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STUDIES ON THE PRIMARY STRUCTURES OF THE EXOCELLULAR D-ALANYL-D-ALANINE PEPTIDASES OF *STREPTOMYCES* STRAIN R61 AND *ACTINOMADURA* STRAIN R39

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The M_r 37 000 D-alanyl-D-alanine peptidase excreted by *Streptomyces* R61 and the M_r 53 000 D-alanyl-D-alanine peptidase excreted by *Actinomadura* R39 are both characterized by a very uneven distribution of the basic (Arg + Lys) amino acid residues. Trypsin degradation of the heat-denatured enzymes generates (1) thirteen soluble peptides which contain from 2 to 28 residues in the case of the R61 enzyme and nineteen soluble peptides which contain 2 to 39 residues in the case of the R39 enzyme; and (2) three large segments or core peptides which, irrespective of the enzymes from which they originate, consist of 50–60, 70–80 and 110–120 residues. About 90% of the basic (Arg + Lys) amino acid residues are recovered in the soluble tryptic peptides. The core peptides represent 62% ($M_r \approx 23\,000$) and 45% ($M_r \approx 24\,000$) of the untreated R61 and R39 enzymes, respectively. One 28-residue soluble peptide isolated from the R61 enzyme represents the N-terminal portion of the protein whose sequence has been established. The penicillin attachment site of the R61 enzyme has been located in one of the core peptides. For the R39 enzyme, indirect evidence shows that the penicillin binding site is probably within one of the soluble peptides.

Introduction

Substantial progress has been made in the elucidation of the mode of action of the β-lactam antibiotics at the molecular level [1]. All the bacteria possess several membrane-bound proteins that serve as penicillin receptors. At least several of these receptors are known to be enzymes and in all cases where the characterization could be made, they appear to be different molecular forms of D-alanyl-D-alanine peptidases performing, with varying efficiencies, transpeptidase, carboxypeptidase and endopeptidase activities [1]. The D-alanyl-D-alanine peptidases are involved in the last stages of bacterial wall peptidoglycan synthesis; they distinguish themselves from the usual proteases and peptidases by their ability to attack peptide bonds extending between two D-centres in an α-position to a free carboxyl group. It has been established that penicillin and other β-lactam antibiotics inactivate several D-alanyl-D-alanine peptidases of Gram-positive bacteria [2–5] by immobilizing them, at least for some time, in the form of penicilloyl-enzyme complexes in which the penicilloyl group is ester-linked to an enzyme serine residue. Both the D-alanyl-D-alanine peptidase of *Streptomyces* R61 (in short

Abbreviation: SDS, sodium dodecyl sulphate.

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the R61 enzyme) [2] and that of *Actinomadura* R39 (in short the R39 enzyme) [5] are serine enzymes. However, they differ from each other by their molecular weights (37 000 for the R61 enzyme and 53 000 for the R39 enzyme), their requirements for D-Ala-D-Ala-terminated carbonyl donor and amino acceptor peptides, and their susceptibilities to inactivation by the β-lactam antibiotics, the R39 enzyme being always much more sensitive than the R61 enzyme [1]. How these differences in specificity and functioning are related to the primary structures of the enzymes is a problem of great importance. In order to obtain a partial answer to this question, the R61 and R39 enzymes and the corresponding [^{14}C]benzylpenicilloyl-enzyme complexes were submitted to trypsin degradation and the peptide fragments thus produced were analysed. The results, which include the N-terminal sequence of the R61 enzyme, are reported in the present paper.

Materials and Methods

Enzymes. The R61 enzyme, the R39 enzyme and the [^{14}C]benzylpenicilloyl-enzyme complexes (specific radioactivity, 59 mCi/mmol) were those used previously [2,6]. Both enzymes were 95% pure. Trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethylketone was purchased from Worthington.

