[Reprinted from Biochemistry, (1971) 10, 2163.]

Penicillin-Sensitive DD-Carboxypeptidase from *Streptomyces* Strain R 61*

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ABSTRACT: The D-alanyl-D-alanine carboxypeptidase excreted by the penicillin-sensitive *Streptomyces* strain R 61 is an anionic protein at pH 6.5. It does not require cations for its action. By comparison with the penicillin-resistant DD-carboxypeptidase from *Streptomyces albus* G, the penicillin-sensitive R 61 enzyme has high K_m values with all the peptides which were used but the alignment of catalytic groups is more effective in promoting hydrolysis of the peptides (high V_{max} values). Moreover, the substrate structures required by the two enzymes show some differences. In contrast to the *albus* G DD-carboxypeptidase which is either not, or very little, affected by penicillins, the R 61 enzyme is inhibited by low dose levels of penicillin G and by cephalothin.

The polymerization of the nascent peptidoglycan within the expanding bacterial wall is believed to be introduced by transpeptidation, *i.e.*, a reaction which catalyzes the transfer of the penultimate C-terminal D-alanine of an acyl-D-alanyl-D-alanine donor peptide to the amino group of an acceptor peptide of the same composition (Tipper and Strominger, 1965; Wise and Park, 1965). In this process, interpeptide bonds are formed and equivalent amounts of D-alanine residues are released from the donor peptides. Concomitantly, peptides from adjacent glycan strands are interconnected giving rise to an insoluble peptidoglycan network. Among the numerous enzymes involved in the synthesis of the wall peptidoglycan, that transpeptidase which is responsible for the peptide cross-linking is known to be the target of the penicillin molecule (Tipper and Strominger, 1965; Strominger, 1969). The transpeptidase is either inactivated through penicilloylation or it is competitively inhibited (or both) because of a structural analogy between penicillin and the D-alanyl-D-alanine backbone of the peptides involved in transpeptidation. The elucidation of the exact molecular basis of penicillin action would require the isolation from the membrane of the transpeptidase protein, a problem which is made difficult because it is not known how much of the *in situ* structural integrity must be retained by both the enzyme and the substrate in order to be operative *in vitro*. Another possible approach to the problem, however, rested upon the recent observation that the D-alanyl-D-alanine carboxypeptidase spontaneously excreted by *Streptomyces albus* G appeared to be the transpeptidase that had undergone solubilization (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1970b,c). Indeed, the substrate requirements of this soluble DD-carboxypeptidase presented a remarkable similarity with those of the transpeptidase, as deduced from the structure of the completed peptidoglycan (Leyh-Bouille *et al.*, 1970a) involved in the peptide bridge closure reaction. *Streptomyces albus* G was insensitive to penicillin and so, too, was its DD-carboxypeptidase. The purpose of the present paper is to describe the isolation, purification, and substrate requirements of a penicillin-sensitive DD-carboxypeptidase excreted by the penicillin-sensitive *Streptomyces* strain R 61 and to compare its properties with those of the penicillin-resistant DD-carboxypeptidase form *Streptomyces albus* G.

Materials and Methods

Analytical Techniques. Amino acids, N-terminal groups (fluorodinitrobenzene technique), and D-alanine (enzymatic procedure) were measured as previously described (Ghuysen *et al.*, 1966, 1968).

Polyacrylamide Gel Electrophoresis. Cylindrical polyacrylamide gels (65 × 8 mm) were prepared with Cyanogum 41 (BDH Ltd., Poole, Dorset, United Kingdom) 8% in buffer and polymerized in the presence of 0.08% ammonium persulfate and 0.08% *N,N,N',N'*-tetramethylethylenediamine. The buffers were prepared from Tris base, brought to the required pH value (7.5 or 8.5) with acetic acid, and diluted to 0.05 M. Before use, the gels were cleared of ionic contaminants by passing a current for 30–45 min. To the enzyme solutions (about 0.1 ml) a few crystals of sucrose were added and the samples were applied to the top of the gels. A potential of 100 V (current about 6–8 mA per tube) was applied for 2 hr. Proteins were stained with Amido Black and excess stain was removed with 7% acetic acid.

Sucrose gradient electrophoresis was carried out as previously described (Dierickx and Ghuysen, 1962) using a K_2HPO_4–KH_2PO_4 buffer, pH 6.45, of 0.01 ionic strength and a sucrose gradient from 0 to 56% (w/v).

