ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2005, p. 4760–4762 Vol. 49, No. 11
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.11.4760–4762.2005

Kinetics Study of KPC-3, a Plasmid-Encoded Class A Carbapenem-Hydrolyzing β-Lactamase

Jimena Alba,[1] Yoshikazu Ishii,[1]* Kenneth Thomson,[2] Ellen Smith Moland,[2] and Keizo Yamaguchi[1]

Department of Microbiology and Infectious Diseases, Toho University School of Medicine, 5–21–16 Omori-nishi, Ota-ku, Tokyo 1438540, Japan,[1] and Center for Research in Anti-Infectives and Biotechnology, Department of Medical Microbiology and Immunology, Creighton University School of Medicine, Omaha, Nebraska 68178[2]

Received 8 February 2005/Returned for modification 12 March 2005/Accepted 3 August 2005

The kinetic activity of KPC-3, a plasmid-encoded class A carbapenemase, was studied. It hydrolyzed penicillins, cephalosporins, carbapenems, and even sulbactam. The best substrate was cephalothin ($k_{cat/K}m$ = 3.48 μM^{-1} s^{-1}). The efficiency of the enzyme was similar for imipenem and meropenem (k_{cat}/K_m, 1.4 and 1.94 μM^{-1} s^{-1}, respectively).

Carbapenem use has increased during the past 2 decades. This is due, in part, to their broad-spectrum of antibacterial activity and their resistance to hydrolysis by extended spectrum β-lactamases (1, 10, 14, 17). However, the appearance of carbapenemases and other carbapenem resistance mechanisms is threatening the effectiveness of this antibiotic class. In gram-negative bacteria, carbapenem resistance has been attributed to three main mechanisms: the combination of high-level production of an AmpC β-lactamase and the loss of outer membrane proteins (5, 13), changes in the affinity of penicillin binding proteins for carbapenems (7, 8), and the production of a carbapenem-hydrolyzing β-lactamase (11, 16). Although clinically significant, carbapenem-hydrolyzing β-lactamases remain rare, but their frequency has been increasing. The β-lactamases involved belong to Ambler molecular classes A, B, and D (16, 19). A small number of class A enzymes have been found to be able to hydrolyze carbapenems (6). They belong to group 2f, as defined by Bush and colleagues (4, 23). They hydrolyze ampicillin and early cephalosporins more efficiently than carbapenems and can be inhibited by clavulanic acid. Class A carbapenemases can be chromosomally encoded (NMC-A, Sme-1 to -3, IMI-1) (9, 15, 21, 22, 24, 27) or plasmid encoded (KPC-1, KPC-2, GES-2) (18, 20, 25, 26, 28, 29, 30).

KPC-type β-lactamases have become one of the most frequently encountered carbapenem-hydrolyzing enzymes on the East Coast of the United States (2). KPC-3 is the most recently reported enzyme in that group (T. Hong, E. S. Moland, B. Abdalhamid, et al., Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1–665, 2003; K. Young, P. Tierno, Jr., L. Tysall, et al., Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2–50, 2003). KPC-3 is closely related to its predecessors, differing by only 1 amino acid from KPC-2 (H272Y) and by 2 amino acids with KPC-1 (S174G, H272Y). It has been recovered from isolates of *Klebsiella pneumoniae* (Young et al., 43rd ICAAC), *Escherichia coli* (Hong et al., 43rd ICAAC), and *Enterobacter cloacae* (3).

* Corresponding author. Mailing address: Department of Microbiology, Toho University School of Medicine, 5-21-16 Omori-nishi, Ota-ku, Tokyo 1438540, Japan. Phone: 81-3-3762-4151, ext. 2396. Fax: 81-3-5493-5415. E-mail: yoishii@med.toho-u.ac.jp.

In this study we purified KPC-3 and subjected the enzyme to kinetic characterization.