Carboxymethylation and trypsin degradation of the R61 and R39 enzymes. Samples of the R61 enzyme (116 nmol) and the R39 enzyme (67 nmol), previously dialyzed against 8 M urea in 0.4 M Tris-HCl buffer, pH 8.6, containing 1 mM EDTA, were treated at room temperature and under nitrogen, with 1 mM dithiothreitol for 10 h and subsequently with 20 mM iodoacetate for 10 min. The samples were dialysed against a 0.5% ammonium bicarbonate solution, boiled for 2 min (which treatment caused the formation of a precipitate in the case of the R61 enzyme) and supplemented with 60 μg trypsin. After 2-h incubation at 37°C, a fresh sample of 60 μg trypsin was added and the mixtures were further incubated for 1 h and freeze-dried. It should be noted that at the end of the incubation, a precipitate was still present in the R61 enzyme digest. This precipitate was eliminated by centrifugation. Increasing the amount of trypsin and/or the duration of the incubation did not alter the results described below. The lyophilized mixtures were dissolved in 200 μl 5% formic acid and submitted to peptide fingerprinting.

Peptide fingerprinting. Peptide mapping was carried out on Whatman 3MM papers essentially as described in Ref. 7. Peptides were first separated by high-voltage electrophoresis at pH 6.5 (90 min; 35 V/cm) using a Gilson High Voltage Electrophorator (model DW) with 10% pyridine/0.5% acetic acid as the electrophoresis buffer. The area of the electrophoretogram containing the neutral peptides was cut out, sewn onto a new paper and submitted to electrophoresis at pH 3.5 (90 min; 50 V/cm) with 0.5% pyridine/5% acetic acid as buffer. The peptides separated at pH 6.5 and pH 3.5 were then submitted to descending chromatography in the solvent 1-butanol/acetic acid/pyridine/water (15 : 3 : 10 : 12, v/v) in a direction at a right angle to that used for the electrophoresis. Peptides were detected by a dilute stain of fluorescamine (F. Hoffmann-LaRoche Inc., Basel) as described in Ref. 8. Since only a minor fraction of the NH_2-terminus of the peptides reacts under these staining conditions, peptides could be further characterized by amino acid analyses.

Polyacrylamide gel electrophoreses. Polyacrylamide gel electrophoresis were performed at pH 7.0 in the presence of 0.1% SDS according to the method described in Ref. 9. Gels contained 10% acrylamide and 0.37% *N,N'*-methylenebisacrylamide. Cytochrome *c* (M_r 13 200), pike parvalbumin III (M_r 10 000) and insulin (M_r 6000) were used as standards.

Amino acid analyses. Peptides were hydrolysed with azeotropic HCl for 24 and 48 h at 110°C. Amino acid analyses were carried out with a Bio-Cal 201 or a Beckman Multichrom 4255 amino acid analyser.

Measurements of radioactivity. A Packard Tri-Carb 2425 Liquid Scintillation Spectrometer and a Packard Radiochromatogram Scanner model 7201 were used for the radioactivity measurements.

Amino acid sequencing. N-terminal sequences were determined using a PS 110 automatic sequencer (Socosi, Paris). The thiazolinones were converted with 1 M HCl and the extraction of the phenylthiohydantoin derivatives was carried out as described in Ref. 10 except that 0.7 ml ethylacetate was used. The phenylthiohydantoin-arginine and phenylthiohydantoin-histidine remaining in the aqueous phase were characterized independently by spot reactions on

