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On the DD-Carboxypeptidase Enzyme System of *Streptomyces* Strain K15

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Streptomyces K15 possesses a set of exocellular and cell-bound D-alanyl-D-alanine carboxypeptidases. Four of them have been isolated to the stage where each enzyme preparation contains one single penicillin-binding protein. The exocellular 54000-M_r enzyme is extremely sensitive to benzylpenicillin and performs low transpeptidase activity on the carbonyl-donor/amino-acceptor tetrapeptide AcLLys(Gly)-DAla-DAla. The exocellular 40000-M_r enzyme and the two lysozyme-releasable 40000-M_r and 38000-M_r enzymes are moderately sensitive to benzylpenicillin and have a high propensity to catalyse dimer formation from the aforementioned tetrapeptide monomer.

The D-alanyl-D-alanine carboxypeptidases are enzymes involved in the crosslinking step of bacterial cell wall synthesis [1]. They catalyse attack, by a suitable exogenous nucleophile, of the carbonyl carbon of the DAla-DAla amide bond of LXaa-DAla-DAla-terminated peptides, where Xaa is most often a diamino acid residue. These enzymes exhibit widely varying molecular weights, sensitivities to β-lactam antibiotics, specificity profiles for the carbonyl-donor peptides and abilities to use amino compounds as nucleophilic acceptors of the LXaa-DAla moieties in transpeptidation reactions [2]. In addition, they can operate by at least two different mechanisms: four serine DD-carboxypeptidases [3–5] and one Zn^{2+} DD-carboxypeptidase [6,7] are known. A survey, carried out under standard but arbitrary conditions, of the activities performed by the culture filtrates obtained from various strains of actinomycetes, led to the isolation of the very highly penicillin-sensitive, 53000-M_r R39 DD-carboxypeptidase (from *Actinomadura* R39) [4], the moderately penicillin-sensitive, 38000-M_r R61 DD-carboxypeptidase (from *Streptomyces* R61) [3], and the highly penicillin-resistant 18000-M_r G DD-carboxypeptidase (from *Streptomyces albus* G) [6–7]. The R61 and R39 enzymes are serine peptidases; they are able to perform transpeptidation reactions. The G enzyme is a Zn^{2+} peptidase; it functions only as a hydrolase. The question whether the R61, R39 and G enzymes were strain-specific or not has been examined. It has been found that *Streptomyces* strain K15 excretes and/or possesses, bound to its cell envelope, a set of DD-carboxypeptidases. On the basis of their penicillin sensitivities and substrate requirements, one of them is a R39-like enzyme and the others are R61-like enzymes.

MATERIALS AND METHODS

DD-Carboxypeptidase/Transpeptidase Assays

The procedures used were those described previously [8,9]. Unless otherwise stated, the substrate concentrations were: 1.8 mM Ac_2LLys-DAla-DAla (DD-carboxypeptidase activity; reaction catalysed: Ac_2LLys-DAla-DAla + H_2O → DAla + Ac_2LLys-DAla; measurements based on the DAla released) and 1.8 mM Ac_2LLys-DAla-DAla + 1.8 mM [^{14}C]Gly-Gly (transpeptidase activity; reaction catalysed: Ac_2LLys-DAla DAla + [^{14}C]Gly-Gly → DAla + Ac_2LLys-DAla-[^{14}C]Gly-Gly; measurements made on the radioactive tetrapeptide formed). The incubations were carried out at 37 °C in 30 µl (final volumes) of 30 mM Tris/HCl pH 8.0 for the exocellular enzymes and 5 mM Tris/HCl pH 7.5 for the lysozyme-releasable enzymes. The specific enzyme activities (DD-carboxypeptidase assays) were expressed in amounts of tripeptide hydrolysed × min^{-1} × mg $protein^{-1}$. DD-Carboxypeptidase activity was also measured by using N^{α}AcLLys-DAla-DAla or UDP-AcMur-LAla-DGlu[(L)A_2pm(L)-DAla-DAla] as carbonyl donors (release of the C-terminal DAla).

