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The β-lactamase of *Enterobacter cloacae* P99

Chemical properties, *N*-terminal sequence and interaction with 6β-halogenopenicillanates

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The β-lactamase of *Enterobacter cloacae* P99 consists of one polypeptide chain of M_r 39000 devoid of disulphide bridges and free thiol groups. It contains an unusually high proportion of tyrosine and tryptophan. The *N*-terminal sequence exhibits overlaps with the tryptic peptide obtained after labelling the active site with 6β-iodopenicillanate. The active-site serine residue is at position 64. The homology with the chromosomal β-lactamase of *Escherichia coli* K12 (*ampC* gene) is lower within the 25 residues of the *N*-terminal portion than around the active-site serine residue. The P99 β-lactamase is inactivated by 6β-bromo- and 6β-iodo-penicillanate, with a second-order rate constant of 110–140 $M^{-1} \cdot s^{-1}$ at 30°C and pH 7.0, a value that is much lower than that observed with class-A β-lactamases.

Since hydrolysis of the β-lactam ring of penicillins and cephalosporins yields biologically inactive compounds, the synthesis of β-lactamases by pathogenic bacterial strains makes them resistant to these antibiotics and represents a problem of major clinical importance.

Three classes of β-lactamases have now been clearly identified, which differ by their mechanistic properties or primary structures (Ambler, 1980; Jaurin & Grundström, 1981). Enzymes of classes A and C contain an active-site serine residue (Knott-Hunziker *et al.*, 1979, 1982*a*), but can be distinguished on the basis of their different primary structures, whereas class-B enzymes are Zn^{2+}-dependent hydrolases (Ambler, 1980). The existence of other classes remains a distinct possibility. For several years, enzymes of class A have received a lot of attention [reviewed by Ambler (1980) and Hill *et al.* (1980)]. Conversely, the identification of class C as a distinct protein family is rather recent. Three members of class C, the chromosomal β-lactamases of *Escherichia coli* K12, *Pseudomonas aeruginosa* and *Enterobacter cloacae* P99 have been identified (Jaurin & Grundström, 1981; Knott-Hunziker *et al.*, 1982*a*; Joris *et al.*, 1984). The present knowledge about these enzymes can be summarized as follows: (1) the three proteins have similar M_r values (39000) and exhibit extensive sequence homology around the active-site serine residue (Joris *et al.*, 1984); (2) the *ampC* gene coding for the *E. coli* enzyme has been sequenced (Jaurin & Grundström, 1981); (3) the pH-dependence and the inhibition of the *Pseudomonas* and *E. coli* enzymes by boronic acids have been studied (Bicknell *et al.*, 1983; Cartwright & Waley, 1984); (4) convincing evidence for the formation of an acyl-enzyme intermediate has been presented (Knott-Hunziker *et al.*, 1982*b*); (5) the *Enterobacter* enzyme has been crystallized (Charlier *et al.*, 1983).

In the present paper we report studies on the chemical properties of the *Enterobacter* β-lactamase and on its interaction with 6β-halogenopenicillanates.

Abbreviations used: SDS, sodium dodecyl sulphate; Nbs_2, 5,5'-dithiobis-(2-nitrobenzoic acid) ('DTNB'); N_2ph, dinitrophenyl ('Dnp').

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Materials and methods

The β-lactamase preparation was that described previously (Charlier *et al.*, 1983). SDS/polyacryl-

amide-gel electrophoresis was performed as described by Laemmli & Favre (1969) and indicated a purity of 93% after staining of the gel with Coomassie Blue R250 and scanning at 500nm with the help of a Beckman DU8 spectrophotometer. The distance migrated by the main band corresponded to an M_r of 39000, a value in agreement with that previously reported (Ross, 1975). Carboxypeptidase Y was from Boehringer (Mannheim, Germany). 6β-Bromopenicillanic acid was the sample synthesized by Claes *et al.* (1985). Nitrocefin, cephalexin and 6β-iodopenicillanate were kindly given by Glaxo Research Group (Greenford, Middx., U.K.), Lilly Research Laboratories (Indianapolis, IN, U.S.A.) and Pfizer Central Research (Sandwich, Kent, U.K.) respectively.