Whatman paper with specific staining [11,12]. The phenylthiohydantoin derivatives present in the combined ethyl acetate extracts were identified by: (i) thin-layer chromatography on Silica plates (Merck 5715) in water-saturated butyl acetate/propionic acid/formamide (200 : 7 : 10, v/v); (ii) gas chromatography [13]; and (iii) high-pressure liquid chromatography using a Siemens S100 chromatograph, a Cecil CE212 detector and the two-column system of Frank and Strubert [14]. The elution was carried out with CH_2Cl_2 containing 5% MeOH/3% Me_2SO/0.15% CH_3COOH/0.2% H_2O for the polar phenylthiohydantoin amino acids and CH_2Cl_2 containing 0.5% MeOH and 160 ppm Me_2SO for the apolar phenylthiohydantoin amino acids. Three different runs (referred to as (a), (b) and (c)) were carried out. In experiment (a), carried out with 44 nmol enzyme, an Edman program [10] and 1 M quadrol as coupling buffer were used. The protein film exhibited a high propensity to undergo detachment from the glass cup during evaporation of ethyl acetate. Since the duration of the drying periods decreased as the number of Edman cycles increased, pressurization of the cup compartment with nitrogen was performed manually at the proper times. Because of an increasing background of phenylthiohydantoin amino acids, the experiment was stopped after cycle 17. In experiment (b), 300 nmol enzyme and 0.14 M quadrol were used. The excess of phenylisothiocyanate and quadrol was eliminated by a single 400-s wash with a mixture of benzene/ethyl acetate (60 : 40, v/v) instead of a 200-s wash with benzene followed by a 300-s wash with ethyl acetate as in experiment (a). An excess of hexafluorobutyric acid was used after cycle 11 in order to deposit the loosened protein film back on the spinning cup. Cyclization of Gln-12 might have occurred, explaining the poor yield of this experiment (see Results). In experiment (c), carried out on 150 nmol enzyme, volatile dimethylbenzylamine (to avoid ethyl acetate extraction) was used as coupling buffer. Protein detachment, however, occurred during benzene evaporation. Reducing the loss of protein was achieved, at least in part, by delivering the hexafluorobutyric acid just before the level of benzene reached the bottom of the cup during the 'prevacuum' step and by omitting the subsequent 'fine vacuum' step. Under these conditions, the NH_2-terminal 28 residues could be identified. Finally, the correct functioning of the sequencer was checked by two control assays carried out with horse myoglobin, before experiment (a) and after experiment (c), respectively.

Results

Soluble tryptic peptides of the R61 and R39 enzymes

The amino acid composition of each of the isolated peptides is deposited in the BBA data bank (see footnote). Peptides found in trace amounts were not considered. The total amino acid compositions of all the soluble peptides isolated are given in Table I.

1. The R61 enzyme possesses 8 lysine and 14 arginine residues, i.e. 22 points of attack by trypsin. Fifteen soluble peptides were isolated (Fig. 1). At pH 6.5, five peptides were acidic (peptides A_1–A_5), nine were basic (peptides B_1–B_9) and only one was neutral (its position in the two-dimensional map is not shown). The isolated peptides contained from 2 (peptide B_6) to 28 (peptide A_5) amino acid residues. (Note, however, that tryptophan and CM-Cys were not included in the amino acid analyses.) Peptide A_1 and peptide A_2 had identical amino acid compositions (16 residues) but exhibited different electrophoretic mobilities, peptide A_1 probably originating from deamidation of peptide A_2 (see, for example, Ref. 15). Peptide A_3 and peptide A_4 also had identical amino acid compositions (20 residues) but exhibited different chromatographic mobilities, probably due to the presence of an oxidized methionine in peptide A_3 (decreased chromatographic mobility).

2. The R39 enzyme possesses 5 lysine and 19 arginine residues, i.e. 24 possible points of attack by trypsin. Nineteen soluble peptides were isolated (Fig. 2). At pH 6.5, nine of them were acidic and ten were basic; no neutral peptide was detected. Bearing in mind that tryptophan and CM-Cys were not analyzed, the isolated peptides contained from 2 (peptide B_1) to 39 (peptide A_4) amino acid residues.

3. The sum of the molecular weights of the soluble peptides represented only 36 and 53% of the molecular weights of the original R61 and R39 enzymes, respectively. Altogether the 'R61' soluble peptides contained all the arginine residues and six out of the eight lysine residues present in the R61 enzyme. The 'R39' soluble peptides contained all the lysine residues and 16 out of the 19 arginine residues