An isolate of *E. coli* (isolate 233) showing reduced susceptibility to carbapenems was referred to Creighton University from Hackensack University Medical Center. It was subsequently found to produce KPC-3 (report in press).

The isolate was grown in 4 liters of Luria-Bertani broth at 37°C (250 rpm) for 8 h, harvested, and suspended in 30 mM Tris-HCl buffer (pH 8.0) containing 30% sucrose. The periplasmic content was extracted as described previously (12). Purification was achieved using a HiPrep 16/10 SP XL column (Amersham Biosciences AB, Uppsala, Sweden) equilibrated in 10 mM acetate buffer (pH 5.0). Fractions displaying β-lactamase activity, observed as the initial rate of hydrolysis of nitrocefin (100 μM) ($\Delta\varepsilon_{482}$ = +10,000 $M^{-1}cm^{-1}$) (Oxoid Ltd., Hampshire, United Kingdom) were obtained after elution with a linear gradient of NaCl (0 to 400 mM). After concentration using Amicon ultrafiltration membranes (Millipore Corporation, Bedford, MA) and overnight dialysis in 10 mM morpholineethanesulfonic acid (MES) buffer (pH 5.5) at 4°C, the sample was reloaded onto a Mono S HR 5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden), equilibrated in MES buffer (pH 5.5), and eluted with a linear gradient of NaCl (0 to 300 mM). The entire purification process was done with an AKTA purifier (Amersham Pharmacia Biotech). The purity of the β-lactamase preparation was controlled using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. The purity achieved was above 90%. The purified enzyme was then dialyzed overnight at 4°C in phosphate buffer (pH 7.0) and concentrated. Four liters of culture yielded a total of 0.27 mg of pure enzyme. After determination of the protein concentration using a Bio-Rad (Richmond, Calif.) protein assay, 20 μg/ml bovine serum albumin was added. The N-terminal sequence was determined using a Procise 492clC-1 protein sequencer (Applied Biosystems, Foster City, Calif.), and the kinetic parameters were determined with the pure enzyme.

All kinetics studies were done by measuring hydrolysis rates with a Shimadzu (Kyoto, Japan) UV-2550 spectrophotometer connected to a personal computer. To determine the kinetic parameters, 6 to 10 different concentrations of each β-lactam were used. Each reported parameter is an average of three

TABLE 1. Comparison of kinetic parameters for KPC-1, KPC-2, and KPC-3[a]

Substrate	K_m or K_i (μM)			k_{cat} (s^{-1})			k_{cat}/K_m (μM^{-1} s^{-1})		
	KPC-1	KPC-2	KPC-3	KPC-1	KPC-2	KPC-3	KPC-1	KPC-2	KPC-3
Ampicillin	130	239	65 (±5)	110	210	77 (±4)	0.9	0.9	1.2
Nitrocefin	NA[b]	NA	42 (±5)	NA	NA	107 (±5)	NA	NA	2.6
Cephaloridine	560	500	261 (±1)	340	530	364 (±4)	0.6	1.1	1.4
Cephalotin	53	82	44 (±3)	75	69	153 (±5)	1.4	0.8	3.5
Cefotaxime	160	220	95 (±8)	14	22	52 (±4)	0.1	0.1	0.5
Ceftazidime	94	NA	88 (±1)	0.1	0.1	3 (±0.06)	0.001	NA	0.03
Cefoxitin[c]	120	180	970 (±65)	0.3	0.3	0.05[d] (±0.001)	0.002	0.002	0.5[d]
Moxalactam[c]	NA	NA	14 (±1.6)	NA	NA	0.008 (±0.0002)	NA	NA	0.05
Meropenem	12	15	4 (±1)	3	4	6 (±0.09)	0.3	0.3	1.4
Imipenem	81	51	23 (±6)	12	15	45 (±0.04)	0.2	0.3	1.9
Sulbactam	NA	NA	30 (±0.9)	NA	NA	4 (±0.1)	NA	NA	0.1