Absorbance measurements (spectra and kinetics) were performed with a Beckman DU8 spectrophotometer. Fluorescence spectra were recorded on a Kontron SFM23 spectrofluorimeter.

Automatic sequence determination was performed by using an Applied Biosystems model 470A gas-phase sequenator. The amino acid phenylthiohydantoin derivatives were identified by reversed-phase h.p.l.c. on an IBM cyanopropyl column (25cm × 0.46cm) with a Waters Intelink system with two 6000A pumps. The gradient and the solvent solutions were similar to those described by Hunkapiller *et al.* (1983). Detection was carried out simultaneously at 269 and 323nm with a Perkin–Elmer LC75 and a Pye–Unicam LC u.v. detector respectively. The *N*-terminal residue was identified, as described by Wittmann-Liebold & Lehmann (1980), by using 4-*NN*-dimethylaminoazobenzene-4′-isothiocyanate ('DABITC'). The protein was reduced and carboxymethylated as described previously (Joris *et al.*, 1983). The *C*-terminal sequence was determined by digesting the native protein with carboxypeptidase Y in 100mM-pyridine adjusted to pH5.5 with acetic acid.

Amino acid analyses were performed, after 24, 48 and 72h hydrolyses in 6M-HCl at 120°C, in a Beckman Multichrom automatic analyser. Values for threonine and serine were back-extrapolated to zero hydrolysis time. Analyses were similarly performed after oxidation with performic acid (Hirs, 1956). The concentration of free thiol groups was determined by reaction with Nbs_2 in the presence of 1% (w/v) SDS, and the number of disulphide bridges by measuring the free thiol groups after reduction of the protein with $NaBH_4$ (Habeeb, 1972).

Several methods were used to determine the number of tryptophan residues, proteins of known sequence being used as standards: hen's-egg white lysozyme, bovine erythrocyte carbonic anhydrase, ovalbumin, bovine trypsinogen, bovine pancreatic ribonuclease and *Streptomyces albus* G DD-peptidase.

(1) U.v. spectra were recorded in 10mM-Tris/HCl buffer, pH8, and, on the basis of the number of tyrosine residues (N_{Tyr}) obtained by analysis of the acid hydrolysates, the number of tryptophan residues (N_{Trp}) was calculated by using the equation:

$$A_{280}^{0.1\%,1cm} = \frac{5700 \times N_{Trp} + 1300 \times N_{Tyr}}{M} \quad (1)$$

where M is the M_r of the proteins (Cantor & Schimmel, 1980).

(2) U.v. spectra obtained after adjusting the pH to 13 with NaOH were analysed as described by Bencze & Schmid (1957).

(3) Titration with *N*-bromosuccinimide was performed as described by Spande & Witkop (1965).

(4) The intensity of fluorescence emission at 355nm ($\lambda_{excitation} = 280$nm) of unfolded proteins was compared with that of free tryptophan in 10mM-Tris/HCl, pH8.0, containing 6M-guanidinium chloride. The linearity of fluorescence intensity with tryptophan concentation was verified between 3 and 30μM. During these experiments, protein concentrations were determined by measuring the number of free amino groups available to dinitrophenylation after total hydrolysis. A value of $7500\,M^{-1}\cdot cm^{-1}$ (in 2M-HCl and at 410nm) was found for the average molar absorption coefficient of a N_2ph-amino acid by recording the spectrum of pure N_2ph-alanine (Serva) and by performing the experiment with a standard mixture of amino acids (Beckman). In the case of ovalbumin, the concentration found by this method was within 2% of the weighed amount, provided that the preparation had been freeze-dried just before weighing. The concentration of the purified P99 β-lactamase was also determined with Coomassie Blue by the Bradford method, with the Bio-Rad Protein Mixture as standard.