TABLE I

OVERALL AMINO ACID COMPOSITION OF THE R61 AND R39 ENZYMES AND OF THE PEPTIDE FRAGMENTS OBTAINED BY TRYPSIN DEGRADATION

CM-Cys, carboxymethylcysteine. n.d., not determined

Residue	R61 enzyme			R39 enzyme		
	Intact protein	Soluble peptides	Core peptides	Intact protein	Soluble peptides	Core peptides
Asx	38	13	25	50	24	26
Thr	38	13	25	34	14	20
Ser	29	6	23	38	25	13
Glx	28	9	19	57	37	20
Pro	11	4	7	25	8	17
Gly	32	13	19	66	45	21
Ala	34	10	24	82	40	42
Val	30	11	19	51	25	26
Met	6	3	3	3	6	0
Ile	9	3	6	10	4	6
Leu	33	8	25	50	25	25
Tyr	13	3	10	10	3	7
Phe	12	3	9	11	3	8
Lys	8	6	2	5	7	0
His	8	4	4	9	3	6
Arg	14	15	0	19	16	3
Trp	4	n.d.	n.d.	6	n.d.	n.d.
CM-Cys	3	n.d.	n.d.	2	n.d	n.d.
Total	350	124	220	528	286	239
M	37400	13500	22900	53500	28000	24000
Polarity (%)	48	52	45	44	41	37

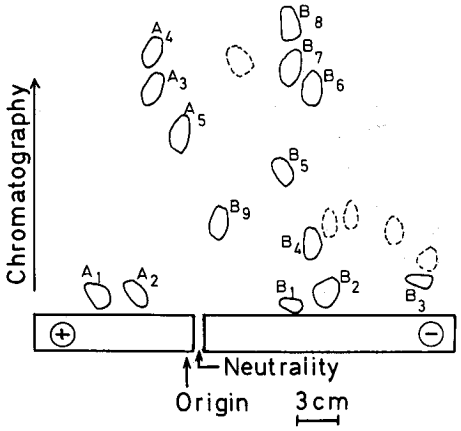

Fig. 1. Fingerprinting of the soluble peptides obtained by trypsin degradation of the R61 enzyme. Horizontal separation is at pH 6.5; vertical separation is by chromatography (for details, see text). The anodic (+) and cathodic (−) sides are indicated. A, acidic peptides; B, basic peptides. The origin and position of the neutral peptides are indicated. Dotted spots, peptides occurring in trace amounts.

present in the R39 enzyme. Both enzymes are thus characterized by a very uneven distribution of the basic amino acids. As a consequence, the tryptic digests should contain large core peptides representing about 62 and 45% of the molecular weights of the R61 and R39 enzymes, respectively (see below). Note that the other polar amino acids did not exhibit such an uneven distribution. On the basis of the criteria of Capaldi and Vanderkooi [16], the soluble peptides had an average polarity only slightly higher than that of the corresponding enzymes.

Identification of peptide A_5 as the N-terminal portion of the R61 enzyme

Fig. 3 gives the NH_2-terminal 28 amino acid residues of the R61 enzyme. Residues beyond position 28 could not be reliably determined (see Materials and Methods). Since an arginine residue occurred at position 28, trypsin cleavage of the enzyme must

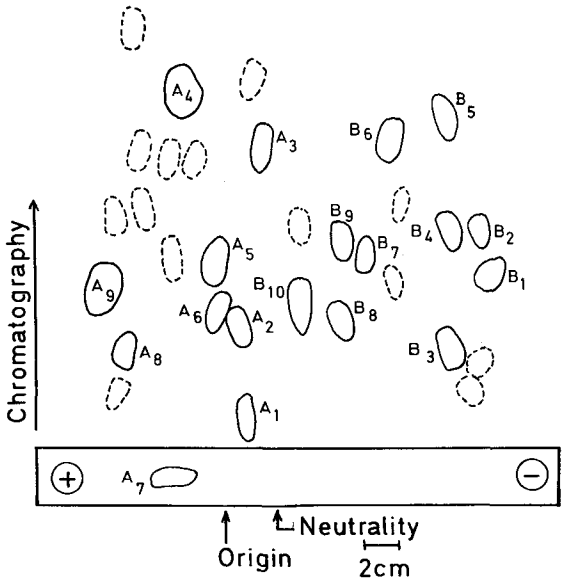

Fig. 2. Fingerprinting of the soluble peptides obtained by trypsin degradation of the R39 enzyme. Use of the symbols is as described in the legend to Fig. 1. The paper zone containing the neutral peptides was not cut out here since no neutral peptide could be detected.

have released a 28 amino acid residue peptide: Asp, 3; Thr, 2; Ser, 1; Gln, 1; Glu, 1; Pro, 3; Gly, 3; Ala, 5; Val, 2; Met, 1; Leu, 4; His, 1 and Arg, 1. Such a composition is exactly that found for peptide A_5, which therefore represents the N-terminal tryptic peptide of the R61 enzyme.