The T/Hy ratio values of reactions, where transpeptidation (T) and hydrolysis (Hy) occurred concomitantly, were determined with the system [^{14}C]Ac_2LLys-DAla-DAla and either A_2pm or Gly-LAla. The reaction products are [^{14}C]-Ac_2LLys-DAla (Hy) and [^{14}C]Ac_2LLys-DAla-A_2pm or [^{14}C]-Ac_2LLys-DAla-Gly-LAla (T). Alternatively, the tetrapeptide [^{14}C]AcLLys(Gly)-DAla-DAla was used both as carbonyl donor and amino acceptor. The reaction products are [^{14}C]-AcLLys(Gly)-DAla (Hy) and [^{14}C]AcLLys-DAla-(DAla)[^{14}C]-AcLLys(Gly)-DAla-Gly.

Exocellular and Lysozyme-Releasable D-Alanyl-D-alanine Carboxypeptidases

Streptomyces K15 was grown in the glycerol/casein medium and the culture filtrate submitted to fractionation as described in [8] (exocellular enzymes). In turn, *Streptomyces* K15 was grown in Merck peptone (batch 7213) medium and the washed mycelium was transformed into protoplasts with lysozyme in a sucrose medium [8]. After centrifugation, the supernatant fraction was used as source of lysozyme-releasable enzymes. The proteins were estimated following the technique of Lowry et al. [10] or by measuring the absorbance of the samples at 280 nm and 260 nm [11].

Separation and Detection of [^{14}C]Benzylpenicillin-Binding Proteins

Samples were successively (a) incubated for 15 min at 37 °C with 0.2 mM [^{14}C]benzylpenicillin (52 Ci/mol; from

Abbreviations. AcMur, *N*-acetylmuramic acid; A_2pm, *meso*-2,2′-diaminopimelic acid; AcLys, N^{α}-acetyllysine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

Amersham); (b) supplemented with 30 µl 25 mM non-radioactive benzylpenicillin in 125 mM Tris/HCl buffer pH 6.8 containing 0.001% bromophenol blue, 2% sodium dodecylsulphate, 20% glycerol and 30% mercaptoethanol; (c) heated at 100 °C for 2 min; and (d) submitted to electrophoresis in polyacrylamide slab gels containing sodium dodecylsulphate [12]. The gels were stained with Coomassie brilliant blue and then examined by fluorography [13, 14]. The penicillin-binding proteins were estimated by microdensitometry of the fluorograms with the help of a Joyce Loebl Mk3CS densitometer. Catalase (M_r 60000), D-amino acid oxidase (M_r 37000) and chymotrypsinogen (M_r 25700) were used as standard proteins of known molecular weight.

Polyacrylamide Gel Electrophoresis at pH 8.4, under Non-denaturing Conditions

After a prerun of 18 h, the electrophoreses were carried out in Tris/glycine buffer pH 8.4 as described in [15], for 90 min and at 3 mA/gel. In some cases the cylindrical gels were sliced into 2-mm sections and each portion was extracted with 100 µl water for 18 h at 4 °C and the DD-carboxypeptidase activity measured on 10-µl samples of the extracts thus obtained.

Anti-(Exocellular R61 Enzyme) Antiserum

The antiserum was that used previously [16] The IgGs were prepared as described in [17].

Detection of [^{14}C]Benzylpenicilloate and [^{14}C]Phenylacetylglycine

[^{14}C]Benzylpenicilloate migrated by electrophoresis at pH 6.5 and on 3MM Whatman paper with a mobility towards the anode, of 25 cm × h^{-1} at 60 V × cm^{-1}. [^{14}C]Phenylacetylglycine migrated by thin-layer chromatography on Polygram Sil G (Macherey-Nagel Co), at 22 °C and using as solvent the mixture chloroform/methanol/acetic acid: 88/10/2, v/v/v), with an R_F of 0.56. Under the same conditions [^{14}C]benzylpenicilloate remained at the origin of the chromatogram.

β-Lactamase

β-Lactamase Rikker was used. 1 unit corresponds to 1 µmol benzylpenicillin hydrolysed × min^{-1}.

