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The K1 β-lactamase of *Klebsiella pneumoniae*

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β-Lactamase K1 was purified from *Klebsiella pneumoniae* SC10436. It is very similar to the enzyme produced by *Klebsiella aerogenes* 1082E and described by Emanuel, Gagnon & Waley [Biochem. J. (1986) **234**, 343–347]. An active-site peptide was isolated after labelling of the enzyme with tritiated β-iodopenicillanate. A cysteine residue was found just before the active-site serine residue. This result could explain the properties of the enzyme after modification by thiol-blocking reagents. The sequence of the active-site peptide clearly established the enzyme as a class A β-lactamase.

INTRODUCTION

Emanuel *et al.* (1986) have recently described the purification and properties of β-lactamase K1 from *Klebsiella aerogenes* 1082E. We had purified an apparently very similar enzyme produced by *Klebsiella pneumoniae* SC10436. The general properties of both enzymes were the same (M_r, isoelectric pH, substrate profile). The *K. pneumoniae* β-lactamase could be inactivated by β-iodopenicillanate, a specific reagent for active-site-serine β-lactamases (De Meester *et al.*, 1986). The goal of the present study was to determine the amino acid sequence around the active site after labelling with that reagent and to compare it with that of the enzyme produced by *K. aerogenes*.

MATERIALS AND METHODS

Materials

A sample of the β-lactamase of *K. aerogenes* 1082E was kindly given by Dr. E. L. Emanuel and Dr. S. G. Waley, University of Oxford, Oxford, U.K. *K. pneumoniae* strain SC10436 was a gift from Dr. R. Sykes and Dr. K. Bush, The Squibb Institute, Princeton, NJ, U.S.A. It was grown in 1-litre conical flasks, each containing 500 ml of 1% (w/v) yeast extract (Difco 0127-01), on a rotatory shaker. A typical preparation consisted of 20 vessels. After 5 h at 37 °C, the cells were harvested by centrifugation, the sediment was resuspended in 250 ml of 50 mM-sodium phosphate buffer, pH 7.0, and the suspension was sonicated (2 × 3 min) and centrifuged at 25000 *g* for 40 min. The sonication–centrifugation cycle was repeated twice, and the three supernatants were combined.

Two methods were used for the purification of the enzyme: successive chromatographies on Sephadex G-75, DEAE-cellulose and DEAE-Sephadex, or the affinity-chromatography procedure described by Cartwright & Waley (1984), with the more-hydrophobic ('A'-type column) support. This latter procedure was easier and the yield was high (75%). Typically, 70 mg of pure enzyme was obtained from 10 litres of culture.

Catalytic properties and M_r

Kinetic parameters were derived from complete time-course analyses. The hydrolysis of benzylpenicillin and carbenicillin was monitored at 230 nm and that of cephaloridine and cephalothin at 260 nm. These experiments were performed in 50 mM-sodium phosphate buffer, pH 7.0, with the use of a Beckman DU-8 spectrophotometer directly connected to an Apple II microcomputer. The optical pathlength was 10 mm and experiments were performed at two or three different concentrations for each substrate. For comparison with the β-lactamase of *K. aerogenes*, the conditions were those used by Emanuel *et al.* (1986), i.e. 0.5 M-NaCl/50 mM-Mops buffer, pH 7.0, at 30 °C.

The M_r was estimated by polyacrylamide-gel electrophoresis in the presence of 0.1% SDS (Laemmli & Favre, 1969). A value of 27000 ± 1000 was found.

Peptide maps

Peptides obtained after trypsin digestion were separated with the help of Pharmacia fast-protein-liquid-chromatography apparatus equipped with a Pro-RPC column (10 cm × 0.5 cm). The enzyme was first reduced and carboxymethylated as described by Bibring & Baxandall (1978). After dialysis and freeze-drying, the samples were redissolved in 100 mM-sodium phosphate buffer, pH 7.0, containing 4 M-urea. The final protein concentration was 0.36 mg/ml. To 100 μl samples were added 4 μg portions of 1-chloro-4-phenyl-3-tosylamido-butan-2-one-('TPCK'-)treated trypsin (Millipore Corp., Freehold, NJ, U.S.A.). After a 4 h incubation at 37 °C, the digestion was stopped by addition of 6 μl of 86% (v/v) H_3PO_4. The samples were then submitted to chromatography on the Pro-RPC column. Buffers contained 1% H_3PO_4 adjusted to pH 2.2 with KOH in water (buffer A) or aq. 60% (v/v) acetonitrile (buffer B). The flow rate was 0.3 ml/min and the gradient was linear from 0 to 100% of buffer B over 60 min after a 2 min washing period with buffer A.