Insoluble tryptic peptides of the R61 and R39 enzymes

In order to recover the insoluble core peptides, samples of the R61 and R39 tryptic digests were analyzed by polyacrylamide gel electrophoresis in the presence of SDS and stained with Coomassie brilliant blue. A scan of these gels is presented in Fig. 4 for the R61 enzyme and in Fig. 5 for the R39 enzyme. In both cases, three peptides were detected and their apparent molecular weights were deduced by comparing their mobilities with those of reference peptides (see Materials and Methods). The tryptic core of the R61 enzyme contained three large peptides of about 6.2, 8.3 and 11.8 kdaltons, and similarly the tryptic core of the R39 enzyme contained three large peptides of 5.6, 6.6 and 11.2 kdaltons. Varying the conditions of enzyme denaturation and trypsin degradation did not alter the gel electrophoresis profiles. The number of the core peptides (three) was close to that of the lysines (two) in the case of the R61 enzyme, and, in the case of the R39 enzyme, equal to that of the arginines (three) that were not found in the analyses of the soluble peptides. Whatever the enzyme, the sum of the molecular weights of the core peptides thus detected (Σ = 26.3 and 23.4 kdaltons; see above) was close to the value (Σ = 22.9 and 24 kdaltons; see Table I) obtained by subtracting the sum of the molecular weights of the soluble peptides from the molecular weight of the intact protein. Hence, to all appearances, D-alanyl-D-alanine

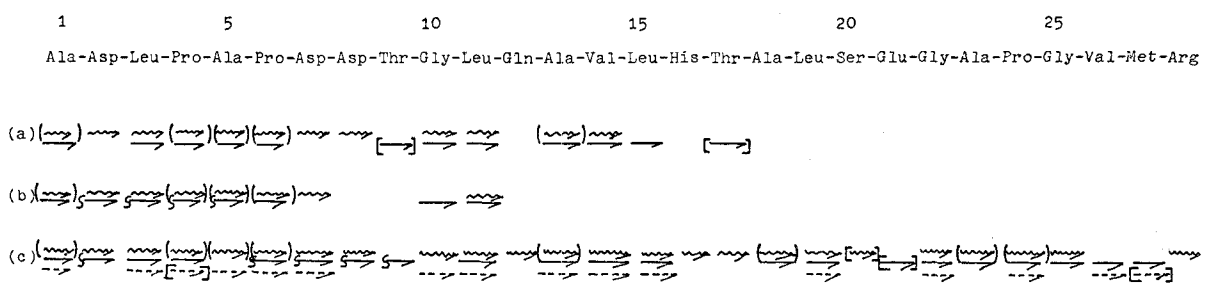

Fig. 3. N-terminal sequence of the R61 eznyme. (a), (b) and (c) refer to three different runs (see Materials and Methods). ~~~~, identified by thin-layer chromatography and for histidine and arginine, by appropriate staining on paper; (~~~~), phenylthiohydantoin-proline or -alanine are not distinguishable from each other by this method; ——›, identified by gas chromatography; S ——›, identified by gas chromatography of the silylated derivative; - - - - -›, identified by high-pressure liquid chromatography. Symbols between square brackets mean that the amounts of phenylthiohydantoin amino acids detected were small but that the qualitative criterion was good enough for an unambiguous identification. Note that each residue, except histidine and arginine, has been identified by at least two different methods.

