

Chromosome-encoded β -lactamases of *Citrobacter diversus*

Interaction with β -iodopenicillanate and labelling of the active site

Gianfranco AMICOSANTE,* Arduino ORATORE,* Bernard JORIS,† Moreno GALLENi,†
Jean-Marie FRÈRE‡§ and Jozef VAN BEEUMEN‡

* Università degli Studi dell'Aquila Dipartimento di Scienze e Tecnologie Biomediche e di Biometria, Cattedre di Chimica Biologica e Biologia Molecolare, Località Collemaggio, I-67100 L'Aquila, Italy, † Laboratoire d'Enzymologie et Service de Microbiologie, Université de Liège Institut de Chimie, B6, B-4000 Sart Tilman (Liège), Belgium, and ‡ Rijksuniversiteit-Gent Laboratorium voor Microbiologie, Ledeganckstraat 35, B-9000 Gent, Belgium

Both forms of the chromosome-encoded β -lactamase of *Citrobacter diversus* react with β -iodopenicillanate at a rate characteristic of class A β -lactamases. The active site of form I was labelled with the same reagent. The sequence of the peptide obtained after trypsin hydrolysis is identical with that of a peptide obtained in a similar manner from the chromosome-encoded β -lactamase of *Klebsiella pneumoniae*.

INTRODUCTION

Two chromosome-encoded β -lactamases produced by a β -lactam-resistant clinical isolate of *Citrobacter diversus* have been isolated and purified to protein homogeneity (Amicosante *et al.*, 1987, 1988). The two enzymes are very similar in most respects but exhibit isoelectric points differing by 0.6 unit. It is not known whether they correspond to different processings of the same precursor or are the products of different genes. The fact that they were strongly inhibited by clavulanate indicated that they were probably active-serine β -lactamases. These enzymes are divided into three classes, A, C and D, on the basis of their primary structures. Although chromosome-encoded β -lactamases produced by Gram-negative bacteria are generally members of class C, the M_r values and substrate profiles of the two *C. diversus* enzymes were closer to those of class A β -lactamases. In the present paper we report the kinetic parameters of the interaction between β -iodopenicillanate and the two enzymes and the amino acid sequence around the active serine residue of form I.

MATERIALS AND METHODS

The enzymes were purified as described elsewhere (Amicosante *et al.*, 1988). Cefazolin was obtained from Sigma Chemical Co. and β -iodopenicillanic acid was a gift from Dr. J. Kemp, Pfizer Central Research, Sandwich, Kent, U.K. β -Iodo[3 H]penicillanic acid (2.1 mCi/mmol) was the sample described previously (De Meester *et al.*, 1985). Inactivation experiments were performed in 500 μ l of 25 mM-sodium phosphate buffer, pH 7.2, containing 0.05 mg of bovine serum albumin/ml and 0.2 M-NaCl. The temperature was 30 °C. Cefazolin (200 μ M) was used as a reporter substrate. With form I 0.14 μ g of enzyme was used, and 0.32 μ g with form II. Experiments were performed on a Beckman DU8 spectrophotometer and the data were analysed with the

help of an Apple II microcomputer as described by De Meester *et al.* (1987a)

The peptide obtained after trypsin degradation of form I was purified by chromatography on a Sephadex G-25 column (100 cm \times 1 cm) equilibrated in 50 mM-NaHCO₃. Elution was performed with the same buffer. Further purification was realized with the help of an f.p.l.c. apparatus (Pharmacia, Uppsala, Sweden) equipped with a PEP-RPC HR 5/5 (0.5 cm \times 5 cm) column. The solvent was 0.1% (v/v) trifluoroacetic acid in water (A) or in acetonitrile/water (7:3, v/v) (B). The flow rate was 0.7 ml/min and the gradient went from 0 to 65% (v/v) of solvent B over a period of 30 min. Each fraction contained 0.5 ml.

The sequence of the purified peptide was determined with a 477 A pulsed-liquid Sequenator (Applied Biosystems, Foster City, CA, U.S.A.) equipped with an on-line 120 A phenylthiohydantoin analyser. Solvents and reagents were also from Applied Biosystems. The Edman degradation was performed for 11 cycles.