Walls and Peptides. The walls of *Corynebacterium poinsettiae* (in which the interpeptide bonds are mediated through C-terminal N^{α}-D-alanyl-D-ornithine linkages; Perkins, 1967; Ghuysen *et al.*, 1970) and most of the peptides used in the present studies were previously described (Leyh-Bouille *et al.*, 1970b). N^{α},N^{ϵ}-Diacetyl-L-lysyl-D-alanyl-D-lysine was prepared by the *N*-hydroxylsuccinimide ester method of Anderson *et al.* (1964). Details of the synthesis will be presented elsewhere (M. Nieto and H. R. Perkins, in prepara-

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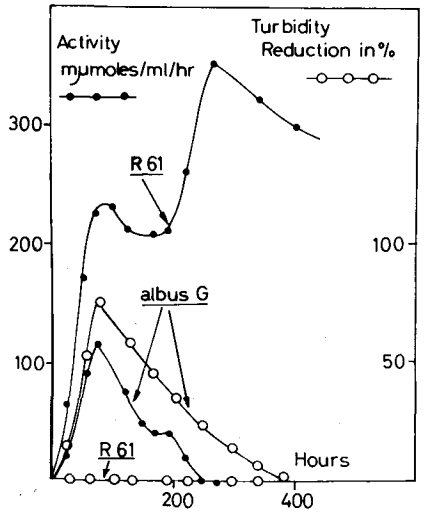


FIGURE 1: Excretion of DD-carboxypeptidase by *Streptomyces albus* G (in peptone medium) and by *Streptomyces* R 61 (in glycerol–casein medium). Strains were grown at 27°, with shaking, in 1-l. flasks containing 250 ml of medium. Samples were removed after increasing times of culture and they were tested with regard to their activities upon N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (liberation of the C-terminal D-alanine residue) and upon isolated walls of *C. poinsettiae* (turbidity reduction). Culture filtrates (12 μl) were incubated at 37° with 50 nmoles of peptide in the presence of 7 μl of 0.1 M Tris-HCl buffer, pH 7.5 (final volume 35 μl), for 6 hr. Results are expressed in nmoles of peptide hydrolyzed per ml of culture filtrate, per hr. Culture filtrates (100 μl) were also incubated at 37° with 500 μg of *C. poinsettiae* walls in the presence of 100 μl of 0.1 M Tris-HCl buffer, pH 7.5 (final volume 500 μl), for 4 hr. Results are expressed in per cent of turbidity reduction. With R 61 culture filtrates, the incubation time was prolonged up to 24 hr.

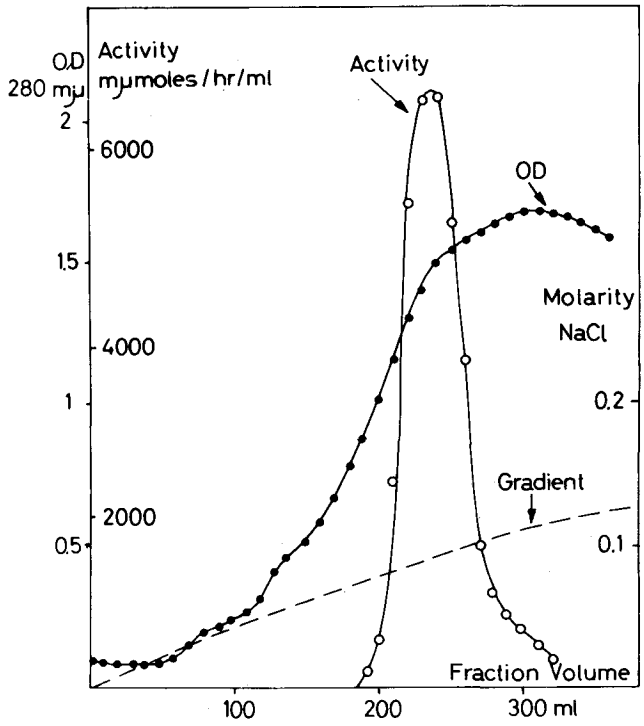


FIGURE 2: Purification of DD-carboxypeptidase from *Streptomyces* R 61. Elution from DEAE-cellulose column with increasing gradient of Tris-NaCl buffer, pH 7.5 (step 2). The curve shows the activity of the effluent fractions on N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (liberation of C-terminal D-alanine). For conditions of chromatography, see text. Fractions (12 μl) were incubated at 37° with 50 nmoles of peptide, in a final volume of 35 μl, for 0.5 hr or 1 hr, depending upon the samples. Results are expressed in nmoles of peptide hydrolyzed per ml of fractions, per hr. OD = optical density of the effluent fractions at 280 mμ.

tion). The peptide N^{α}-(acetyl-L-alanyl-γ-D-glutamyl)-N^{ϵ}-acetyl-L-lysyl-D-alanyl-D-alanine was a gift from Dr. K. H. Schleifer (Botanisches Institut der Universität, Munich, Germany).