Bacterial Cell Walls

The walls of *Streptomyces* K15 were prepared and the primary structure of the wall peptidoglycan polymer was investigated using the techniques applied previously to *Streptomyces* strain R61 and *albus* G [18].

RESULTS

The Wall Peptidoglycan of Streptomyces *K15*

The wall peptidoglycan had the same primary structure as that found in other *Streptomyces* strains [18, 19], i.e. a structure in which glycan strands are substituted by L-alanyl-D-γ-glutamyl-(L$_1$)LL-diaminopimelyl(L$_1$)-D-alanine peptides and where these peptide units are in turn crosslinked via D-alanylglycyl-(L$_2$)LL-diaminopimelic acid linkages. One should note, however, that the position of the amide group in the *Streptomyces* K15 peptidoglycan was not determined [20].

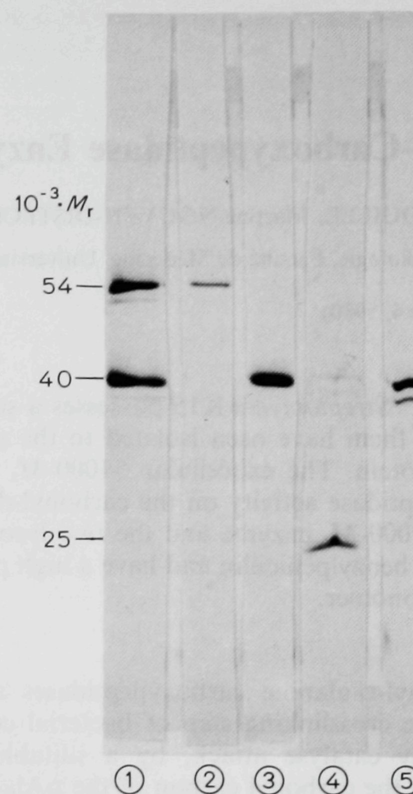

Fig. 1. *Fluorograms obtained after polyacrylamide slab gel electrophoresis in sodium dodecylsulphate of the exocellular and lysozyme-releasable* DD-*carboxypeptidases previously labelled with [^{14}C]benzylpenicillin.* Track 1: the partially purified preparation of exocellular enzymes as described in [8]. Track 2: the purified exocellular 54000-M_r DD-carboxypeptidase. Track 3: the purified exocellular 40000-M_r DD-carboxypeptidase. Track 4: the supernatant fraction obtained after lysozyme treatment of the mycelium (see text). Track 5: the purified lysozyme-releasable 40000-M_r and 38000-M_r DD-carboxypeptidase (as obtained after affinity chromatography). Amounts of proteins submitted to electrophoresis: 35 µg and 200 µg for tracks 1 and 4 respectively; 5 µg in the other cases

*Partial Purification of the Exocellular 54000-*M$_r$ *and 40000-*M$_r$ DD-*Carboxypeptidases*

The partially purified enzyme preparation (specific activity: 330 nmol × min^{-1} × mg protein^{-1}) contained two [^{14}C]-benzylpenicillin-binding proteins exhibiting molecular weights of 54000 and 40000 respectively. Under conditions of penicillin saturation and on the basis of densitometry measurements, they occurred in a proportion of 43% and 57% respectively (Fig. 1, track 1). Each major penicillin-binding protein was accompanied by a 'satellite' penicillin-binding protein of smaller molecular weight (52000 and 38000 respectively) occurring in trace amounts; they were not further studied. The benzylpenicillin concentrations at which extent of labelling was 50% of the maximum value, were 0.064 µM for the 54000-M_r protein and 0.26 µM for the 40000-M_r protein.