RESULTS

Inactivation by β -iodopenicillanate

Preliminary experiments indicated that the inactivation was complete at an inactivator/enzyme ratio of 1:1. No re-activation of the enzyme occurred after elimination of the excess of inactivator by dialysis and prolonged incubation at 30 °C. The results of inactivation experiments are shown in Table 1. The fact that the $k_{+2}/[C]$ ratio remained constant with $[C]$ indicated that the highest inactivator concentration used was well below the value of K . Under those conditions, only the ratio k_{+2}/K could be computed, after correction for the protection by the reporter substrate (Frère *et al.*, 1982):

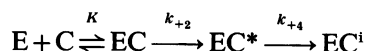
$$\frac{k_{+2}}{K} = \frac{k_1 \cdot K_m + [S]}{[C] \cdot K_m}$$

§ To whom correspondence should be addressed.

Table 1. Inactivation of the enzyme by β -iodopenicillanate

Apparent first-order rate constants (k_i) were measured for various inactivator concentrations ([C]) in the presence of 200 μ M-cefazolin. The k_i values were directly derived from the time-courses of cefazolin hydrolysis (De Meester *et al.*, 1987a).

[C] (μ M)	k_i (s^{-1})	$k_i/[C]$ ($M^{-1}\cdot s^{-1}$)
Form I		
2.16	0.0094 ± 0.0010	4350 ± 400
3.2	0.0123 ± 0.0015	3840 ± 600
4.2	0.0185 ± 0.0010	4400 ± 240
Average: $k_i/[C] = 4200 \pm 330 M^{-1}\cdot s^{-1}$ $k_{+2}/K = 45800 \pm 3600 M^{-1}\cdot s^{-1}$		
Form II		
2.16	0.0098 ± 0.0002	4450 ± 100
3.2	0.0145 ± 0.0015	4500 ± 500
4.2	0.018 ± 0.001	4250 ± 250
Average: $k_i/[C] = 4400 \pm 150 M^{-1}\cdot s^{-1}$ $k_{+2}/K = 48000 \pm 2000 M^{-1}\cdot s^{-1}$		

**Scheme 1. Interaction between enzyme (E) and β -iodopenicillanate (C)**

EC* is the acyl-enzyme and ECⁱ is the irreversibly inactivated enzyme after rearrangement of the β -iodopenicilloyl moiety.

where K_m and [S] are respectively the K_m for and the concentration of the reporter substrate. Values of 40×10^3 – $50 \times 10^3 M^{-1}\cdot s^{-1}$ were found for the second-order rate constants characteristic of the inactivation, i.e. k_{+2}/K (Scheme 1).

Labelling of the active site

The enzyme (form I; 1 mg, i.e. about 30 nmol), dissolved in 1 ml of 50 mM-sodium phosphate buffer, pH 7.0, containing 1 M-NaCl, was incubated with β -iodo[³H]penicillanate (final concn. 30 μ M) for 60 min at 30 °C, which resulted in complete loss of activity. The sample was dialysed against 50 mM-sodium phosphate

buffer, pH 7.0, containing 8 M-urea and 1 mM-dithiothreitol. The u.v.-absorption spectrum exhibited maxima at 320 and 280 nm and the ratio A_{320}/A_{280} was 0.55 ± 0.02 . On the basis of a molar absorption coefficient of $12000 M^{-1}\cdot cm^{-1}$ for the enzyme-bound dihydrothiazine chromophore (De Meester *et al.*, 1986), one could compute that 30 nmol of enzyme had bound 42 ± 2 nmol of β -iodopenicillanate. This discrepancy, indicating that the value of A_{320} was about $25 \pm 5\%$ too high, might be due to an overestimation of the ϵ_{280} value of the protein, resulting in an underestimation of its concentration.