Enzymic activity

The β-lactamase activity was routinely determined with cephalexin (specific activity = $100\,\mu mol\cdot min^{-1}\cdot mg$ of enzyme^{-1}; $K_m = 70\,\mu M$) or nitrocefin (specific activity = $500\,\mu mol\cdot min^{-1}\cdot mg$ of enzyme^{-1}, $K_m = 56\,\mu M$) as substrates. These values were determined by continuously recording the decrease of absorbance at 260nm (cephalexin) or the increase of absorbance at 482nm (nitrocefin) of 100μM solutions of substrate and analysing the results by the integrated equation given by Dixon & Webb (1964) which was rearranged as follows:

$$\frac{1}{t}\ln\frac{A_\infty - A_0}{A_\infty - A_t} = \frac{1}{K'_m}\left(V' - \frac{|A_t - A_0|}{t}\right) \quad (2)$$

where A_0 and A_∞ were the initial and final absorbances respectively, $|A_t - A_0|$ the absolute value of $A_t - A_0$ and K'_m and V' the kinetic parameters expressed in absorbance and absorbance variation per unit time respectively. The real values of $A_t - A_0$ were positive in the case of nitrocefin and negative in the case of cephalexin. The absorbance values were directly transmitted from the Beckman DU8 spectrophotometer to an Apple II microcomputer via a RS232 interface. The values of the kinetic parameters were obtained with the help of a linear-regression program. During these experiments, the pure enzyme was found to lose activity spontaneously and rapidly when solutions of low concentration ($<10\,\mu g \cdot ml^{-1}$) were conserved, even at 0°C. Stabilization was obtained by adding bovine serum albumin ($1\,mg \cdot ml^{-1}$) to the buffers used for dilution. Under these conditions, no spontaneous inactivation of the enzyme was recorded during the time course of substrate hydrolysis. No loss of activity was observed at higher enzyme concentrations. Attempts to measure a possible DD-carboxypeptidase activity were performed using Ac_2-L-Lys-D-Ala-D-Ala (UCB Bioproducts, Braine, Belgium) and Ac_2-L-Lys-Gly-D-Ala (a gift from Professor H. R. Perkins, Department of Microbiology, University of Liverpool, Liverpool, U.K.) as potential substrates. The release of D-alanine was assayed by using the D-amino acid oxidase–peroxidase coupling system (Frère *et al.*, 1976).

Reaction with 6β-halogenopenicillanates was monitored by measuring the increase in A_{325} (Loosemore *et al.*, 1980) or the progressive decrease in the velocity of hydrolysis of 100 μM-nitrocefin as described by Knott-Hunziker *et al.* (1980) and Frère *et al.* (1982*a*). All the experiments involving the determination of the enzymic activity or rates of inactivation were done in 50 mM-sodium phosphate, pH 7.0, and at 30°C.

Results

Amino acid composition: spectra

Table 1 shows the amino acid composition of the protein. In agreement with the negligible amount of cysteic acid found after performic oxidation, titration with Nbs_2 revealed 0.29 free thiol groups per enzyme molecule and the same result was obtained after reduction with $NaBH_4$. These experiments indicated the absence of free cysteine and of disulphide bridges in the molecule. The protein concentration of a sample exhibiting an $A_{280}^{1\,cm}$ of 0.79 was 0.41 and $0.44\,mg \cdot ml^{-1}$ as determined by the Coomassie Blue and the dinitrophenylation methods respectively. The A_{280} was thus about twice as high as that usually observed. This result was explained by the rela-

Table 1. *Amino acid composition of the β-lactamase of Enterobacter cloacae P99*
The percentages in the last column are based on the lower values for histidine and methionine. Abbreviation used: Met-O_2, methionine sulphone.