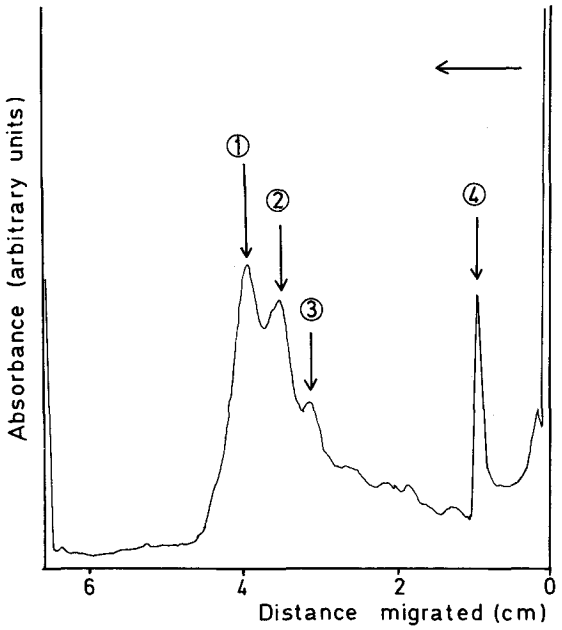

Fig. 4. Polyacrylamide gel electrophoresis of the core peptides of the R61 enzyme. Densitometer tracing. The enzyme (50 μg, 1.3 nmol) was dialysed against 0.5% NH_4HCO_3, boiled for 2 min and digested by 1.5% (w/v) trypsin for 2 h at 37°C. The sample was freeze-dried, dissolved in 10 mM Na_2CO_3 containing 0.3% SDS, supplemented with 20 μg bovine serum albumin and maintained at 37°C during 10 min. The sample was then supplemented with 1 drop of glycerol and deposited on the gel. After 7 h electrophoresis (8 mA per gel), the gels were stained with Coomassie blue and scanned at 265 nm. The arrows (1–3) indicate the position of the peptides. The corresponding molecular weights are 6200 for peptide 1, 8300 for peptide 2 and 11 800 for peptide 3. Arrow No. 4 indicates the position of bovine serum albumin used as internal standard.

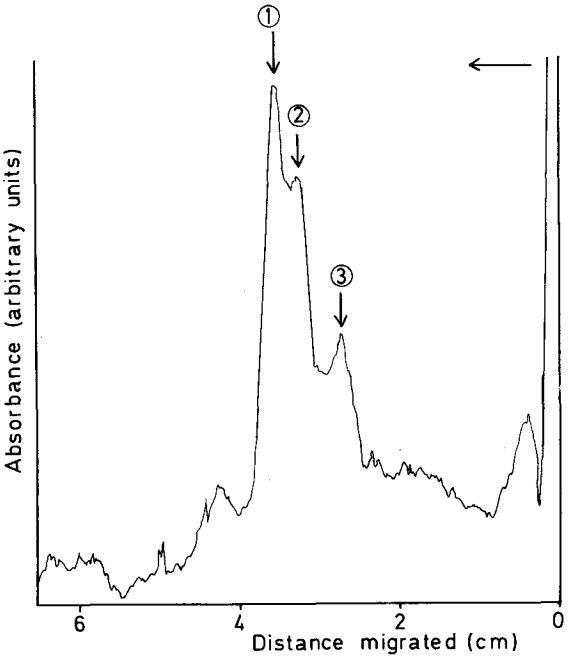

Fig. 5. Polyacrylamide gel electrophoresis of the core peptides of the R39 enzyme. Densitometer tracing. For details, see text and legend of Fig. 4. In the present case, the electrophoresis was carried out for 7 h at 8 mA per gel. The arrows (1–3) indicate the position of the peptides. The molecular weights are 5600 for peptide 1, 6600 for peptide 2 and 11 200 for peptide 3.

peptidases of different molecular weights (37 000 and 53 000) yield core peptides of very similar lengths, when subjected to trypsin degradation.

Assignment of the penicillin binding sites of the R61 and R39 enzymes to one of the tryptic peptides

In previous studies, pronase had been used to degrade the [^{14}C]benzylpenicilloyl-R61 or -R39 enzyme complexes and short peptides containing the active serine residue had been isolated, namely, a tripeptide Val-Gly-Ser in the case of the R61 enzyme [2], and a heptapeptide Leu-Pro-Ala-Ser-Asn-Gly-Val in the case of the R39 enzyme [5]. The question therefore arose as to which of the tryptic fragments contained these penicillin attachment sites.