Purification and sequencing of active-site peptides

After labelling with β-iodo[^{3}H]penicillanate and digestion (see the Results section), the samples were first

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filtered through a Sephadex G-25 (fine grade) column (115 cm × 1 cm) in 50 mM-NH_4HCO_3. The radioactive fractions were pooled and freeze-dried, and the residue was redissolved in water. Final purification of the labelled peptide was performed by chromatography on a Pro-RPC column with the help of the fast-protein-liquid-chromatography apparatus. Buffers contained 10 mM-NH_4HCO_3 in water (buffer A) or in aq. 60% (v/v) acetonitrile (buffer B). After labelling with β-iodo[^{3}H]-penicillanate, reaction of the free thiol group with 5,5′-dithiobis-2-nitrobenzoate and trypsin digestion, the samples were filtered through the Sephadex G-25 column. Final purification was obtained by chromatography on a Pep-RPC column (10 cm × 0.5 cm). Buffers contained 0.1% trifluoroacetic acid in water (buffer A) or in aq. 60% (v/v) acetonitrile (buffer B). Amino acid composition of the peptides was determined after a 16 h hydrolysis with azeotropic HCl, by using a Dionex D-300 analyser equipped with a Waters column and a Spectra Physics detector. The *N*-terminal residue was identified by using the micro dansyl method of Hartley (1970).

Performic acid oxidation was performed on the dry residue obtained after freeze-drying the peptide solution. The oxidizing agent consisted of performic acid vapours as described by Brown & Hartley (1966).

The amino acid sequence of the active-site peptide was determined with an Applied Biosystems 470-A gas-phase Sequenator, with methanolic HCl as the converting agent for the amino acid thiazolinones. The amino acid phenylthiohydantoin derivatives were analysed off-line the sequencer on a Waters h.p.l.c. installation equipped with a 4.6 mm × 250 mm cyanopropyl column (IBM, Danbury, CT, U.S.A.). The gradient program was that suggested by Dr. B. C. Touchstone (A.B.I. user's bulletin no. 3).

Thiol-group determination

Thiol-group determination was performed with 5,5′-dithiobis-2-nitrobenzoate in the presence (Habeeb, 1972) or in the absence of 2% (w/v) SDS. The same determination was also performed after reduction of the possible disulphide bridges by $NaBH_4$.

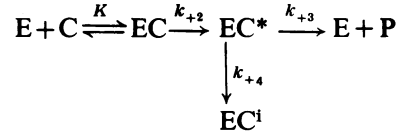
The methyl ester on the 6-carboxylic group of 2,3-dihydro-2,2-dimethyl-1,4-thiazine-3,6-dicarboxylic acid was prepared by incubating 10 mg of β-iodopenicillanic acid for 72 h at 20 °C in 2 ml of anhydrous methanol. The extent of the reaction was determined by measuring the absorbance at 305 nm of a diluted sample.

RESULTS

Comparison with the K1 enzyme from *K. aerogenes* described by Emanuel *et al.* (1986)

Kinetic data. Table 1 compares the kinetic parameters for some substrates determined for the two enzymes under identical conditions. We have reported (De Meester *et al.*, 1986) that the *K. pneumoniae* β-lactamase interacted with β-iodopenicillanate according to a branched pathway (Scheme 1). The ratio k_{+3}/k_{+4} was found to be characteristic of a given enzyme. This ratio was determined by measuring the exact amount of β-iodopenicillanate necessary to inactivate a given quantity of enzyme completely (De Meester *et al.*, 1986). A value of 65±5:1 was found for both enzymes in 50 mM-sodium phosphate buffer, pH 7.0. Moreover, the ratio k_{+2}/K was also determined by using the reporter-substrate method (De Meester *et al.*, 1986), with nitrocefin as the reporter substrate and very large β-iodopenicillanate/enzyme ratios (80000–60000:1). Values of 97000±5000 and 103000±6000 $M^{-1} \cdot s^{-1}$ were found for the *K. pneumoniae* and the *K. aerogenes* enzymes respectively.

Peptide maps. Peptide maps obtained after trypsin digestion of the reduced and carboxymethylated proteins are presented in Fig. 1. The maps are clearly identical (Figs. 1*a* and 1*b*) when obtained with separate samples



Scheme 1. Branched-pathway interaction between enzyme and β-iodopenicillanate

E represents enzyme, C, β-iodopenicillanate, EC* the covalent intermediate, probably the acyl-enzyme, and EC^i the irreversibly inactivated enzyme after re-arrangement of the acyl moiety of β-iodopenicillanate into the dihydrothiazine chromophore, i.e.