	No. of residues			
	Experimentally obtained	Nearest integer	Total molecular mass (Da)	Percentage composition [(residues/total no. of residues) × 100]
Lys	22.8	23	2948	6.3
His	6.3	6 or 7	822–959	1.6
Arg	12.8	13	2031	3.5
Cys	0	0	0	0
Asx	32.8	33	3765	9.0
Thr	25.8	26	2628	7.1
Ser	18.1	18	1568	4.9
Glx	39	39	5050	10.6
Pro	21.2	21	2040	5.7
Gly	33.6	34	1941	9.3
Ala	45.6	46	3271	12.5
Val	30.1	30	2972	8.2
Met-O_2	2.3	2 or 3	262–393	0.5
Ile	14.3	14	1584	3.8
Leu	32.1	32	3622	8.7
Tyr	13.2	13	2121	3.5
Phe	7.9	8	1177	2.2
Trp	–	9	1676	2.5
Total	–	367–369	39478–39746	99.9

tively large content of tyrosine and tryptophan residues.

Table 2 displays the results obtained by using four different methods to determine the number of tryptophan residues. Short of the complete sequence, none of the methods appeared to be totally reliable (see the Discussion section), but the mean values were within one residue of the theoretical number. A value of nine tryptophan residues was accepted for the P99 β-lactamase, but a slightly higher value (ten) could not be excluded.

The maximum of the fluorescence spectrum was at 338nm, not far from that of free tryptophan (Fig. 1). The u.v.-absorption spectrum displayed a maximum at 280nm. A characteristic feature was the rather large value of the A_{280}/A_{250} ratio, which could again be attributed to the high tyrosine+tryptophan content of the molecule.

N-Terminal sequence

Fig. 2 gives the amino acid sequence of the first 57 residues of *E. cloacae* P99 β-lactamase, determined with 5nmol of carboxymethylated protein using the gas-phase sequenator. The repetitive yields of the Edman degradation, calculated from the amount of valine at positions 3, 11 and 25 were, respectively, 98.3% (Val-3 versus Val-11), 93.1% (Val-3 versus Val-25) and 88.5% (Val-11 versus Val-25). Between Leu-8 and Leu-19, this factor was found to be 93.4%, and between Ala-9 and Ala-13, 84.1%.

C-Terminal sequence

Digestion of 3nmol with carboxypeptidase Y yielded the following *C*-terminal sequence: -Glu-

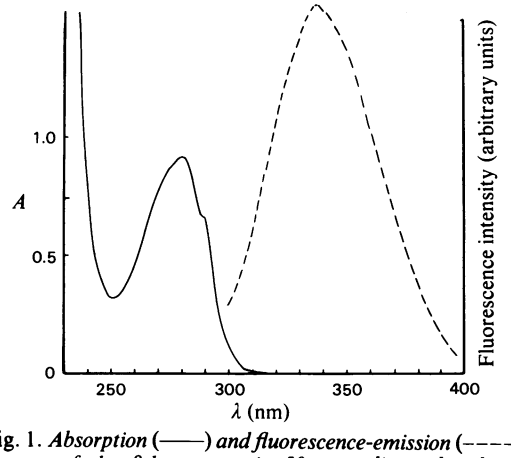

Fig. 1. *Absorption* (——) *and fluorescence-emission* (----) *spectra of the β-lactamase in 50mM-sodium phosphate, pH7.0*

Absorption: protein concn. 0.5mg·ml^{-1}; fluorescence emission: protein concn. 0.045mg·ml^{-1}, $\lambda_{excitation}$ 280nm (the maximum of the excitation spectrum was at 285nm).

Table 2. *Number of tryptophan residues*

Standard protein	Theoretical no. of residues		Method	No. of residues					
				...U.v. spectrum in alkali		A_{280} pH8.0 (Trp)*	Fluorescence (Trp)	*N*-Bromosuccinimide (Trp)	Mean ± S.D. (Trp)
	Tyr	Trp		Tyr	Trp				
Lysozyme	3	6		5	4.6	6.1	4.7	5.4	5.2±0.7
Carbonic anhydrase	8	5		6.5	5	5.6	5.7	N.D.	5.4±0.4
Ovalbumin	10	3		5.7	3.8	4.3	2.5	N.D.	3.5±0.9
Trypsinogen	10	4		9.5	6.4	4.1	5.1	3.6	4.8±1.2
Ribonuclease	6	0		5.3	<0.3	0.1	<0.2	0.26	0.2±0.1
DD-peptidase	7	4		4.9	4.1	3.0	4.5	3.1	3.7±0.7
Average error (%)†				30	19	14	15	14	13
P99 β-lactamase	13‡			16.5	8	9.8	9.2	9.8	9.2±0.8