[a] Kinetic values used for KPC-1 and KPC-2 were reported by H. Yigit et al. (29, 30).
[b] NA, not available.
[c] K_i values were obtained using 100 μM nitrocefin as a reporter substrate.
[d] k_{cat} values were obtained by hydrolyzing a low concentration of substrate with a high concentration of enzyme.

separate measurements. All kinetic parameters were acquired by measuring the initial hydrolysis rate of the β-lactam under study at a constant temperature of 30°C, using 50 mM phosphate buffer (pH 7.0). Analysis of the data was done using the Hanes-Woolf linearization of the Michaelis-Menten equation. For all poor substrates, the competitive inhibition constant (K_i) was determined by competition experiments between the tested β-lactam and 100 μM nitrocefin. Hydrolysis of the substrate at a concentration 10 times the K_m (K_i) value or higher yielded the catalytic constants (k_{cat}) for poor substrates. The k_{cat} value for cefoxitin was obtained using 100 μM substrate with 1.1×10^{-7} M enzyme.

The N-terminal sequence of KPC-3 was determined as LT NLVAEPFAKLE. Table 1 shows a comparison between previously reported k_{cat}, K_m (K_i), and k_{cat}/K_m values for KPC-1 and KPC-2 (29, 30) and the parameters obtained for KPC-3 in this study. KPC-3 hydrolyzed penicillins, cephalosporins, and carbapenems. Among the substrates tested, the highest hydrolytic efficiency was seen with nitrocefin and cephalothin (k_{cat}/K_m, 2.55 μM^{-1} s^{-1} and 3.48 μM^{-1} s^{-1}, respectively). The k_{cat} and K_m values for cephaloridine were very high (k_{cat}, 364 s^{-1}; K_m, 261 μM). Imipenem and meropenem were hydrolyzed by KPC-3 with good efficiencies (k_{cat}/K_m, 1.94 μM^{-1} s^{-1} and 1.40 μM^{-1} s^{-1}, respectively), which were similar overall to those exhibited for ampicillin and cephaloridine. The substrate profiles for the three enzymes were similar overall, although the catalytic efficiency of KPC-3 appeared to be somewhat higher with some substrates, including oxyiminocephalosporins and carbapenems. One of the notable differences is the behavior of this enzyme with ceftazidime. The catalytic activity (k_{cat}) with ceftazidime (3.0 s^{-1} ± 0.01) was approximately 30 times higher than those of KPC-1 and KPC-2 (0.1 s^{-1} for both). Due to this, KPC-3 was 30 times more efficient than KPC-1 toward this substrate. Moreover, KPC-3 showed a lower affinity for cefoxitin than the other enzymes. The present findings, therefore, suggest that the amino acid substitution that differentiates KPC-3 from KPC-2 (H272Y) could have a functional significance. A molecular modeling analysis based on the structure of the TOHO-1 enzyme (25), now called CTX-M-44, suggested that the H272Y mutation (which would be at a position similar to that of R274 in TOHO-1) could influence the positions of R209, which interacts with the substrate carboxylate. Further investigation will be necessary to clarify these matters.

We thank Tao Hong of Hackensack University Medical Center for providing us with the resistant *E. coli* strain.

J.A. was supported by a grant from the Japan Health Sciences Foundation. This work was supported by a grant from the Ministry of Health, Labor, and Welfare of Japan (H15-Shinko-09) and by Toho University project research grants 15-4 and 16-6.