The sample was treated with 5,5'-dithiobis-(2-nitrobenzoic acid) (final concn. 30 mM), incubated at 30 °C for 30 min, exhaustively dialysed against water and freeze-dried. The dry powder was dissolved in 0.5 ml of sodium phosphate buffer, pH 7.0, containing 8 M-urea and incubated at 37 °C for 1 h. Trypsin (50 μ g in 0.5 ml of phosphate buffer) was added and digestion was performed at 37 °C for 4 h. After a second addition of 50 μ g of trypsin, the mixture was further incubated at 37 °C for 4 h. The sample was then applied to the Sephadex G-25 column, and the radioactive fractions were pooled and freeze-dried. The powder was dissolved in 0.5 ml of solvent A and applied to the PEP-RPC column. Two groups of radioactive fractions were separated: fractions 44+45 contained 2.3 nmol and fractions 56+57 contained 2.4 nmol of radioactive peptides. The fractions were separately freeze-dried. The u.v.-absorption spectrum of the solution obtained after addition of 0.3 ml of 10 mM-NH₄HCO₃ to fraction 56 exhibited a maximum at 320 nm. After the addition of 2-mercaptoethanol (final concn. 0.33 mM) the intensity of that maximum decreased and a second chromophore appeared, centred at 412 nm. This result, corresponding to those obtained with the β -lactamases of *Streptomyces albus* G (De Meester *et al.*, 1987b) and *Klebsiella pneumoniae* (Joris *et al.*, 1987), indicated that the peptide probably contained a cysteine residue.

Sequence of the peptide

Fig. 1 shows the sequence of the peptide as obtained with the 477 A Sequenator starting with 0.19 nmol of material (fractions 56+57). It was not possible, from the sequence data alone, to decide which of the two serine residues was bound to the dihydrothiazine chromophore. We have noted, however, that the yield in serine phenylthiohydantoin derivative was about 1.5-fold

	F	A	M	C	S*	T	S	K	Identity	References
<i>Citrobacter diversus</i>	F	A	M	C	S	T	S	K		
<i>Klebsiella pneumoniae</i>	F	A	M	C	S	T	S	K	8/8	(1)
<i>Escherichia coli</i> (RTEM)	F	P	M	M	S	T	F	K	5/8	(2)
<i>Staphylococcus aureus</i>	F	A	Y	A	S	T	S	K	6/8	(2)
<i>Bacillus licheniformis</i>	F	A	F	A	S	T	I	K	5/8	(2)
<i>Streptomyces albus</i> G	F	P	M	C	S	V	F	K	5/8	(3), (4)

Fig. 1. Sequence of the β -iodopenicillanate-labelled peptide of the *C. diversus* β -lactamase and comparison with the corresponding peptides of some class A β -lactamases

The initial yield for Phe-1 of the *C. diversus* sequence was 66%. Cysteine was recognized by its typical phenylthiohydantoin decomposition pattern. For comparison with class C and OXA-2 β -lactamases see Joris *et al.* (1987). References: (1) Joris *et al.* (1987); (2) Ambler (1980); (3) Dehottay *et al.* (1986); (4) De Meester *et al.* (1987b).

larger at position 7 than at position 5, and also that the ratio of serine phenylthiohydantoin derivative to the addition product of dithiothreitol (dithiothreitol was present in the buffers) and to dehydroserine phenylthiohydantoin derivative was about 1:15 at position 5 and 1:1 at position 7. The latter value is found in most cases with unmodified serine residues. We have observed a similar phenomenon when comparing the results of one Edman degradation cycle on phosphoserine and serine. It is therefore very likely that the residue at position 5 in the peptide was modified. Moreover, the analogy with the other class A β -lactamases and the occurrence of the characteristic sequence



indicated that the first residue of serine is the active one.