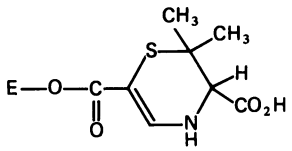


Table 1. Kinetic parameters for the β-lactamases isolated from *K. aerogenes* and *K. pneumoniae*

Buffer...	50 mM-Mops, pH 7.0, containing 0.5 M-NaCl				50 mM-Sodium phosphate, pH 7.0	
	K. aerogenes enzyme		*K. pneumoniae* enzyme		*K. pneumoniae* enzyme	
Substrate	K_m (μM)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}$ (s^{-1})
Benzylpenicillin	188±6	1170±20	192±3	1200±50	55±3	1210±50
Carbenicillin	305±10	180±10	323±13	210±10	125±5	248±10
Cephaloridine	165±10	260±12	165±6	240±10	103±4	305±9
Cephalothin	252±12	290±7	265±9	360±9	66±3	358±12
Nitrocefin	–	–	–	–	5±1*	500±12

* Obtained by substrate competition with benzylpenicillin as substrate.

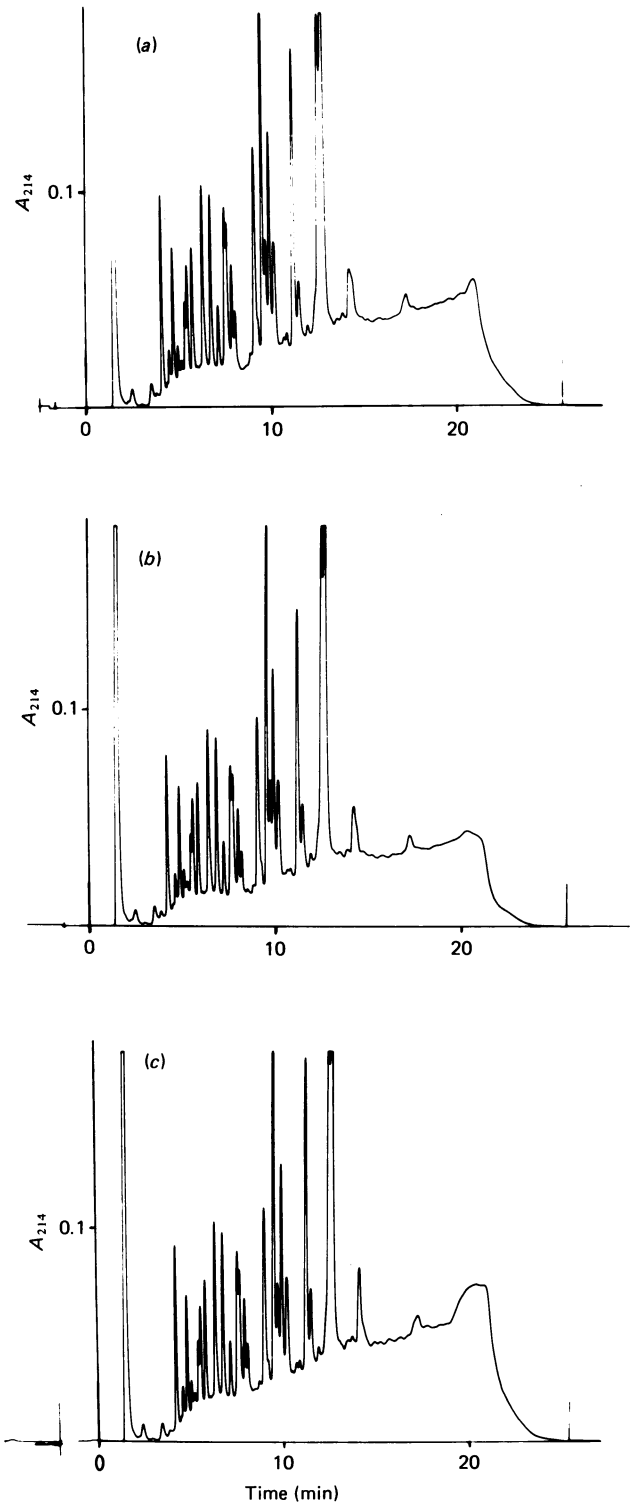


Fig. 1. Peptide maps obtained after tryptic digestions of reduced and carboxymethylated *K. aerogenes* and *K. pneumoniae* β-lactamases