* Based on eqn. (1), the number of tyrosine residues given in column 1 being used.
† The values for ribonuclease have not been included since all the methods clearly indicate the absence of tryptophan residues.
‡ Based on the analysis of the acid hydrolysates.

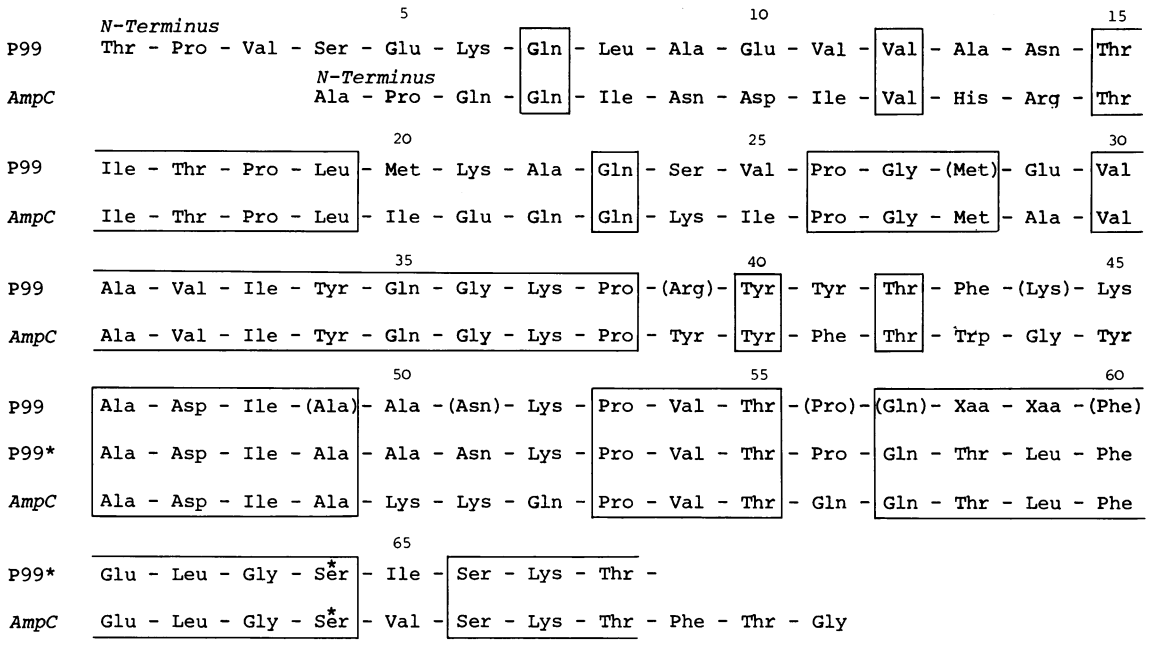

Fig. 2. *N-Terminal sequence of the P99 β-lactamase and comparison with that of the chromosomal β-lactamase of E. coli K12 (ampC; Jaurin & Grundström, 1981)*

The evidence for the residues at the positions 28, 39, 44, 49, 51, 56, 57 and 60 was rather weak in terms of peak heights on the h.p.l.c. chromatograms, but there was no indication for residues other than those indicated. Starting at position 46, the previously determined sequence of the tryptic peptide containing the active-site serine-64 (Joris *et al.*, 1984) is also shown (P99*). Residues at positions 58 and 59 could not be identified.

Leu-Ala-OH. An equimolecular mixture of Tyr, Lys, Gly and Val was obtained for the residues preceding Glu-.

