgCl_2$, 0.25 mM PMSF, 2 mM EDTA and 1 µg/ml DNase and sonicated on ice for 15 min. After removal of cell debris, cell wall and cell membrane by differential centrifugation at 4°C, the cell-free lysate was concentrated by ultrafiltration using a M_r 10,000 cut-off membrane and loaded onto a 500 ml Sephadex G-75 column equilibrated in 50 mM sodium phosphate, pH 7 containing 5% (v/v) ethanediol, 5% (v/v) glycerol and 1 mM EDTA. Active fractions were pooled, the pH was adjusted to 6 with HCl and loaded onto a 14 ml Q-Sepharose Fast Flow column equilibrated in 20 mM Bis Tris, 5% glycerol, 5% ethanediol, 0.05 mM dithioerythritol (DTE), 1 mM EDTA, pH 6 [Buffer A]. After washing the column with buffer A, the beta-lactamase was eluted with a gradient of 0-0.2 M NaCl in buffer A. Active fractions were pooled, dialyzed against 20 mM Bis Tris, pH 6.3 and loaded onto a Mono P HR-5/20 chromatofocusing column coupled to a FPLC system. The enzyme was eluted with a pH gradient of 6.3 to 4 using Polybuffer 74. The active fractions were dialyzed against 50 mM sodium phosphate buffer, pH 7, containing 5% (v/v) each of ethanediol and glycerol, 1 mM EDTA and 0.05 mM DTE. Protein was estimated according to the method of Lowry et al (9). The purity of the enzyme was checked and its molecular mass was determined by SDS-PAGE (10).

Determination of beta-lactamase activity. Beta-lactamase activity was routinely monitored at 30°C using the chromogenic cephalosporin, nitrocefin (11) at a concentration of 150 µM in 50 mM sodium phosphate buffer, pH 7. Hydrolysis was monitored spectrophotometrically at 482 nm ($\Delta\varepsilon$ =15,000). One unit of beta-lactamase activity represents the amount of enzyme hydrolysing 1 µmole of nitrocefin per min.

N-terminal sequencing. 10 nmol protein was run on a SDS polyacrylamide gel and electrotransferred onto an Immobilon P (Millipore) membrane followed by N-terminal amino acid sequencing on a 477A pulse-liquid sequenator (Applied Biosystems) with on-line analysis of the amino acid phenylthiohydantoin derivatives by reverse phase HPLC.

Detection of beta-lactamase activity by SDS-PAGE and isoelectric focusing (IEF). Purified beta-lactamase was subjected to SDS-PAGE. After electrophoresis, the gel was incubated for 4 h at 30°C in phosphate buffer containing 1% (v/v) Triton X-100 to obtain renaturation of the beta-lactamase. The gel was then overlaid with 1% agarose containing

nitrocefin at a concentration of 50 μg/ml and kept at room temperature in the dark for 15 min. Beta-lactamase activity was detected by the development of a pink color in a light yellow background. For IEF, purified protein was applied on the surface of a 5% polyacrylamide gel containing ampholyte (pH 3.5 to 9.5) and focusing was carried out at 10°C and 25 W for 90 min. Beta-lactamase activity was detected by overlay with nitrocefin.

Kinetic studies. Enzyme activity was monitored spectrophotometrically at wavelengths that gave a maximum in the difference spectrum of the hydrolyzed antibiotics against the unhydrolyzed one. The kinetic parameters k_{cat} and k_{cat}/K_m of the substrates were obtained either by continuously monitoring the complete time courses of hydrolysis in 100 mM sodium phosphate buffer, pH 7 at 30°C and fitting the data to the integrated Michaelis Menten equation (12), or by using the initial rates of hydrolysis at different substrate concentrations. The K_i values were determined by monitoring the initial rates of hydrolysis of the reporter substrate nitrocefin in the presence of various concentrations of beta-lactams. Data were analyzed using the software ENZFITTER (Elsevier Biosoft). Enzymatic activity was also monitored by observing nitrocefin hydrolysis in the presence and in the absence of the non-beta-lactam inhibitors, EDTA (1 mM), PMSF (1 mM), borate (0.5 to 1 mM) and p-hydroxymercuribenzoate (pHMB) (500 μM). All results represent the mean of three separate determinations (S.D. $\leq$ 10%).