(*a*) β-Lactamase of *K. aerogenes* described by Emanuel *et al.* (1986); (*b*) β-lactamase of *K. pneumoniae* described in the present study; (*c*) equimolecular mixture of both proteins. A 36 μg portion of each reduced and carboxymethylated enzyme was dissolved in 100 μl of 100 mM-sodium phosphate buffer, pH 7.0, containing 4 M-urea, and 4 μg of 1-chloro-4-phenyl-3-tosylamidobutan-2-one-treated trypsin was added. The conditions for the chromatography are described in the Materials and methods section.

of each enzyme, and also, more convincingly, when obtained with an equimolecular mixture of the proteins (Fig. 1*c*). The amino acid compositions of the two proteins (not shown) did not differ significantly.

Thiol-group determination

The enzyme (22 μM) was incubated for 4 h at 25 °C with 1 mM-dithiothreitol in a total volume of 415 μl of 50 mM-sodium phosphate buffer, pH 7.0. The solution was then dialysed against 100 ml of the same phosphate buffer. To 150 μl of the dialysed solution, 100 μl of 100 mM-Na_2HPO_4 and 10 μl of a 10 mM solution of 5,5′-dithiobis-2-nitrobenzoate in ethanol were added. The absorbance at 412 nm immediately increased to 0.07, a value identical with that observed when the dialysis buffer was similarly treated. The absorbance, however, continued to increase, but very slowly (about 0.015 in 10 min). Upon addition of 20 μl of a 20% SDS solution, the absorbance rapidly increased to 0.244 and stabilized within 5 min. In the case of the dialysis buffer, addition of the same amount of the SDS solution only resulted in an increase of the absorbance to 0.09. On the basis of a value of $\epsilon = 14130\ M^{-1}\cdot cm^{-1}$ for the Ellman chromophore, it was computed that the addition of SDS made 0.92 thiol group per enzyme molecule accessible to the reagent. After treatment of the protein with $NaBH_4$, reaction with 5,5′-dithiobis-2-nitrobenzoate indicated the presence of 1.4 thiol groups per enzyme molecule, which suggested the absence of disulphide bridges in the native enzyme. The enzyme thiol group was also modified by 5,5′-dithiobis-2-nitrobenzoate in the presence of 5 M-guanidinium chloride, as described by Emanuel *et al.* (1986). In agreement with those authors, it was found that the K_m value of the enzyme exhibited a 4-fold increase, after removal of the guanidinium chloride by dialysis.

Isolation and analysis of active-site peptides

As observed with other β-lactamases, the k_{+3}/k_{+4} ratio decreased with increasing ionic strength. In consequence, and in order to consume a minimum of ^3H-labelled β-iodopenicillanate, inactivations of large quantities of enzyme were performed in 50 mM-sodium phosphate buffer, pH.7.0, containing 1 M-NaCl. In a first experiment, 10 mg of enzyme at a concentration of 2.4 mg/ml was treated with a 5-fold molar excess of β-iodo[^3H]-penicillinate. The fully inactivated sample was dialysed exhaustively against 50 mM-sodium phosphate buffer, pH 7.0, and freeze-dried. The residue was dissolved in 500 μl of 8 M-urea and incubated at 37 °C for 60 min. The total volume was then adjusted to 2 ml by addition of 100 mM-NH_4HCO_3 containing 0.1 mM-$CaCl_2$. Then 1 mg of trypsin was added and digestion was performed for 60 min at 37 °C. The labelled peptide was purified by chromatography on Sephadex G-25 (K_D approx. 0.25) and the Pro-RPC column as described above. Three radioactive peaks containing respectively 15, 60 and 25% of the total radioactivity were separated on the reverse-phase column, and only the second one was subsequently analysed. The *N*-terminal residue was identified on 1.0 nmol. Only phenylalanine was found. The amino acid composition, determined after acid hydrolysis of 1 nmol, is given in Table 2. The results indicated that the peptide was rather large (about 35 residues) and that it contained two lysine and one arginine residues, which strongly suggested an incomplete digestion. The large M_r was in

Table 2. Amino acid composition of the large (1) and small (2) active-site peptides obtained by tryptic digestion of *K. pneumoniae* β-lactamase