The 54000-M_r and 40000-M_r penicillin-binding proteins were separated from each other by two successive filtrations on a 110-ml column of Sephadex G-100. The preparation containing the 54000-M_r enzyme (Fig. 1; track 2) had a specific activity of 0.5 µmol × min^{-1} × mg protein^{-1} and the

Table 1. *Hydrolysis (*DD*-carboxypeptidase assays) of* $N^{\alpha},N^{\varepsilon}$-*Ac₂LLys-DAla-DAla (peptide 1),* N^{α}-*AcLLys-DAla-DAla (peptide 2) and UDP-AcMur-LAla-DGlu[(L)A₂pm(L)-DAla-DAla] (peptide 3)*
With the K15 enzymes all the experiments were carried out at 37 °C and in 35 µl, final volumes, of 30 mM Tris/HCl buffer, pH 8, for the exocellular enzymes or 5 mM Tris/HCl buffer, pH 7.5, for the lysozyme-releasable enzymes. For the determination of the K_m and V parameters the exocellular 54000-M_r enzyme (0.1 µg protein) was incubated for 25 min with [14C]Ac₂LLys-DAla-DAla at concentrations ranging from 0.08 mM to 0.35 mM. The exocellular 40000-M_r enzyme (0.45 µg) and the lysozyme-releasable 38000 – 40000-M_r enzymes (0.66 µg) were incubated for 45 min and 30 min respectively, with Ac₂LLys-DAla-DAla at concentrations ranging from 4.7 mM to 25 mM. The turnover numbers of the K15 exocellular enzymes were estimated assuming a degree of purity of 1.65 % for the 54000-M_r enzyme preparation and 3.75 % for the 40000-M_r enzyme preparation (see text)

Strain	Enzymes	Kinetic parameters for peptide 1			Specific activities as determined at a 1.8 mM concentration of		
		K_m	V	turnover number	peptide 1	peptide 2	peptide 3
		mM	µmol × min⁻¹ × mg protein⁻¹	min⁻¹	µmol × min⁻¹ × mg protein⁻¹		
K15	exocellular 54000-M_r	0.1	0.5	1600 (?)	0.37	0.37	0.27
	exocellular 40000-M_r	14	2	2100 (?)	0.24	0.03	0.02
	lysozyme-releasable 38000 – 40000-M_r	12	1.7	–	0.15	0.006	n.d.
R39	exocellular 53000-M_r [a]	0.8	20	1050	13.2	32	20.8
R61	exocellular 38000-M_r [a]	12	86	3300	10.1	0.04	0.04

[a] For a recent review on the R39 and R61 enzymes, see [2]. For more specific information, see [15, 21 – 23].

preparation containing the 40000-M_r enzyme (Fig. 1; track 3) had a specific activity of 0.25 µmol × min⁻¹ × mg protein⁻¹. When expressed with respect to the original partially purified preparation, the enzyme recoveries were 20 % and 10 % respectively.

In spite of their different molecular weights, the 54000-M_r and 40000-M_r DD-carboxypeptidase had the same electrical charge at pH 8.4. As shown by polyacrylamide gel electrophoresis under non-denaturing condition, DD-carboxypeptidase activity and penicillin-binding ability were, in both cases, associated with one single protein migrating 2.4 cm towards the anode (after 90 min at 3 mA/gel).

*Efficacy of Hydrolysis of DAla-DAla-Terminated Peptides by the Exocellular 54000-*M*ᵣ and 40000-*M*ᵣ DD-Carboxypeptidase (Table 1)*

On Ac₂LLys-DAla-DAla, the 54000-M_r enzyme characterized itself by a low K_m value (0.1 mM) and the 40000-M_r enzyme by a high K_m value (14 mM). The turnover numbers indicated in Table 1 were roughly estimated on the basis that the 54000-M_r and 40000-M_r enzyme preparations were 1.65 % and 3.75 % pure respectively (see below). Replacement of Ac₂LLys-DAla-DAla by equivalent concentrations (1.8 mM) of N^{α}AcLLys-DAla-DAla or UDP-AcMur-LAla-DGlu[(L)-A₂pm(L)-DAla-DAla] had no or little effect on the specific activity of the 54000-M_r enzyme but caused a 10-fold decreased specific activity of the 40000-M_r enzyme (Table 1).