Culture Media. The two following media were used: (A) peptone medium containing 1% peptone oxoid, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% $NaNO_3$, and 0.05% KCl; (B) glycerol–casein medium containing per liter of final volume: 20 g of glycerol, 40 ml of 10% casein solution (w/v), and 50 ml of salt suspension. The salt suspension contained per liter: 5 g of NaCl, 1 g of $CaCO_3$, 1 g of $MgSO_4 \cdot 7H_2O$, 10 g of K_2HPO_4, and 1 g of $FeSO_4 \cdot 7H_2O$. The casein was previously dissolved at 70° with the help of KOH (0.04 g/g of casein). The solution was then cooled and neutralized.

Enzyme Unit. By definition one unit catalyzes the hydrolysis of 1 nmole of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine per hr at 37° when 4 μmoles of peptide are incubated with the enzyme in 30 μl of 0.01 M Tris-HCl buffer, pH 7.5 (substrate concentration = 10 × K_m; *vide infra*).

Experimental Section

Selection of Streptomyces Strain R 61 and Conditions of Culture. As a result of a survey carried out on 65 local wild strains, only two *Streptomyces* (strains R 61 and K 27) were found sensitive to low concentrations of penicillin G and related antibiotics (Leyh-Bouille *et al.*, 1970a). *Streptomyces* R 61 and *Streptomyces* K 27 grew well in both peptone and glycerol–casein media. Neither medium, however, was suitable for excretion by strain K 27 of a DD-carboxypeptidase active on N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine. Strain R 61 excreted a DD-carboxypeptidase active on this peptide solely in the glucose–casein medium although the peptone medium had been used previously for the production of the DD-carboxypeptidase from the penicillin-resistant strain *albus* G (Ghuysen *et al.*, 1970). Figure 1 shows the excretion of DD-carboxypeptidase activity by *S. albus* G (in peptone) and by *S.* R 61 (in glycerol–casein). Maximal production of mycelia (1.5 g dry weight, per liter) was obtained after about 60–80 hr of culture in both cases. The carboxypeptidase activity of the *S. albus* G culture was maximal after about the same time and then it progressively disappeared. The *S.* R 61 culture exhibited two peaks of carboxypeptidase activity, the second one occurring after about 300 hr of culture. No corresponding increase in mycelia production was observed. Large amounts of free amino acids were present in the 100- to 200-hr cultures and they subsequently disappeared, suggesting that part of the bacterial population resumed growth from autolysis products. The *S.* R 61 culture filtrates were more active on N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine than those of *S. albus* G but, in marked contrast with the latter, they did not exhibit any lytic activity on walls of *C. poinsettiae*.

Isolation and Purification of DD-*Carboxypeptidase from Streptomyces R 61. S.* R 61 was grown in the presence of glycerol–casein (3 l.), with shaking, in 1-l. flasks containing 250 ml of medium, at 27°, for about 125 hr (Figure 1) and the mycelia were removed by centrifugation. All the manipulations hereinafter described were carried out at 4°.

STEP 1. The enzyme was adsorbed from the culture filtrate

TABLE I: Isolation and Purification of the R 61 DD-Carboxypeptidase.

Steps[a]	Relative Specific Activity[b]	Total Activity	Recovery (%)	Total Protein in mg
Culture filtrate	585	3,930,000	100	6725
1	2,580	3,330,000	82	1290
2	83,000	2,910,000	74	35
3	310,000	350,000	9	1.125

[a] See text. [b] N^{α},N^{ϵ}-Diacetyl-L-lysyl-D-alanyl-D-alanine (200 nmoles) was incubated with the enzyme preparation, at 37°, in 35 μl (final volume) of 0.01 M Tris buffer, pH 7.5, and the D-alanine liberated after increasing times of incubation was measured. The activities were estimated under the conditions required to liberate 20% of D-alanine that is liberated at completion of the reaction. Specific activity is expressed in nanoequivalents of D-alanyl-D-alanine linkage hydrolyzed per hour, per milligram of protein. Under the above conditions, the peptide concentration (6 mM) is about 0.5 the K_m value (*vide infra*). The proteins were estimated by measuring the amount of total NH_2 groups available to fluorodinitrobenzene after 6 N HCl hydrolysis (100°, 20 hr), using bovine serum albumin as standard. The final preparation, after step 3, exhibited an absolute specific activity of 890,000 units/mg of protein, as determined by using a peptide concentration (120 mM) equivalent to 10 × K_m value.