DISCUSSION

The β -lactamases that belong to class C exhibit a very definite and characteristic substrate profile. On that basis alone it was reasonable to assume that the two *C. diversus* β -lactamases were not members of that class (Amicosante *et al.*, 1988). Moreover, the M_r values were also much more in agreement with those generally reported for class A enzymes. De Meester *et al.* (1986) have suggested that the second-order rate constant for the inactivation of a β -lactamase by β -iodopenicillanate might represent a good classification criterion. On that basis the two *C. diversus* β -lactamases also behaved as class A enzymes. Finally, the sequence of the tryptic peptide that carried the dihydrothiazine label after reaction with the same inactivator was very similar to that of various corresponding peptides of class A enzymes. Indeed, as shown by Fig. 1, the peptide was identical with that obtained from the β -lactamase of *K. pneumoniae*, and for each amino acid a corresponding identical residue could be found in one or more of the other known class A enzymes (Fig. 1). The complete identity with the *K. pneumoniae* peptide is indeed remarkable, and it should be noted that this latter enzyme is also a chromosome-encoded β -lactamase produced by a Gram-negative strain. The meaning of this finding cannot be further interpreted at the present stage. That the *K. pneumoniae* enzyme is, however, different from the *C. diversus* β -lactamase is demonstrated by the fact that, with the former enzyme and β -iodopenicillanate, a branched pathway was observed (De Meester *et al.*, 1986). Surprisingly, the presence of the

two sulphur-containing residues Met-Cys just before the active serine residue is also found in the enzyme excreted by *Streptococcus albus* G, a very distant Gram-positive strain. Although, in contrast with class C, class A contains enzymes whose substrate profiles are extremely variable, it seems that a high ($> 2000 \text{ m}^{-1} \cdot \text{s}^{-1}$) second-order rate constant for the inactivation by β -iodopenicillanate remains a good criterion for deciding that an enzyme belongs to class A, as suggested by De Meester *et al.* (1986).

The work in Liège was supported by the Fonds de la Recherche Scientifique Médicale (Contract no. 3.4507.83), an Action Concertée with the Belgian Government (Convention 86/91-90), a convention with the Région Wallonne (C2/C16/Conv. 246/20428), the Fonds de Recherche de la Faculté de Médecine ULg and a contract with E.E.C. (BAP-0197-B). The sequence data obtained in Gent present the research result of the Belgian National incentive programme on Fundamental Research in Life Sciences, initiated by the Belgian State-Prime Minister's Office-Science Policy Programming. G.A. is indebted to Italian C.N.R. and Professor R. Strom (University of Rome) for financial support for a 3-month stay in Liège.

REFERENCES

- Ambler, R. P. (1980) *Philos. Trans. R. Soc. London B* **289**, 321-331
- Amicosante, G., Marinucci, M. C., Franceschini, N., Tizzani, M. I., Oliva, B. & Oratore, A. (1987) *J. Chromatogr.* **403**, 366-372
- Amicosante, G., Oratore, A., Franceschini, N., Maccarrone, M., Strom, R., Galleni, M. & Frère, J.-M. (1988) *Biochem. J.* **254**, 885-890
- Dehottay, P., Duez, C. & Dusart, J. (1986) *Proc. Int. Symp. Biology of Actinomycetes 6th*, Debrecen, 1985, 385-387
- De Meester, F., Frère, J.-M., Piette, J. L. & Vanderhaeghe, H. (1985) *J. Labelled Compd. Radiopharm.* **22**, 415-425
- De Meester, F., Frère, J.-M., Waley, S. G., Cartwright, S. J., Virden, R. & Lindberg, F. (1986) *Biochem. J.* **239**, 575-580
- De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frère, J.-M. & Waley, S. G. (1987a) *Biochem. Pharmacol.* **36**, 2393-2403
- De Meester, F., Joris, B., Lenzini, M. V., Dehottay, P., Erpicum, T., Dusart, J., Klein, D., Ghuysen, J. M., Frère, J.-M. & Van Beeumen, J. (1987b) *Biochem. J.* **244**, 427-432
- Frère, J.-M., Dormans, C., Duyckaerts, C. & De Graeve, J. (1982) *Biochem. J.* **207**, 437-444
- Joris, B., De Meester, F., Galleni, M., Frère, J.-M. & Van Beeumen, J. (1987) *Biochem. J.* **243**, 561-567

Received 31 May 1988/27 June 1988; accepted 4 July 1988