*Efficacy of Transpeptidation by the Exocellular 54000-*M*ᵣ and 40000-*M*ᵣ DD-Carboxypeptidases (Table 2)*

With both enzymes, A₂pm was a better acceptor than Gly-LAla for the Ac₂LLys-DAla moiety of Ac₂LLys-DAla-DAla. At concentrations of Ac₂LLys-DAla-DAla equivalent to 1× (for the 54000-M_r enzyme) or 0.5 and 0.25× (for the 40000-M_r enzyme), the corresponding K_m values, and in the presence of equimolar amounts of A₂pm or Gly-LAla, the 40000-M_r enzyme had a higher transpeptidase efficacy than the 54000-M_r enzyme. Similarly, the ability of the 40000-M_r enzyme to catalyse dimer formation from [14C]AcLLys(Gly)-DAla-DAla (6.6 mM) was 10-fold higher than that of the 54000-M_r enzyme (at equivalent DD-carboxypeptidase activity). Note that the N^{ε}-(D-alanylglycyl)-L-lysine linkage formed by transpeptidation is structurally related to the D-alanylglycyl-LL-diaminopimelic acid linkages, which serve as interpeptide bonds in the wall peptidoglycan of *Streptomyces* K15.

*Reaction between Benzylpenicillin and the Exocellular 54000-*M*ᵣ and 40000-*M*ᵣ DD-Carboxypeptidases (Table 3)*

Irrespective of their mechanistic properties, the DD-carboxypeptidases (E) react with penicillin (I) according to $E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + P(s)$ where K = dissociation constant, k_2 and k_3 = first-order reaction constants and P(s) = degradation products [1, 2]. The values of the k_2/K and k_3 parameters, which govern the interaction between benzylpenicillin and the enzyme under consideration, were estimated according to Frère et al. [25, 26]. Preliminary experiments showed that the benzylpenicillin concentrations required to inhibit the DD-carboxypeptidase activity by 50 % were 0.014 µM for the 54000-M_r enzyme and 0.14 µM for the 40000-M_r enzyme. In addition, no β-lactamase activity could be detected under the conditions used for the determination of the k_2/K and k_3 values.

a) The k_3 *Parameter.* Complex EI*, formed with the 54000-M_r enzyme, had a long half-life of 780 min ($k_3 = 1.5 \times 10^{-5}\ s^{-1}$). Complex EI*, formed with the 40000-M_r enzyme, had a short half-life of 24 min ($k_3 = 4.8 \times 10^{-4}\ s^{-1}$).

b) The k_2/K *Parameter.* The propensity to form complex EI* was very high with the 54000-M_r enzyme ($k_2/K = 175000\ M^{-1}\ s^{-1}$) and moderate with the 40000-M_r enzyme ($10000\ M^{-1}\ s^{-1}$). Note that because of the very high k_2/K ratio value and the very low k_3 value, which characterize the 54000-M_r enzyme, both the benzylpenicillin concentration required to inhibit the enzyme activity by 50 % and the [14C]benzyl-

Table 2. *Efficacy of transpeptidation. Determination of the T/Hy ratio values*
D = carbonyl-donor peptide; A = amino-acceptor peptide; T = transpeptidation; Hy = hydrolysis. T and Hy were expressed as percentages of [^{14}C]Ac_2LLys-DAla-DAla or [^{14}C]AcLLys(Gly)-DAla-DAla utilized. With the K15 enzymes, all the experiments were carried out at 37 °C and in 35 µl, final volumes, using the same buffers as those indicated in the footnote of Table 1. For the experiments involving [^{14}C]Ac_2LLys-DAla-DAla, the 54000-M_r enzyme (0.1 µg), the exocellular 40000-M_r enzyme (0.45 µg) and the lysozyme-releasable 38000 − 40000-M_r enzymes (2.6 µg) were incubated for 30, 60 and 30 min respectively, with the tripeptide donor and either A_2pm or Gly-LAla at the indicated concentrations. For the experiments involving [^{14}C]AcLLys(Gly)-DAla-DAla, the exocellular 54000-M_r enzyme (0.8 µg), the exocellular 40000-M_r enzyme (1 µg) and the lysozyme-releasable 38000 − 40000-M_r enzymes (2.6 µg) were incubated for 1 h, 1 h and 30 min respectively, in the presence of 6.6 mM AcLLys-(Gly)-DAla-DAla