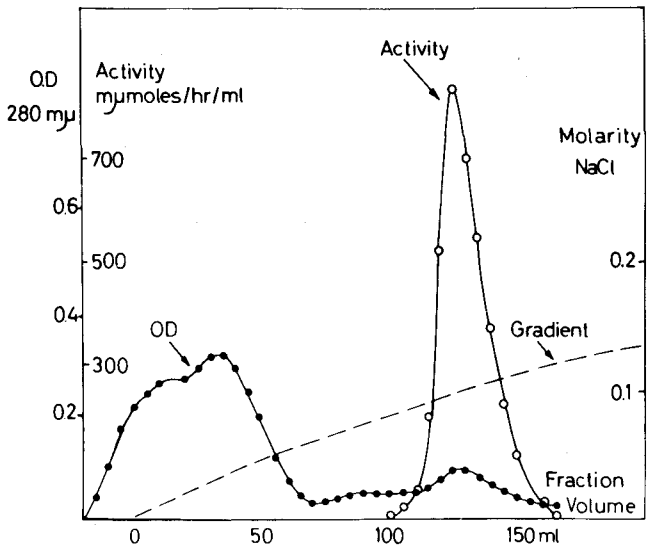


FIGURE 3: Purification of DD-carboxypeptidase from *Streptomyces* R 61. Elution from DEAE-cellulose column with increasing gradient of Tris-NaCl buffer, pH 7.5 (step III). See Figure 2. Effluent fractions (from 5 to 12 μl) were incubated at 37°, with 50 nmoles of peptide, in a final volume of 35 μl, for 1 hr. OD = optical density at 280 mμ.

on 60 g of Amberlite XE 64 H^+ by adjusting the pH to 4 with acetic acid. The amberlite-adsorbed enzyme complex was suspended in 0.1 M K_2HPO_4 and the pH of the suspension was brought to 8 by dropwise addition of concentrated ammonia. The resin was removed by filtration, the filtrate was clarified by centrifugation, and the volume of the supernatant (240 ml) was reduced to 15 ml by dialysis against dry Carbowax 4000. The concentrated extract was clarified by centrifugation and solid $(NH_4)_2SO_4$ was added up to 30% saturation. The precipitate was collected by centrifugation, suspended in water, and dialyzed against water for 4 hr. The solution (17 ml) was clarified by centrifugation.

STEP 2. The enzyme solution was diluted 10-fold (final volume: 170 ml) with a 0.05 M Tris buffer pH 8 and 50 g (dry weight) of DEAE-cellulose, previously equilibrated against the same buffer, was added. The suspension was stirred for 1 hr. The resin-adsorbed enzyme complex was collected by filtration and suspended in 170 ml of 0.05 M Tris buffer, pH 8, supplemented with 0.1 M NaCl. After centrifugation, the treatment was repeated once. The pooled Tris–NaCl extracts were concentrated by dialysis against Carbowax and then dialyzed against 0.05 M Tris buffer, pH 8, for 5 hr (final volume 53 ml). The dialyzed solution was filtered through a 125-ml column of DEAE-cellulose previously equilibrated against 0.05 M Tris buffer, pH 8. The column was washed with the same buffer until the effluent contained only traces of material absorbing at 280 mμ. The enzyme was then eluted from the resin with an increasing gradient of Tris–NaCl buffer, pH 8 (mixing flask, at constant volume: 500 ml of 0.05 M Tris buffer, pH 8; solution added: 0.05 M Tris buffer and 0.25 M NaCl) (Figure 2). The active fractions were pooled and concentrated by dialysis against Carbowax to a final volume of 5 ml. At this stage, the DD-carboxypeptidase was found to be contaminated by *N*-acetylmuramyl-L-alanine amidase activity (as shown by the hydrolysis of the D-lactyl-L-alanine linkage in the disaccharide peptide N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine from *Staphylococcus aureus*) and by aminopeptidase activity (as shown by the hydrolysis of the synthetic peptide glycyl-glycyl-L-alanine). All attempts to separate the 3 enzyme activities by altering the conditions of DEAE-cellulose chromatography failed.