Residue	Peptide 1			Peptide 2		Peptide 1–2(peptide 2)
	Amount (nmol)	No. of residues (Lys = 2) (calc.)	No. of residues (Lys = 2) (rounded off)	Amount (nmol)	No. of residues (rounded off)	No. of residues (rounded off)
Lys	0.64	2.0	2	1.0	1.0	0
His	0.13	0.4	1			1
Arg	0.29	0.9	1			1
Asx	0.92	2.9	3			3
Thr	0.99	3.1	3	0.95	1	1
Ser	1.18	3.7	4	1.6	2	0
Glx	0.69	2.15	2			2
Gly	0.74	2.3	2	0.5	0	2
Ala	1.33	4.15	4	1.0	1	2
Val	0.57	1.8	2			2
Met or Met(O_2)	0.54 / –	1.7 / –	2 / –	0.95	1	0
Ile	0.37	1.15	1			1
Leu	0.79	2.5	3			3
Tyr	0.16	0.5	1			1
Phe	0.57	1.8	2	0.7	1	0
$Cys(O_3H)$*	0.42	1.3	2	1.2	1	0
Total			35		8	19

* Under our conditions, a ninhydrin-positive compound with the same chromatographic behaviour as cysteic acid was produced during the HCl hydrolysis of peptide 1 that had not been submitted to performic oxidation. No cysteic acid seemed to be present in the peptide, since the corresponding phenylhydantoin was not found on sequencing. Peptide 2 was hydrolysed after performic oxidation as explained in the Materials and methods section. Similarly, 65 nmol of the methyl ester of 2,3-dihydro-2,2-dimethyl-3,6-dicarboxylic acid was submitted to performic oxidation followed by 6 M-HCl hydrolysis. On analysis, two ninhydrin-positive compounds were found: the first one behaved as cysteic acid and the area of the peak corresponded to 20 nmol (30%); the second behaved as glycine and the area of the peak corresponded to 11 nmol (16%). These results might explain the slight excess of cysteic acid found in peptide 2 and the presence of some of the contaminating glycine.

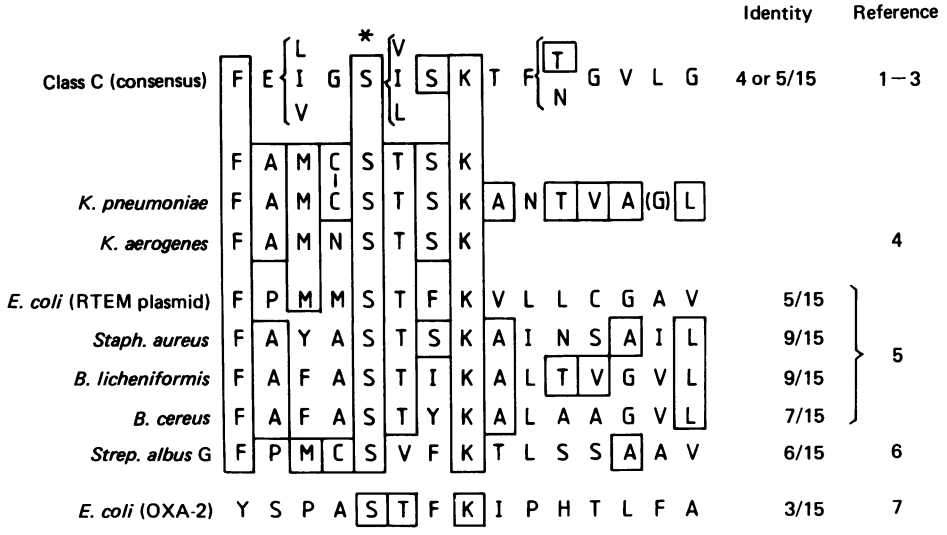


Fig. 2. Partial structure of the large active-site peptide of *K. pneumoniae* β-lactamase as determined by sequential degradation

The identification of the fourth residue as cysteine rests upon the determination of the composition of the smaller peptide. Two runs were performed, respectively with 2.0 and 1.6 nmol of peptide, on the basis of the radioactivity. The sequence is compared with those of the *K. aerogenes* enzyme and of various active-site-serine β-lactamases. Boxes surround residues that are identical in the *K. pneumoniae* β-lactamase and in one or several of the other enzymes, from *Escherichia coli* RTEM plasmid, *Staphylococcus aureus*, *Bacillus licheniformis*, *Bacillus cereus*, *Streptomyces albus* G and *Escherichia coli* R46 plasmid (OXA-2). References: (1) Jaurin & Grundström (1981); (2) Lindberg & Normark (1986); (3) Joris *et al.* (1985, 1986) and M. Galleni (unpublished work); (4) Emanuel *et al.* (1986); (5) Ambler (1980); (6) Dehottay *et al.* (1986); (7) Dale *et al.* (1985).