Strain	Enzymes	D = [^{14}C]Ac_2LLys-DAla-DAla A = A_2pm or Gly-LAla								A and D = [^{14}C]-AcLLys(Gly)-DAla-DAla (6.6 mM)		
		[D]	A/D ratio	A = A_2pm			A = Gly-LAla			T	Hy	T/Hy
				T	Hy	T/Hy	T	Hy	T/Hy			
		mM		%			%			%		
K15	exocellular 54000-M_r	0.1	1	0.5	11.8	0.04	0.35	12.6	0.03	2.2	27.3	0.08
		0.1	5	1.9	11	0.17	0.6	12	0.05			
		0.1	15	3.8	8	0.47	0.9	10	0.09			
	exocellular 40000-M_r	7.7	1	4.2	1.4	3	1.4	1.5	0.9	22	26.5	0.84
		3.7	1	7.1	6.9	1	4	8	0.5			
	lysozyme-releasable 38000 − 40000-M_r	3.7	1	5.4	8.4	0.64	3	8	0.37	9	30	0.30
R39	exocellular 53000-M_r[a]	0.86	1	1.4	19	0.08	no transpeptidation reaction			no transpeptidation reaction		
		0.86	3.9	5.4	19	0.28						
R61	exocellular 38000-M_r[a]	2.8	1.4	20.4	23.8	0.86	4.6	8.9	0.5	9.6	35	0.27

[a] For a recent review on the R39 and R61 enzymes, see [2]. For more specific information, see [9,24].

Table 3. *Interaction between benzylpenicillin (I) and the DD-carboxypeptidase (E)* $E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + Ps$
The enzymes were incubated at 37 °C in 50 mM Hepes/HCl buffer pH 8.0 (exocellular enzymes) and in 7 mM Tris/HCl buffer pH 7.5 (lysozyme-releasable enzyme). For the determination of k_3, the 54000-M_r enzyme (2 µg) was incubated in 25 µl buffer (see above) at 37 °C in the absence and in the presence of benzylpenicillin (at a final concentration of 0.1 mM for the 54000-M_r enzyme and of 0.2 mM for the others). For the determination of k_2/K, the 54000-M_r enzyme (0.8 µg), the 40000-M_r exocellular enzyme (3.6 µg) and the lysozyme-releasable enzyme (8 µg) were incubated at 37 °C in 100 µl buffer (see above) in the absence and in the presence of various concentrations of benzylpenicillin

Strains	Enzymes	k_2/K	k_3	Half-life of complex EI*
		$M^{-1} \times s^{-1}$	s^{-1}	min
K15	exocellular 54000-M_r	175000	1.5×10^{-5}	780
	exocellular 40000-M_r	10000	4.8×10^{-4}	24
	lysozyme-releasable 40000-M_r + 38000-M_r doublet	16000	2.3×10^{-4}	50
R39	exocellular 53000-M_r[a]	300000	2.8×10^{-6}	4100
R61	exocellular 38000-M_r[a]	12000	1.4×10^{-4}	82

[a] For a recent review on the R39 and R61 enzymes, see [2].

penicillin concentration at which the extent of radioactive labelling is 50% of the maximal value are virtually equivalent to the half-concentration of the enzyme present in the preparation.