STEP 3. The crude enzyme solution was filtered in water on a 220-ml column of Sephadex G-50. DD-Carboxypeptidase, aminopeptidase, and *N*-acetylmuramyl-L-alanine amidase were all excluded from the gel. The active fractions were pooled (11 ml) and then submitted to sucrose gradient electrophoresis in phosphate buffer, pH 6.45, of 0.01 ionic strength, for 19 hr, under 10 V/cm. Before electrophoresis, the enzyme was injected in the column at the 20% sucrose level. The anode was at the top of the gradient (0% sucrose) and the cathode at the bottom of it. Under these conditions, the *N*-acetylmuramyl-L-alanine amidase did not migrate. The DD-carboxypeptidase migrated to the anode with a mobility of 0.37 mm/hr per V per cm (methyl orange, used as a marker, had a mobility of 1.5 mm/hr per V per cm). No aminopeptidase activity could be detected in the fractions collected after electrophoresis. Part of the sucrose was removed from the electrophoretically purified DD-carboxypeptidase by concentration against Carbowax, followed by dialysis against 0.05 M Tris buffer, pH 8, for 5 hr (final volume 80 ml). The solution was then filtered through a 50-ml column of DEAE-cellulose equilibrated against the same buffer, and the enzyme was eluted from the resin with an increasing gradient of Tris–NaCl buffer, pH 8 (mixing flask: 250 ml of 0.05 M Tris buffer, pH 8; solution added: 0.05 M Tris buffer and 0.25 M NaCl) (Figure 3). The pooled active fractions were concentrated by dialysis against Carbo-

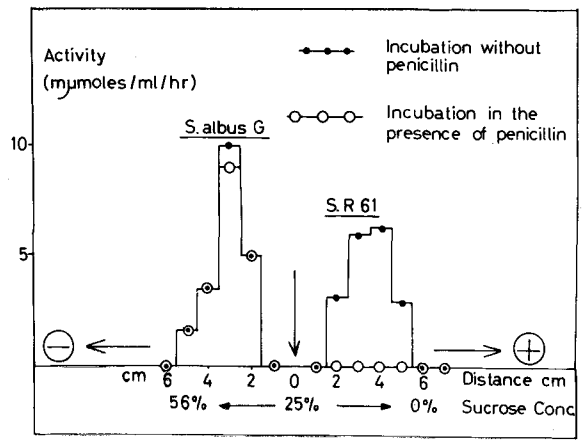


FIGURE 4: Sucrose gradient electrophoresis at pH 6.45 of DD-carboxypeptidases from *S. albus* G and from *S.* R 61: gradient, from 0 to 56% sucrose (w/v); buffer, K_2HPO_4–KH_2PO_4, pH 6.45, of 0.01 ionic strength. *Albus* G enzyme (50 μg in protein) and R 61 enzyme (75 μg in protein) were dissolved in 1.5 ml of phosphate buffer containing 25% (w/v) of sucrose. The solution was injected in the gradient at the 25% sucrose level (arrow in the figure). Anode was at the top of the column and cathode at the bottom of it. Electrophoresis was carried out at 4° under 6 V/cm for 19 hr. Fractions of 5 ml (corresponding to sections 1-cm thick of the sucrose gradient) were collected. Aliquots (21 μl) were incubated at 37° with 50 nmoles of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine, in the presence of 7 μl of 0.1 M Tris-HCl buffer, pH 8, in a final volume of 35 μl, for 6 hr, in the absence or in the presence of penicillin G (final concentration 10 μg/ml). Free D-alanine was measured. Results are expressed in nmoles of peptide hydrolyzed per ml of fraction, per hr.