Attempts to detect a peptidase activity

The enzyme exhibited a rather high esterase activity towards esters whose structure was similar to that of DD-carboxypeptidase substrates (Pratt & Govardhan, 1984). We failed to detect any peptidase activity after a 120min incubation of 30μg of enzyme in the presence of 2mM-Ac_2-L-Lys-D-Ala-D-Ala or Ac_2-L-Lys-Gly-D-Ala, which indicated that the peptidase specific activity of the enzyme, if it existed, was lower than 1nmol·min^{-1}·mg of $enzyme^{-1}$.

Interaction with β-halogenopenicillanates

No turnover of 6β-halogenopenicillanate was detected. This conclusion rested on the following observations.

(1) Titration experiments (see below) indicated that a 1:1 ratio of β-iodopenicillanate to enzyme resulted in complete inactivation.

(2) On reaction with 6β-bromo- or 6β-iodopenicillanate, a new absorption band appeared in the near-u.v. region. The maximum was 325nm and the ε_{325} was 11000–12000M^{-1}·cm^{-1}, close to the value observed by Loosemore *et al.* (1980) with the *Bacillus cereus* β-lactamase I. Hydrolysis of the halogenopenicillanates would yield 2,3-dihydro-2,2-dimethyl-1,4-thiazine-3,6-dicarboxylate (Frère *et al.*, 1982*b*), whose absorption maximum is at 305nm (ε_{305} 8200M^{-1}·cm^{-1}), with substantial residual absorption at 325nm (ε_{325} 3500M^{-1}·cm^{-1}). If turnover occurred, one would thus expect a shift of the absorption maximum to 305nm and an increase in A_{325}.

Titration with β-iodopenicillanate

To 500μl of enzyme solution containing 0.49mg of protein/ml, 30μl of 0.25M-6β-iodopenicillanate was added in six portions of 5μl each. The A_{325} was continuously monitored and, after each addition, a 10μl sample was withdrawn the residual activity in which was determined after suitable dilution. Fig. 3 shows the results of the titration experiment. According to the linear regression, the end point was at 24.2μl, indicating a starting concentration of enzyme of 12.1μM, i.e. 0.47mg·ml^{-1} (on the basis of an M_r of 39000). According to these data, the enzyme was thus 96%

Table 3. *Rate of reaction with 6β-iodo- and 6β-bromo-penicillanates as monitored directly at 325nm*

Inactivator	Inactivator concn. ([C]) (μM)	$10^3 \times k_a$ (s^{-1})	k_a/[C] ($M^{-1} \cdot s^{-1}$)
6β-Bromopenicillanate	52	5.9	113
	26	3.0	115
6β-Iodopenicillanate	117	12.9	110
	229	25.7	112
	335	37.4	112

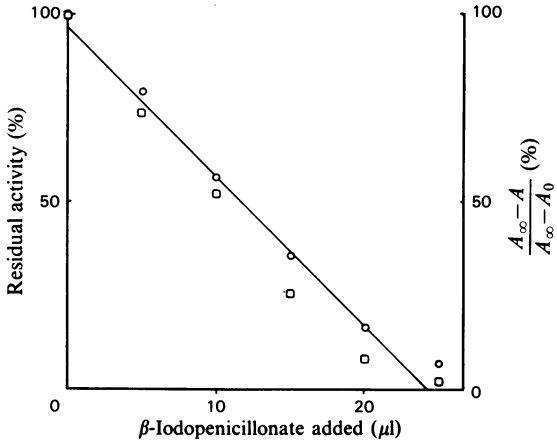

Fig. 3. *Titration of the β-lactamase with β-iodopenicillanate*

After each addition of 6β-iodopenicillanate, a 10μl sample was withdrawn and diluted 200-fold. The residual activity (○) was determined by adding 20μl of the diluted solution to 450μl of 100μM-cephalexin and monitoring the decrease in A_{260}. The reaction between the enzyme and 6β-iodopenicillanate was also monitored directly by measuring the A_{325} of the mixture (□). A_0, A_∞ and A were, respectively, the absorbance of the starting enzyme solution at 325nm, that after the sixth (and last) 5μl addition of 6β-iodopenicillanate and that observed after completion of the reaction after each individual addition. The line was drawn by linear regression using the 12 experimental points.

pure, a value in good agreement with that obtained by gel electrophoresis (93%).