c) Nature of the Reaction Products (Ps). Breakdown of the native complexes EI* formed by interaction between benzylpenicillin and the serine R61 and R39 DD-carboxypeptidases, proceeds via fragmentation of the bound penicilloyl moiety, resulting in the release of phenylacetylglycine [2,27]. The 54000-M_r enzyme (420 µl containing 84 µg proteins) and the 40000-M_r enzyme (160 µl containing 72 µg proteins) were dialysed at 4 °C against 250 ml 50 mM Hepes/HCl buffer, pH 8.0, and then incubated for 15 min at 37 °C with 0.02 mM (for the 54000-M_r enzyme) or 0.06 mM (for the 40000-M_r enzyme) [^{14}C]benzylpenicillin. The two preparations were separately filtered on a 25-ml column of Sephadex G-25 equilibrated against the Hepes buffer. Assuming that benzylpenicillin binds stoichiometrically to the enzymes, the radioactivities recovered in the excluded fractions indicated that the enzyme preparations were about 1.65% pure (the 54000-M_r enzyme) and 3.75% pure (the 40000-M_r enzyme). These fractions were then incubated at 37 °C for 43 h (the 54000-M_r enzyme) and 7 h (the 40000-M_r enzyme), causing an enzyme recovery of 83% and 100% respectively. The preparations thus obtained were lyophilized, the residues dissolved in 200 µl of 1 M HCl and the solutions extracted once with 200 µl and twice with 100 µl ethyl acetate. Analysis of the extracts (after evaporation) by thin-layer chromatog-

raphy (see Materials and Methods) revealed the presence, in each case, of one single radioactive compound, whose mobility was characteristic of [^{14}C]phenylacetylglycine. It represented about 70–80% of the total radioactivity of the fractions obtained after Sephadex filtration (i.e. of the radioactive complexes EI*).

Effect of the Anti-(Exocellular R61 Enzyme) on the Exocellular 54000-M_r *and 40000-*M_r *DD-Carboxypeptidases*

As shown in [16], the antiserum prepared against the R61 serine DD-carboxypeptidase inhibited the activity of this enzyme by at least 80% and had no effect on the activity of the R39 serine DD-carboxypeptidase. Incubation of the 54000-M_r enzyme (0.04 μg) and the 40000-M_r enzyme (0.08 μg) of *Streptomyces* K15 with 1 μl antiserum for 1 h at 37 °C in 30 μl 10 mM sodium phosphate buffer, pH 7.0, inhibited the activity of the 54000-M_r enzyme by perhaps 4% (a value which was within the limits of experimental errors) and that of the 40000-M_r enzyme by 55%.

Partial Purification of the Lysozyme-Releasable 40000–38000-M_r *DD-Carboxypeptidases*

The lysozyme-releasable enzyme preparation (specific activity: 0.12 nmol × min^{-1} × mg protein^{-1}; see Materials and Methods) contained (Fig. 1, track 4) two minor 40000-M_r and 38000-M_r penicillin-binding proteins and a major 25000-M_r penicillin-binding protein. This latter protein proved to be a plasma membrane contaminant (unpublished results). Fractionation with solid $(NH_4)_2SO_4$ at 4 °C resulted in the precipitation of the DD-carboxypeptidase activity within the protein fraction obtained at 40–80% $(NH_4)_2SO_4$ saturation. After solubilization of the precipitate in 33 mM Tris/HCl buffer pH 7.5 and dialysis against the same buffer, the preparation had a specific activity of 0.35 nmol × min^{-1} × mg protein^{-1}. In terms of DD-carboxypeptidase activity, the yield was of 135%. At this stage, 70% of the enzyme activity was inhibited by the anti-(exocellular R61 enzyme) serum. The preparation (30 ml containing a total activity of 310 nmol × min^{-1}) was then purified by immunoaffinity chromatography on a 4-ml column of Sepharose 6B (Pharmacia) previously coupled with purified anti-(exocellular R61 enzyme) IgGs. The technique was that of Marquet et al. [17] except that 3 mg IgG was used/ml activated Sepharose. The fractions eluted from the column at pH 10.5 and pH 11.5 (0.2 M carbonate buffer) were pooled, dialysed against 33 mM Tris/HCl buffer, pH 7.5, and concentrated by ultrafiltration. This preparation (18% recovery) had a specific activity of 215 nmol × min^{-1} × mg protein^{-1}. It contained only the 40000-M_r and 38000-M_r penicillin-binding proteins occurring in the proportion of 3 to 1. Finally, polyacrylamide gel electrophoresis at pH 8.4 under non-denaturing conditions permitted isolation of two fractions exhibiting DD-carboxypeptidase activity. One of them was located at 1 cm from the top of the gel toward the anode; it contained only the 40000-M_r penicillin-binding protein. The other fraction was located at 3.9 cm; it contained only the 38000-M_r penicillin-binding protein.