agreement with the relatively low K_D observed after filtration on the Sephadex G-25 column. Sequence analysis, performed with 3 nmol, yielded the results displayed in Fig. 2. It should be noted that the molarity of the peptide was computed on the basis of the radioactivity, i.e. of the amount of covalently bound dihydrothiazine. The fourth residue could not be identified with certainty, and after the eighth step the yield decreased dramatically. On the basis of these results, we hypothesized that the isolated peptide might have resulted from the formation of a disulphide bond between two active-site peptides, a small one resulting from tryptic cleavage after lysine-8 and a larger one resulting from incomplete digestion and cleavage after arginine-27. That arginine was the 27th residue of the large peptide was deduced on the basis of the amino acid composition (peptide 1)−(peptide 2). An arginine residue is found in a homologous position in the class A β-lactamase produced by *Bacillus licheniformis* (Ambler, 1980). The unidentified residue could have been cystine. The other radioactive peptides detected by fast protein liquid chromatography might have been dimers of the small and large peptides.

To clarify the situation, we performed a new tryptic digestion after blocking of the thiol groups as explained below. The enzyme (3.5 mg, 120 nmol) at a concentration of 3 mg/ml was dialysed against 50 mM-sodium phosphate buffer, pH 7.0, containing 1 M-NaCl. To the solution was added 0.6 μmol of β-iodo[^{3}H]penicillanate and the mixture was incubated at 30 °C for 30 min. The solution was then dialysed against 50 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol and 8 M-urea (u.v.-absorption spectrum is shown in Fig. 3*a*); to this was then added 5,5′-dithiobis-2-nitrobenzoate at a final concentration of 2.7 mM and the mixture was subsequently incubated at 30 °C for 30 min. After exhaustive dialysis against water, a u.v.-absorption spectrum was recorded (Fig. 3*b*) and the solution was freeze-dried. The yellow powder thus obtained was dissolved in 200 μl of 100 mM-NH_4HCO_3 containing 8 M-urea and the solution was incubated at 37 °C for 1 h. After the addition of 0.3 mg of trypsin in 200 μl of water, digestion was performed at 37 °C for 2 h. The sample was then filtered through the Sephadex G-25 column. Two main groups of radioactive fractions also containing a chromophore absorbing at 325 nm were obtained. A first one (about 20% of the radioactivity) exhibited a K_D of 0.27. It probably contained incompletely digested peptide and was not further analysed. The second one (80% of the radioactivity) exhibited a K_D of 0.73. The ratio of radioactivity to A_{325} remained essentially constant in those fractions, which were pooled and freeze-dried. (A yellow band, probably corresponding to the contaminating dithiothreitol–5,5′-dithiobis-2-nitrobenzoate adduct, was strongly adsorbed on the support and was eluted well after the salts.) The freeze-dried powder was dissolved in 300 μl of water (u.v.-absorption spectrum: Fig. 3*c*). Final purification of the peptide was obtained by chromatography on the Pep-RPC column (Fig. 4). The fractions (22 and 23) corresponding to the major radioactive peak were pooled and freeze-dried, the powder was dissolved in 0.3 ml of 10 mM-NH_4HCO_3 and a u.v.-absorption spectrum was recorded (Fig. 3*d*). On the basis of the radioactivity, a concentration of 10 μM in the sample was computed for the dihydrothiazine group (arising from the re-arrangement of the acyl enzyme