REFERENCES

1. **Bradford, P. A.** 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. **14:**933–951, table of contents.
2. **Bradford, P. A., S. Bratu, C. Urban, M. Visalli, N. Mariano, D. Landman, J. J. Rahal, S. Brooks, S. Cebular, and J. Quale.** 2004. Emergence of carbapenem-resistant *Klebsiella* species possessing the class A carbapenem-hydrolyzing KPC-2 and inhibitor-resistant TEM-30 β-lactamases in New York City. Clin. Infect. Dis. **39:**55–60.
3. **Bratu, S., D. Landman, M. Alam, E. Tolentino, and J. Quale.** 2005. Detection of KPC carbapenem-hydrolyzing enzymes in *Enterobacter* spp. from Brooklyn, New York. Antimicrob. Agents Chemother. **49:**776–778.
4. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
5. **Cao, V. T., G. Arlet, B. M. Ericsson, A. Tammelin, P. Courvalin, and T. Lambert.** 2000. Emergence of imipenem resistance in *Klebsiella pneumoniae* owing to combination of plasmid-mediated CMY-4 and permeability alteration. J. Antimicrob. Chemother. **46:**895–900.
6. **Drusano, G. L., H. Lode, and J. R. Edwards.** 2000. Meropenem: clinical response in relation to in vitro susceptibility. Clin. Microbiol. Infect. **6:**185–194.
7. **Edwards, R., and D. Greenwood.** 1996. Mechanisms responsible for reduced susceptibility to imipenem in *Bacteroides fragilis*. J. Antimicrob. Chemother. **38:**941–951.
8. **Fernandez-Cuenca, F., L. Martinez-Martinez, M. C. Conejo, J. A. Ayala, E. J. Perea, and A. Pascual.** 2003. Relationship between β-lactamase production, outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates of *Acinetobacter baumannii*. J. Antimicrob. Chemother. **51:**565–574.
9. **Henriques, I., A. Moura, A. Alves, M. J. Saavedra, and A. Correia.** 2004. Molecular characterization of a carbapenem-hydrolyzing class A β-lactamase, SFC-1, from *Serratia fonticola* UTAD54. Antimicrob. Agents Chemother. **48:**2321–2324.
10. **Jones, R. N.** 1998. Important and emerging β-lactamase-mediated resistances in hospital-based pathogens: the Amp C enzymes. Diagn. Microbiol. Infect. Dis. **31:**461–466.
11. **Livermore, D. M., and N. Woodford.** 2000. Carbapenemases: a problem in waiting? Curr. Opin. Microbiol. **3:**489–495.
12. **Ma, L., J. Alba, F.-Y. Chang, M. Ishiguro, K. Yamaguchi, L. K. Siu, and Y. Ishii.** 2005. Novel SHV-derived extended-spectrum β-lactamase, SHV-57, that confers resistance to ceftazidime but not cefazolin. Antimicrob. Agents Chemother. **49:**600–605.
13. **Martinez-Martinez, L., A. Pascual, S. Hernandez-Alles, D. Alvarez-Diaz,**