Concerning the R61 enzyme, previous studies had also shown that after trypsin degradation of the heat-denatured [^{14}C]benzylpenicilloyl-enzyme complex, the radioactive label remained attached to an insoluble peptide [2]. Difficulties in attempting to identify which of the core peptides contained the penicillin attachment site were not expected since heat denaturation of the [^{14}C]benzylpenicilloyl-R61 complex and degradation by various proteolytic treatments resulted in an increased stability of the penicilloyl moiety (Table II). Contrary to expectations, when the heat-treated and trypsin-degraded [^{14}C]benzylpenicilloyl-R61 complex was submitted to polyacrylamide gel electrophoresis in the presence of SDS most of the radioactivity co-migrated with Bromophenol blue. A very small proportion of radioactive label was found associated, apparently, with a 12-kdalton

TABLE II

EFFECTS OF VARIOUS DENATURATION AND DEGRADATION PROCEDURES ON THE STABILITY OF THE ESTER-LINKED [^{14}C]BENZYLPENICILLOYL-R61 ENZYME (OR PEPTIDE) COMPLEX AND ON THE NATURE OF THE RELEASED PRODUCTS

[^{14}C]Benzylpenicilloate was characterized both by paper electrophoresis at pH 6.5 and by chromatography as described in Ref. 2 (using authentic benzylpenicilloate as internal standard). Note that treatment with guanidinium chloride in water resulted in an increased stability but that the nature of the nucleophile played an important role; replacement of water by methanol resulted in a much faster release of the benzylpenicilloyl residue, probably in the form of an α-methyl ester of benzylpenicilloate.

Procedure	Half-life	Released products
Native	100 min (37°C)	phenylacetylglycine + *N*-formyl-D-penicillamine
Heat-denatured	80 h (37°C)	benzylpenicilloate
Heat-denatured and trypsin-treated	35 h (37°C)	benzylpenicilloate
Heat-denatured and trypsin- and pronase-treated	35 h (37°C)	benzylpenicilloate
Denatured in a 6 M guanidium-HCl aqueous solution	several days (20°C)	benzylpenicilloate
Denatured in a saturated solution of guanidium-HCl in methanol	100 min (20°C)	α-methylester of benzylpenicilloate (tentatively)

peptide but the activity was too low to permit a definite identification. Other fractionation procedures were therefore investigated. The heat-denatured and trypsin-degraded [^{14}C]benzylpenicilloyl-R61 enzyme complex (1 ml containing 40 nCi radioactive label, i.e. about 0.8 nmol) was supplemented with SDS (0.1% final concentration) and filtered through a 150-ml column of Sephadex G-75 previously equilibrated against 2 mM sodium phosphate buffer, pH 6.5, containing 0.1% SDS. About 40% of the radioactivity was eluted at a K_D value of 0.15 and 60% at a K_D value of 1 (salt volume). Fraction $K_D = 1$ consisted of [^{14}C]benzylpenicilloate. By reference to standard peptides (cytochrome *c*, pike parvalbumin III and insulin), fraction $K_D = 0.15$ consisted of an 11-kdalton benzylpenicilloyl-peptide. (It is known that in the presence of SDS the proteins fix large quantities of detergent and adopt a rod-shaped conformation which increases their radius of gyration [17]).

Concerning the R39 enzyme, fingerprinting of the [^{14}C]benzylpenicilloyl-enzyme complex under the conditions described above did not permit detection of any radioactively labelled peptide, thus confirming previous observations [5] that heat denaturation and proteolytic degradation of the complex greatly labilized the penicilloyl group. However, a short incubation in the presence of a large amount of trypsin followed by paper electrophoresis at pH 6.5 of the degradation products permitted detection of a negatively charged radioactive peptide exhibiting a mobility of 7 cm · h^{-1} at 60 V/cm, thus suggesting that the penicillin attachment site was part of one of the soluble peptides produced by trypsin treatment. This peptide was not characterized.

Discussion

The present studies have led to the following observations.