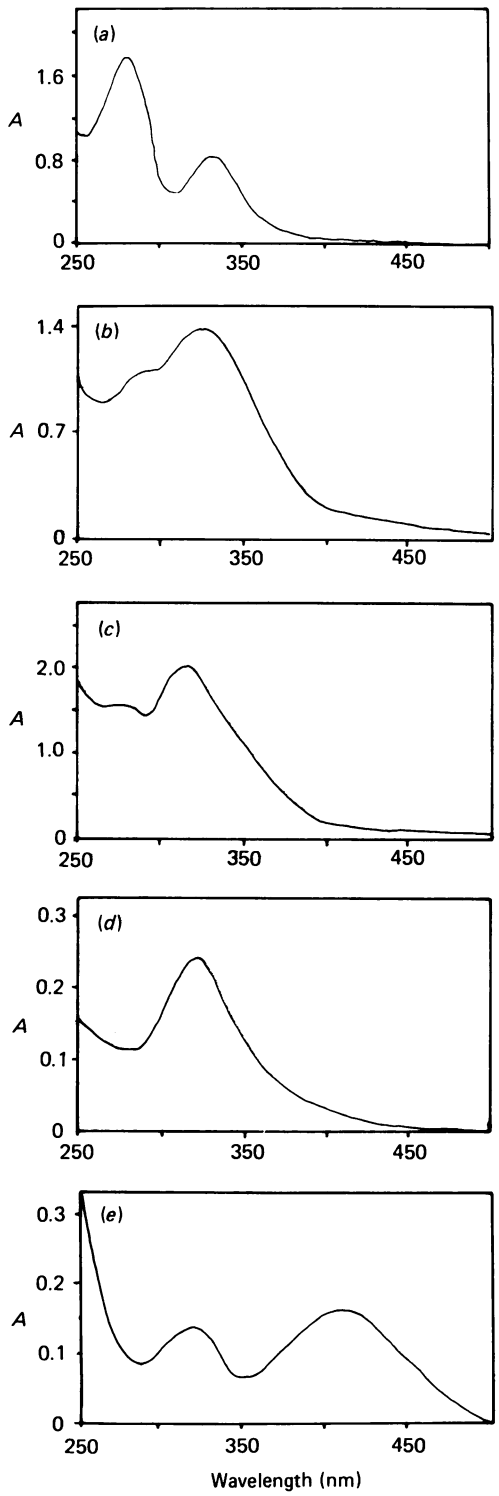


Fig. 3. U.v.-absorption spectra of *K. pneumoniae* β-lactamase after (*a*) inactivation by β-iodo[^{3}H]penicillanate and (*b*) further treatment with 5,5′-dithiobis-2-nitrobenzoate and dialysis, (*c*) of the fractions containing the smaller radioactive peptide after gel filtration, (*d*) of the radioactive peptide after fast-protein-liquid-chromatographic purification, and (*e*) of the same peptide after the addition of 2-mercaptoethanol

The high value of A_{325} observed in (*b*) was due to contamination by the adduct formed between dithiothreitol and Ellman's reagent. This adduct did not dialyse well and adhered to the dialysis bag. It was eliminated during the filtration on Sephadex G-25.

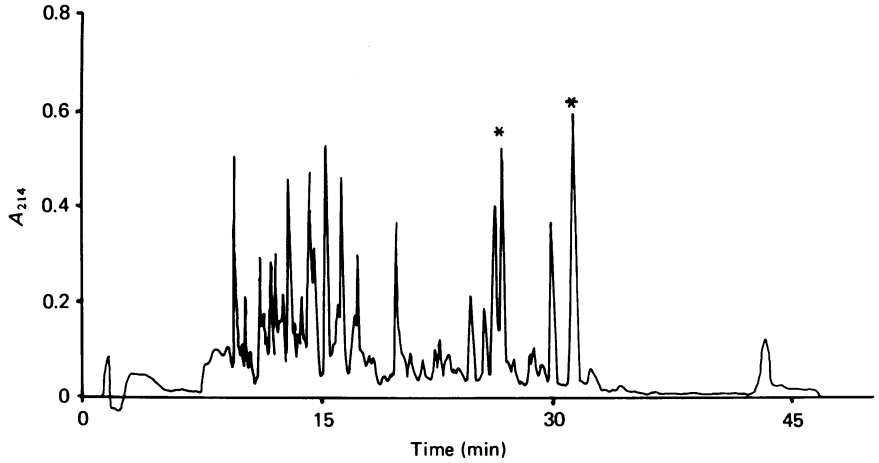
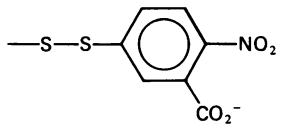


Fig. 4. Purification of the small active-site peptide of *K. pneumoniae* β-lactamase on the Pep RPC column

After injection of the sample, elution was first performed with 1 ml of buffer A. The concentration of buffer B was linearly increased to 20% over 4 ml, then to 70% over 16 ml and finally to 100% over 1 ml. Fractions (0.5 ml) were collected and the flow rate was 0.5 ml/min. Detection was at 215 nm. The peaks marked with asterisks were radioactive and exhibited absorbance at 320 nm. The first (fraction 18) and second (fractions 22+23) peaks contained 25% and 75% of the radioactivity respectively. The first peak was not further analysed. It could have been produced by decarboxylation of the dihydrothiazine chromophore, a phenomenon that has been observed (Cohen & Pratt, 1980) to happen under acidic conditions.