Properties of the Lysozyme-Releasable DD-Carboxypeptidases

The isolated 38000-M_r and 40000-M_r enzymes had the same 12 mM K_m value for the hydrolysis of Ac_2LLys-DAla-DAla; they were half-saturated at the same 0.26 μM [^{14}C]-benzylpenicillin concentration and their DD-carboxypeptidase activity was inhibited by 50% at the same 0.28 μM benzylpenicillin concentration. Hence, in spite of their slightly different molecular weights and their pronounced different electrical charges, it appeared that the two enzymes under consideration had, most probably, identical enzymatic properties. The data of Tables 1–3 were obtained with the unfractionated enzyme preparation as purified by affinity chromatography. It should also be mentioned that the enzyme activity was inhibited by 90% by the anti-(R61 enzyme) serum. All these observations showed that the lysozyme-releasable 38000-M_r and 40000-M_r enzymes were closely related to the exocellular 40000-M_r DD-carboxypeptidase.

DISCUSSION

Streptomyces K15 excretes two DD-carboxypeptidases during growth and possesses at least two cell-bound DD-carboxypeptidases that can be released in a water-soluble form after lysozyme treatment of the mycelium in an osmotically protected medium. The DD-carboxypeptidases under consideration were separated from each other and purified to the stage where each enzyme preparation contained one single penicillin-binding protein. They were compared with the R39 and R61 DD-carboxypeptidases, which originate from *Actinomadura* R39 and *Streptomyces* R61 respectively (see the introduction). The exocellular 54000-M_r DD-carboxypeptidase excreted by *Streptomyces* K15 has a low K_m value (0.1 mM) for Ac_2LLys-DAla-DAla; its activity is not or little influenced by the occurrence of charged groups at the lateral chain of the L-residue which precedes the C-terminal DAla-DAla sequence; it has a very low propensity to catalyse transpeptidation reactions on the tetrapeptide AcLLys(Gly)-DAla-DAla; it is exceedingly sensitive to benzylpenicillin (a property which is due to both a very high k_2/K value and a very low k_3 value); and, finally, its activity is not inhibited by the anti-(R61 enzyme) serum. In all these respects the exocellular 54000-M_r DD-carboxypeptidase of *Streptomyces* K15 closely resembles the 53000-M_r DD-carboxypeptidase which is excreted by *Actinomadura* R39. In spite of their different electrical charges and, for two of them, their slightly different molecular weights, the 40000-M_r exocellular and the 40000-M_r and 38000-M_r lysozyme-releasable DD-carboxypeptidases of *Streptomyces* K15 are closely related enzymes. In contrast to the exocellular 54000-M_r enzyme, these latter enzymes have a high K_m value (about 12 mM) for Ac_2LLys-DAla-DAla; their activities are much decreased by the presence of charged groups at the end of the lateral chain of the L-residue of the carbonyl donor peptide; they have a high propensity to catalyse transpeptidation reaction with the carbonyl donor-amino acceptor tetrapeptide AcLLys(Gly)-DAla-DAla; they are moderately sensitive to benzylpenicillin (a property which is due to both a moderate k_2/K value and a rather high k_3 value); and their activities are inhibited by the anti-(R61 enzyme) serum. In all these respects these exocellular and/or lysozyme-releasable DD-carboxypeptidases of *Streptomyces* K15 closely resemble the 38000-M_r DD-carboxypeptidase, which is excreted by *Streptomyces* R61.

The investigations described above did not lead to the discovery of any novel DD-carboxypeptidase/transpeptidase but demonstrated that enzymes of this class exhibiting complementary activities, in particular with respect to their penicillin sensitivities and abilities to perform transpeptidation

reactions, can be produced by a same *Streptomyces* strain. The penicillin-binding proteins 4 and 5 of *Escherichia coli* [28] and *Proteus mirabilis* [29] are also DD-carboxypeptidases/transpeptidases of compensating activities.

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