Results

Purification of the beta-lactamase. Following chromatofocusing, the enzyme was purified 239-fold compared to the cell lysate with a specific activity of 8125 U/mg protein. Following SDS-PAGE, the purified enzyme appeared as a single band of M_r 25,000 (Fig. 1). After IEF, the purified enzyme focused at pH 4. The beta-lactamase band was confirmed by development of a pink color after overlay with nitrocefin.

The high degree of similarity of the N-terminal sequence with a stretch of amino acids present near the C-terminal ends of seven beta-lactamases from Gram-negative bacteria was intriguing (Fig. 2). It raised the possibility of horizontal gene transfer between these distantly related species and mycobacteria.

Kinetic properties. The results of the kinetic analyses of interaction with beta-lactams, are summarized in Table 1. Nitrocefin was the best substrate among the cephalosporins tested, with k_{cat}=352 s^{-1} and k_{cat}/K_m= 2.27$\mu M^{-1}s^{-1}$. Cephaloridine, cefacetrile, cefazolin, cephalothin, cefamandole and cephalosporin C were good substrates. The oximinocephalosporins cefotaxime, cefuroxime and cefoperazone were also hydrolyzed. Among the penicillins, benzylpenicillin was hydrolyzed with the greatest efficiency. Ampicillin and amoxycillin were not hydrolyzed at detectable levels. Using nitrocefin as reporter substrate, the carbapenem, imipenem, and the monobactam, aztreonam, showed high K_i values of 460 and

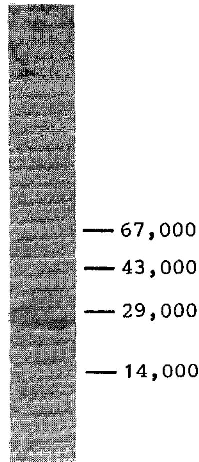

Fig. 1. SDS-PAGE of the purified M. smegmatis beta-lactamase

Mycobacterium smegmatis SN_2	**1**	**PEKNLGIVMLANKSYPNPA**	**19**
Escherichia coli K12 ampC	350	PEKELGIVMLANKNYPNPA	367
Enterobacter clocae P99 ampC	330	PEKQIGIVMLANTSYPNPA	348
Enterobacter clocae Q908R ampC	330	PEKQIGIVMLANTSYPNPA	348
Enterobacter clocae MHN 1 ampC	330	PEKQIGIVMLANKSYPNPA	348
Citrobacter freundii GN 346 ampC	330	PEKNLGIVMLANKSYPNPR	348
Citrobacter freundii OS60 ampC	330	PEKNLGIVMLANKSVPNPA	348
Serratia marcescens ampC	322	PAKNIAVEMLANKWFPN	338
Pseudomonas aeruginosa ampC	357	PGRDLGLVILANRNYPN	373
Yersinia enterocolitica ampC	357	PEEKVGIVMLANKNYPNP	374
Klebsiella pneumoniae	350	PARGIAIVMLANRNYP	365

Fig. 2. Comparison of the N-terminal sequence of M. smegmatis SN_2 enzyme with the C-terminal sequences of other beta-lactamases.

900 uM, respectively. The oxacephems moxalactam and flomoxef were not hydrolyzed and did not inhibit nitrocefin hydrolysis upto a concentration of 1 mM.

Inhibition by 6-beta-iodopenicillanate (6-BIP), clavulanate and sulbactam; and interaction with non-beta-lactam compounds. Inhibition by 6-BIP, sulbactam and clavulanate was time-dependent (data not shown). The K_i values were 0.5, 0.75 and 2.5 uM for 6-BIP, clavulanate and sulbactam respectively. 6-BIP was the most efficient inactivator with a second-order rate constant of inactivation (k_{+2}/K) of 9700 $M^{-1}s^{-1}$. The enzyme was not inhibited by the metal ion chelator EDTA (1 mM). Borate inhibited the

Table 1.
Kinetic properties of the M. smegmatis beta-lactamase

Antibiotic	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Benzylpenicillin	29	28	0.95
Carbenicillin	54	7.48	0.14
Ticarcillin	17.5	3.4	0.19
Oxacillin*	6	N.D.	N.D.
Cloxacillin*	20	N.D.	N.D.
Nitrocefin	158	352	2.27
Cefazolin	43	95	2.21
Cephalosporin C	20	21.6	1.01
Cephalothin	16.7	11	0.66
Cefacetrile	22.5	45.5	2.01
Cefamandole	79	33	0.42
Cefuroxime	35	3.5	0.10
Cefotaxime*	110	2.2	0.02
Cefoperazone	27.3	14	0.51
Cefsulodin	144.6	39	0.27
Cefaclor	53.6	21.8	0.41
Cephaloridine	51	39	0.72
Imipenem*	460	N.D.	N.D.
Aztreonam*	900	N.D.	N.D.