formed by interaction of β-iodopenicillanate with the enzyme). Assuming that the dihydrothiazine was the only chromophore in the solution, a molar absorption coefficient of 24600 $M^{-1} \cdot cm^{-1}$ at 325 nm was calculated, which was considerably higher than that usually observed for that chromophore (12500 $M^{-1} \cdot cm^{-1}$). The high absorbance could be explained by the presence of the



chromophore, obtained upon reaction with 5,5′-dithiobis-2-nitrobenzoate. In agreement with that hypothesis, addition of an excess of mercaptoethanol (1.2 mM, final concentration) resulted in a dramatic change in the spectrum: the absorbance around 320 nm decreased and a new absorption band appeared centred around 410 nm, corresponding to the free Ellman chromophore (Fig. 3*e*). At this stage, the concentrations of the dihydrothiazine and of the free Ellman chromophore could be computed to be 11.1 and 11.3 μM respectively. These values were thus in excellent agreement with that derived above from the radioactivity of the sample. The only *N*-terminal residue, identified as above, was phenylalanine. The amino acid composition, determined after performic acid oxidation and acid hydrolysis of 1.5 nmol, is given in Table 2. This composition exactly corresponded to that of the sequenced large peptide before the decrease in the repetitive yield, if the fourth residue was assumed to be cysteine.

DISCUSSION

Clearly, the comparison of most of the properties of the β-lactamases of *K. pneumoniae* SC10436 and *K. aerogenes* 1082E indicated that both proteins were very similar. However, a striking difference was found in the sequence of the active-site peptide: we identified the residue immediately preceding the active-site serine residue as the sole cysteine residue present in the *K. pneumoniae* enzyme. At the same position in the *K. aerogenes* enzyme Emanuel *et al.* (1986) found an asparagine residue. This means that, after tryptic digestion of the reduced and carboxymethylated protein, at least two peptides should have been different. Indeed, in the active-site octapeptide, carboxymethylcysteine (in the *K. pneumoniae* β-lactamase) should replace asparagine (in *K. aerogenes*). Conversely, the sole carboxymethylcysteine residue should be present in another peptide of the *K. aerogenes* enzyme, and this other peptide should be different in the *K. pneumoniae* enzyme, since both enzymes contain only one cysteine residue and no disulphide bridge. It is possible that, under the acidic conditions utilized for obtaining peptide maps, a carboxymethylcysteine-containing peptide would not behave very differently from a similar peptide containing asparagine instead of carboxymethylcysteine. It also remains possible that the peptides that would allow differentiation between the two enzymes were eluted in the large peak found at the void volume of the column. Indeed, our chromatograms show 27 major peptides, out of 40 possible tryptic peptides, computed on the basis of the content of lysine and arginine. A last possibility would be that the peptide isolated by Emanuel *et al.* (1986) was not the active-site peptide, but a nearly identical peptide arising from another part of the protein. Indeed, those authors did not label the active-site serine residue before the tryptic digestion step. However, such a near-duplication of an active-site peptide in a β-lactamase would be a quite unique phenomenon.

In both native proteins the free thiol group was not easily accessible to 5,5′-dithiobis-2-nitrobenzoate. Utilization of a strong denaturing agent permitted modification of the thiol group and activity was recovered upon renaturation with a significant modification of the affinity for the substrate. This could easily be explained by the presence of the cysteine residue near the active site. The kinetic data of Emanuel *et al.* (1986) suggested that the rate-determining step in the hydrolysis of benzyl-

penicillin by the K1 β-lactamase was the cleavage of the β-lactam ring, and thus the formation of the acyl-enzyme. Under these conditions the K_m would mainly reflect the value of the dissociation constant of the non-covalent Michaelis complex, and it would be easy to visualize an impaired binding of the substrate due to the presence of a bulky group near the active site. It may be noted that the presence of a cysteine residue just before the active-site serine residue is not unique to our K1 β-lactamase. Indeed, a cysteine residue in the same position was found in the extracellular β-lactamase of *Streptomyces albus* G, another class A enzyme, both by sequencing of an active-site peptide (F. De Meester, unpublished work) and as deduced from the nucleotide sequence of the gene (Dehottay *et al.*, 1986). In that case, a peptide labelled both by Ellman's reagent and by β-iodopenicillanate was also obtained.

In conclusion, the determination of the sequence of the peptide isolated after tryptic digestion of the β-iodopenicillinate-labelled enzyme clearly agrees with the hypothesis that the K1 β-lactamase is a class A enzyme. Our enzyme could, however, be slightly different from that isolated and described by Emanuel *et al.* (1986). More extended sequence data or the establishment of the structure of the gene might decide between the various possibilities presented in this discussion.

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