Rate of inactivation by β-halogenopenicillanates

1. *Direct recording of appearance of the chromophoric group.* To 250μl of 11.5μM-β-lactamase, 6β-bromo- or 6β-iodo-penicillanate was added (final concns. 26–335μM). The mixture was maintained at 30°C and the A_{325} continuously monitored. The results (Table 3) indicated that, in both cases, the pseudo-first-order rate constants, k_a, were proportional to the concentration of inactivator [C] and that the second-order rate constants, k_a/[C], were similar for both compounds. No lag was observed before the establishment of the first-order phenomenon.

Table 4. *Reaction with 6β-iodopenicillanate in the presence of 100μM-nitrocefin*

Concn. ([C]) (μM)	$10^3 \times (k_a)_{nit}$ (s^{-1})	$(k_a)_{nit}$/[C] ($M^{-1} \cdot s^{-1}$)
35	1.9	54
70	4.1	59
139	7.0	50
209	10.7	51

2. *Inactivation in the presence of nitrocefin.* The enzyme (20μl at a concentration of 0.27μg·ml^{-1}) was added to 200μl of 100μM-nitrocefin containing 0–209μM-6β-iodopenicillanate. In the absence of inactivator, the hydrolysis rate of nitrocefin was constant for several minutes. The values of $(k_a)_{nit}$, the apparent rate constant for the inactivation (Table 4) were computed as described by Frère *et al.* (1982*a*,*b*). The value of the second-order rate constant, $(k_a)_{nit}$/[C] did not vary with [C].

Discussion

The amino acid composition of the β-lactamase of *E. cloacae* P99 is characterized by the absence of cysteine and disulphide bridges and a relatively high proportion of tyrosine and tryptophan residues, which results in an intense absorption spectrum in the near-u.v. ($A_{280}^{1\%,1cm} = 18.0$, $\varepsilon = 71\,000 M^{-1} \cdot cm^{-1}$). During this investigation we compared various methods for the determination of the number of tryptophan residues. The utilization of proteins whose primary sequence was known allowed us to draw the following conclusions: (1) All methods yielded results with rather large errors (14–19%). The error for tyrosine as determined from the u.v. spectrum in alkali was even worse (30%). (2) the mean values obtained for the standard proteins were always within one tryptophan residue of the theoretical number; (3) the estimation of protein concentration by the dinitrophenylation method indicated that standard proteins were often strongly hydrated and

that a freeze-drying step was necessary before weighing.

The *N*-terminal sequence has been partially established. It overlaps with the sequence of the tryptic peptide, previously isolated and sequenced (Joris *et al.*, 1984), that contains the active-site serine residue, labelled by cloxacillin and 6β-iodopenicillanate. This serine residue is at position 64. Sequence information for 57 out of the first 60 residues of the β-lactamase was obtained during one single run on the gas-phase sequenator. Since the h.p.l.c. gradient used did not differentiate the phenylhydantoin derivative of methionine from diphenylthiourea (Hunkapiller *et al.*, 1983), it could not be excluded *a priori* that methionine residues might occur at positions 39 and 44. However, since single residues were clearly identified at the other positions of the *N*-terminal sequence, the presence of heterogeneities at those two positions was unlikely.