* K_m values for these antibiotics were determined as K_i using nitrocefin as reporter substrate; N.D., not determined.

beta-lactamase with a K_i of 1 mM. pHMB at 500 uM failed to inhibit the enzyme, suggesting the absence of any catalytically important thiol residue.

Discussion

The mycobacterial beta-lactamases have usually been reported to be cell-bound [4]. The enzyme reported here was also cell-associated. Since the M. smegmatis enzyme was not inhibited by EDTA, it is obviously not a metallo-beta-lactamase. Considering the k_{cat} values, the enzyme hydrolyzed benzylpenicillin at a rate approximately 30% less than that observed for cephaloridine, and therefore appeared to behave as a cephalosporinase according to the classification scheme of Bush et al. (13). Among the

penicillins, only benzylpenicillin appeared to be a good substrate, a characteristic which has been reported to be associated with occasional cephalosporinases (13). Cephalosporinases may be classified either in group 1 or group 2e, depending on the inhibitor profile (13). Group 1 enzymes are inhibited by aztreonam but not by clavulanate, whereas group 2e enzymes are inhibited by clavulanate but not aztreonam. In this case, aztreonam behaved as a poor inhibitor with a high K_i value, whereas clavulanate was a good inhibitor. The enzyme therefore behaved like a group 2e cephalosporinase.

This enzyme is obviously distinct from the M. fortuitum beta-lactamase, an enzyme with a pI of 5, capable of hydrolyzing ampicillin and inactivated by thiol-group modification (7). Whether it is also distinct from the enzyme previously reported from M. smegmatis ATCC607 which reportedly hydrolyzes ampicillin and is of estimated M_r 29,000 (14) remains unclear. We suggest this enzyme represents one member of a novel class of mycobacterial beta-lactamases.

Acknowledgements

This work was supported in part by the CEE contract CI1*-CT92-001. D.B. was supported by a fellowship from the Council of Scientific and Industrial Research, Government of India.

REFERENCES

1. Bloom, B.R. and Murray, C.L.J. (1992) Science **257**, 525-526.
2. Cohn, D.L., Bustreo, F. and Raviglione, M.C. (1997) Clin. Infect. Dis. **24** (Supp 1), S121-130.
3. Jarlier, V., Gutmann, L. and Nikaido, H. (1991) Antimicrob. Agents Chemother. **35**, 1937-1939.
4. Kasik, J. (1979) Beta-lactamases (Hamilton-Miller, J. and Smith, J. eds.), pp. 339-350, Academic Press, London.
5. Nadler, J.P., Berger, J., Nord, J.A., Cofsky, R. and Saxena, M. (1991) Chest **99**, 1025-1026.
6. Wong, C.S., Palmer, G.S. and Cynamon, M.H. (1988) J. Antimicrob. Chemother. **22**, 863-866.
7. Amicosante, G., Franceschini, N., Segatore, R., Oratore, A., Fattorini, L., Orefici, G., Van Beeumen, J. and Frere, J.M. (1990) Biochem. J. **271**, 729-734.
8. Hackbarth, C.J., Unsal, I. and Chambers, H.F. (1997) Antimicrob. Agents Chemother. **41**, 1182-1185.
9. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193**, 265-275.
10. Laemmli, U.K. (1970) Nature **227**, 680-685.
11. O'Callaghan, C.H., Morris, A., Kirby, S.M. and Shingler, A.H. (1972) Antimicrob. Agents Chemother. **1**, 283-288.
12. De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C. and Frere, J.M. (1987) Biochem. Pharmacol. **36**, 2393-2403.
13. Bush, K., Jacoby, G.A. and Medeiros, A.A. (1995) Antimicrob. Agents Chemother. **39**, 1211-1233.
14. Kaneda, S. and Yabu, K. (1983) Microbiol. Immunol. **27**, 191-193.