A. I. Suarez, J. Tran, V. J. Benedi, and G. A. Jacoby. 1999. Roles of β-lactamases and porins in activities of carbapenems and cephalosporins against *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **43:**1669–1673.
14. **Mushtaq, S., Y. Ge, and D. M. Livermore.** 2004. Comparative activities of doripenem versus isolates, mutants, and transconjugants of *Enterobacteriaceae* and *Acinetobacter* spp. with characterized β-lactamases. Antimicrob. Agents Chemother. **48:**1313–1319.
15. **Nordmann, P., S. Mariotte, T. Naas, R. Labia, and M. H. Nicolas.** 1993. Biochemical properties of a carbapenem-hydrolyzing β-lactamase from *Enterobacter cloacae* and cloning of the gene into *Escherichia coli*. Antimicrob. Agents Chemother. **37:**939–946.
16. **Nordmann, P., and L. Poirel.** 2002. Emerging carbapenemases in Gram-negative aerobes. Clin. Microbiol. Infect. **8:**321–331.
17. **Philippon, A., G. Arlet, and G. A. Jacoby.** 2002. Plasmid-determined AmpC-type β-lactamases. Antimicrob. Agents Chemother. **46:**1–11.
18. **Poirel, L., I. Le Thomas, T. Naas, A. Karim, and P. Nordmann.** 2000. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β-lactamase, and the class 1 integron In*52* from *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **44:**622–632.
19. **Poirel, L., and P. Nordmann.** 2002. Acquired carbapenem-hydrolyzing β-lactamases and their genetic support. Curr. Pharm. Biotechnol. **3:**117–127.
20. **Poirel, L., G. F. Weldhagen, T. Naas, C. De Champs, M. G. Dove, and P. Nordmann.** 2001. GES-2, a class A β-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. Antimicrob. Agents Chemother. **45:**2598–2603.
21. **Pottumarthy, S., E. S. Moland, S. Juretschko, S. R. Swanzy, K. S. Thomson, and T. R. Fritsche.** 2003. NmcA carbapenem-hydrolyzing enzyme in *Enterobacter cloacae* in North America. Emerg. Infect. Dis. **9:**999–1002.
22. **Queenan, A. M., C. Torres-Viera, H. S. Gold, Y. Carmeli, G. M. Eliopoulos, R. C. Moellering, Jr., J. P. Quinn, J. Hindler, A. A. Medeiros, and K. Bush.** 2000. SME-type carbapenem-hydrolyzing class A β-lactamases from geographically diverse *Serratia marcescens* strains. Antimicrob. Agents Chemother. **44:**3035–3039.
23. **Rasmussen, B. A., and K. Bush.** 1997. Carbapenem-hydrolyzing β-lactamases. Antimicrob. Agents Chemother. **41:**223–232.
24. **Rasmussen, B. A., K. Bush, D. Keeney, Y. Yang, R. Hare, C. O'Gara, and A. A. Medeiros.** 1996. Characterization of IMI-1 β-lactamase, a class A carbapenem-hydrolyzing enzyme from *Enterobacter cloacae*. Antimicrob. Agents Chemother. **40:**2080–2086.
25. **Shimamura, T., A. Ibuka, S. Fushinobu, T. Wakagi, M. Ishiguro, Y. Ishii, and H. Matsuzawa.** 2002. Acyl-intermediate structures of the extended-spectrum class A β-lactamase, Toho-1, in complex with cefotaxime, cephalothin, and benzylpenicillin. J. Biol. Chem. **277:**46601–46608.
26. **Smith Moland, E., N. D. Hanson, V. L. Herrera, J. A. Black, T. J. Lockhart, A. Hossain, J. A. Johnson, R. V. Goering, and K. S. Thomson.** 2003. Plasmid-mediated, carbapenem-hydrolysing β-lactamase, KPC-2, in *Klebsiella pneumoniae* isolates. J. Antimicrob. Chemother. **51:**711–714.
27. **Vourli, S., P. Giakkoupi, V. Miriagou, E. Tzelepi, A. C. Vatopoulos, and L. S. Tzouvelekis.** 2004. Novel GES/IBC extended-spectrum β-lactamase variants with carbapenemase activity in clinical enterobacteria. FEMS Microbiol. Lett. **234:**209–213.
28. **Yang, Y. J., P. J. Wu, and D. M. Livermore.** 1990. Biochemical characterization of a β-lactamase that hydrolyzes penems and carbapenems from two *Serratia marcescens* isolates. Antimicrob. Agents Chemother. **34:**755–758.
29. **Yigit, H., A. M. Queenan, G. J. Anderson, A. Domenech-Sanchez, J. W. Biddle, C. D. Steward, S. Alberti, K. Bush, and F. C. Tenover.** 2001. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **45:**1151–1161.
30. **Yigit, H., A. M. Queenan, J. K. Rasheed, J. W. Biddle, A. Domenech-Sanchez, S. Alberti, K. Bush, and F. C. Tenover.** 2003. Carbapenem-resistant strain of *Klebsiella oxytoca* harboring carbapenem-hydrolyzing β-lactamase KPC-2. Antimicrob. Agents Chemother. **47:**3881–3889.