When compared with the *E. coli* chromosomal β-lactamase (*ampC* gene), the P99 enzyme has three additional residues at the *N*-terminus. A high degree of homology had previously been observed near the active-site serine residues of both enzymes (residues 46–68 of P99; 78% homology). The homology for residues 26–45 was still very high (70%), but that for residues 4–25 was clearly much lower (36%). It had already been observed with some penicillin-binding proteins that the highest degree of homology was found near the penicillin-binding serine residue (Frère & Joris, 1985). It was difficult to compare the *C*-termini with the short stretch of sequence determined in the present work. The β-lactamase did not exhibit any detectable peptidase activity towards two peptides. At a 2mM substrate concentration, the peptidase activity was less than $1\,nmol\cdot min^{-1}\cdot mg$ of $enzyme^{-1}$, which is at least 10000-fold lower than the esterase activity observed by Pratt & Govardhan (1984) with a similar concentration of phenylacetylglycyl-D-mandelate.

The inactivation by 6β-bromo- and 6β-iodo penicillanate appeared to follow a simple unbranched pathway. In that respect the P99 enzyme was thus similar to the *B. cereus* β-lactamase I (class A; Knott-Hunziker *et al.*, 1979) and differed from the *Actinomadura* R39 and *S. albus* G β-lactamases (undetermined class; Frère *et al.*, 1982*b*). If any turnover occurred, its velocity was less than 10% of that of inactivation. The inactivation was thus analysed on the basis of the simple model:

$$E+C \overset{K}{\rightleftharpoons} E\cdot C \xrightarrow{k_{+2}} E\text{–}C^* \xrightarrow{k_{+4}} EC^i \quad (3)$$

where E·C would be the Michaelis complex (dissociation constant K) E–C* the acyl–enzyme intermediate and EC^i the rearranged covalent complex containing the dihydrothiazine chromophore. [The constant for the third step was called k_{+4} to retain the notation used previously (Frère *et al.*, 1982*b*) for an enzyme that also catalysed the hydrolysis of 6β-iodopenicillanate.] That the k_{+2} step was probably rate-limiting was suggested by the pseudo-first-order rate of appearance of the chromophore. With 6β-iodopenicillanate, formation of E–C* was measured in the presence of nitrocefin. In the presence of substrate, the first-order rate constant for the inactivation is given by eqn. (4):

$$(k_a)_{nit} = \frac{k_{+2}[C]}{[C]+\left(1+\frac{[S]}{K_m}\right)K} \quad (4)$$

where [S] is the concentration of substrate. The direct proportionality between [C] and $(k_a)_{nit}$ showed that [C] was negligible when compared with $K(1+[S]/K_m)$ and that eqn. (4) could be simplified and rearranged to give eqn. (5):

$$\frac{k_{+2}}{K} = \frac{(k_a)_{nit}}{[C]}\left(1+\frac{[S]}{K_m}\right) \quad (5)$$

On the basis of the data of Table 4, a value of $145\,M^{-1}\cdot s^{-1}$ was thus calculated for k_{+2}/K, which was not significantly different from that obtained by directly monitoring the appearance of EC^i. These results indicated that the rearrangement to the chromophoric complex

$$E\text{–}C^* \xrightarrow{k_{+4}} EC^i$$

was probably not rate-limiting, i.e. that the rearrangement was faster than the acylation step. When the appearance of the chromophoric complex was monitored, similar second-order rate constants were obtained for 6β-iodo- and 6β-bromopenicillanate. It seemed that the expulsion of the halogen, which occurred during the rearrangement step, did not influence the velocity of the overall reaction. The value of k_{+2}/K was very low compared with those measured by Knott-Hunziker *et al.* (1980) and Loosemore *et al.* (1980) for the β-lactamase I of *B. cereus* ($13–18\,mM^{-1}\cdot s^{-1}$) and by Frère *et al.* (1982*b*) for the β-lactamases of *Streptomyces albus* G ($\cong 100\,mM^{-1}\cdot s^{-1}$) and *Actinomadura* R39 ($\cong 1\,\mu M^{-1}\cdot s^{-1}$). It is possible that this relatively low second-order rate constant is a characteristic of class-C enzymes, but it will be necessary to measure the k_{+2}/K values with other class-C enzymes before any generalization can be proposed.

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