(1) The R61 and R39 D-alanyl-D-alanine peptidases possess 22 and 24 possible points of attack by trypsin, respectively. Trypsin proteolysis yields 13 soluble peptides and three core peptides from the R61 enzyme and, similarly, 19 soluble peptides and three core peptides from the R39 enzyme. Considering that some of the small peptides thus produced contain more than one lysine and/or arginine residue (pentapeptide B_3 from the R61 enzyme, for example, consists of one lysine and four arginine residues), it is clear that altogether these small fragments comprise the great majority (85% or more) of all the potential points of attack by trypsin. Yet these fragments represent only 36% (in the case of the R61 en-

zyme) and 53% (in the case of the R39 enzyme) of the masses of the correponding proteins. Both R61 and R39 enzymes thus characterize themselves by a very uneven distribution of the lysine and arginine residues (while the other amino acids are almost equally distributed between the small and large tryptic fragments).

(2) Peptide A_5 from the R61 enzyme contains 28 amino acids and represents the N-terminal portion of this enzyme. Peptide A_5 does not contain the active serine residue and, at this time, its sequence cannot be compared with certainty with the known N-terminal portion of the DD-carboxypeptidase of *Bacillus subtilis* where the active serine occurs at position 36 [4].

(3) The R61 and R39 enzymes consist of single polypeptide chains. In spite of their different molecular weights (37 000 and 53 000, respectively), there exist in each enzyme three large segments (or core peptides) of about 50–60, 65–80 and 110–120 residues, respectively, which escape trypsin action. This feature suggests that, structurally, the R61 and R39 enzymes differ from each other mainly in those regions which do not form the core peptides and are specifically enriched in the basic amino acid residues. Under trypsin action, however, the active serine residue remains part of one of the core peptides in the case of the R61 enzyme, while it is probably released in the form of a small soluble peptide in the case of the R39 enzyme.

(4) The present studies, as well as others [5], amply demonstrate that the stability of the ester bond which links the benzylpenicilloyl group to the residue much depends on the environmental conditions. In particular, the benzylpenicilloyl-peptide fragment obtained after trypsin degradation of the benzylpenicilloyl-enzyme complex is, somehow, greatly labilized under conditions of polyacrylamide gel electrophoresis in SDS. In all cases, fragmentation of the benzylpenicilloyl group [2] is a mechanism of release of the bound metabolite which occurs only with native complexes.

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References

1 Ghuysen, J.M. (9180) in Topics in Antibiotic Chemistry (Sammes, P.G., ed.), Vol. 5, pp. 9–117, Ellis Horwood Ltd., Chichester
2 Frère, J.M., Duez, C., Ghuysen, J.M. and Vandekerckhove, J. (1976) FEBS Lett. 70, 257–260
3 Georgopapadakou, N., Hammarström, S. and Strominger, J.L. (1977) Proc. Natl. Acad. Sci. USA 74, 1009–1012
4 Yocum, R.R., Waxman, D., Rasmussen, J.R. and Strominger, J.L. (1979) Proc. Natl. Acad. Sci. USA 76, 2730–2734
5 Duez, C., Joris, B., Frère, J.M., Ghuysen, J.M. and Van Beeumen, J. (1981) Biochem. J. 193, 83–86
6 Fuad, N., Frère, J.M., Ghuysen, J.M., Duez, C. and Iwatsubo, M. (1976) Biochem. J. 155, 623–629
7 Ryle, A.P., Sanger, F., Smith, L.P. and Kitai, R. (1955) Biochem. J. 60, 541–556
8 Vandekerckhove, J. and Van Montagu, M. (9174) Eur. J. Biochem. 44, 279–288
9 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
10 Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80–91
11 Yamada, S. and Itano, H.A. (1966) Biochim. Biophys. Acta 130, 538–540
12 Pauly, H. (1904) Z. Physiol. Chemie 42, 508–518
13 Van Beeumen, J., Van Damme, J. and De Ley, J. (1976) I.U.B. Congress of Biochemistry, Hamburg, Abstr. p. 188
14 Frank, G. and Strubert, W. (1973) Chromatographia 6, 522–524
15 Ambler, R. (1963) Biochem. J. 89, 349–378
16 Capaldi, R.A. and Vanderkooi, G. (1962) Proc. Natl. Acad. Sci. USA 69, 930–932
17 Reynolds, J.A. and Tanford, C. (1970) J. Biol. Chem. 245, 5161–5165