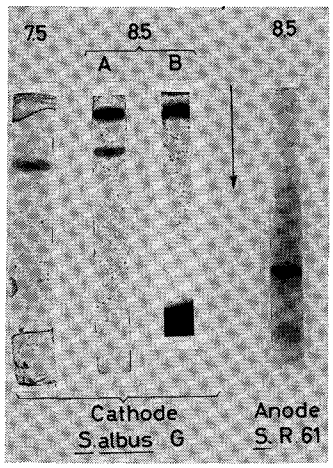


FIGURE 5: Polyacrylamide gel electrophoresis of DD-carboxypeptidases from *S. albus* G and from *Streptomyces* R 61. Samples in sucrose solution were applied directly to the top of cylindrical gels of polyacrylamide (8%). The buffer was Tris acetate (0.05 M in Tris) at pH 7.5 or 8.5 as shown on the figure. A potential of 100 V was applied for 2 hr. The gels were then extruded and sliced lengthwise. *S. albus* G enzyme after stage 4 of purification (Ghuysen *et al.*, 1970) was run at pH 8.5. Print A shows the half-gel stained for protein with Amido Black and for print B the other half-gel was applied to a thin layer of agar containing dried cells of *C. poinsettiae* and incubated at 35° for 75 min. The dark band is a zone of lysis. *S. albus* G enzyme after stage 5 of purification (Ghuysen *et al.*, 1970) was run at pH 7.5 and stained for protein. *Streptomyces* R 61 enzyme after stage 3 of purification (see text) was run toward the anode at pH 8.5 and stained for protein. The arrow indicates the direction of movement of the protein bands. In all cases, activity upon N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine was found associated with the main protein band.

wax (final volume: 5 ml). Finally, filtration in water on Seph adex G-50 (at this stage, the enzyme entered the gel and was eluted just before the salts) yielded the purified enzyme.

Table I gives the actual recoveries and improvements in specific activities, as determined with the help of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine. The final enzyme preparation contained 890,000 DD-carboxypeptidase units per mg of protein (using a substrate concentration = 10 × K_m; see Materials and Methods).

Sucrose Gradient and Polyacrylamide Gel Electrophoresis of Carboxypeptidases from S. R 61 and S. albus G. Aliquots of DD-carboxypeptidase from *S. albus* G (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1970b,c) and from *S.* R 61 were mixed together and the solution was submitted to electrophoresis in sucrose gradient at pH 6.45 (Figure 4). Under these conditions, the *albus* G enzyme was cationic and the R 61 enzyme was anionic. The specific detection of each of the two DD-carboxypeptidases was made possible by incubating the fractions collected after electrophoresis with N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine in the absence and in the presence of penicillin G (10 μg/ml). The activity of the *albus* G enzyme was virtually unaffected by penicillin whereas that of the R 61 enzyme was completely abolished.

Both enzymes were also studied by polyacrylamide gel electrophoresis. Samples were added (with a little added sucrose) to 8% gel with 5% cross-linking at pH 7.5 or pH 8.5 in 0.05 M (with respect to Tris) Tris-acetate buffer. After electrophoresis, the gel was sliced lengthwise. One half was stained for protein and the other half was cut into 2-mm slices from the top end. Each slice was eluted overnight at 2° either with Tris-HCl buffer (0.02 M) of the same pH value used for electrophoresis (*albus* G enzyme) or with water (R 61 enzyme). Aliquots of the eluates were tested against N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine. With the *albus* G enzyme, a similar half gel was also applied to the surface of a thin layer of agar containing cells of *C. poinsettiae* (1% agar in 0.05 M Tris-HCl buffer, pH 7.5, and 0.005 M $MgCl_2$; brought to 100° and mixed with a suspension of dried *C. poinsettiae* cells; final concentration 1.5–2 mg/ml). After incubation for 75 min in a humid chamber at 37°, the gel was removed and the zones of lysis were observed. The top of the gel was the cathode with the R 61 enzyme and the anode with the *albus* G enzyme. In a trial experiment carried out at pH 8.5 with this latter enzyme, reversed polarity was used, but no protein entered the gel. As shown in Figure 5, each of the two enzyme preparations contains mainly one protein. The *albus* G enzyme is cationic even at pH 8.5. The R 61 enzyme is anionic at pH 7.5.

pH and Salts Requirements of the R 61 Enzyme. pH optimum of the reaction was between 5 and 9, in the presence of acetate, phosphate, or Tris-HCl buffers (Figure 6). Optimum ionic strength was between 0.002 and 0.01 for Tris-HCl buffer pH 7.5 (Figure 7) and for acetate or phosphate buffers pH 5.4. Unlike the DD-carboxypeptidase from *S. albus* G which is activated by Mg^{2+} and Ca^{2+} (Ghuysen *et al.*, 1970), the enzyme from *S.* R 61 does not require cations for its action. Mg^{2+} (0.002 M) when added to 0.01 M Tris-HCl buffer, pH 7.5, did not activate the reaction. The noninvolvement of cations in the reaction was also shown by the facts that sodium ethylenediaminetetraacetate and cation-complexing buffers as phosphate did not inhibit the reaction (Figure 7).

Substrate Requirements of the Enzyme. These were studied on peptides with the general structure X→L-R_3→R_2→R_1(OH). The influence exerted by the nature of the C-termina]

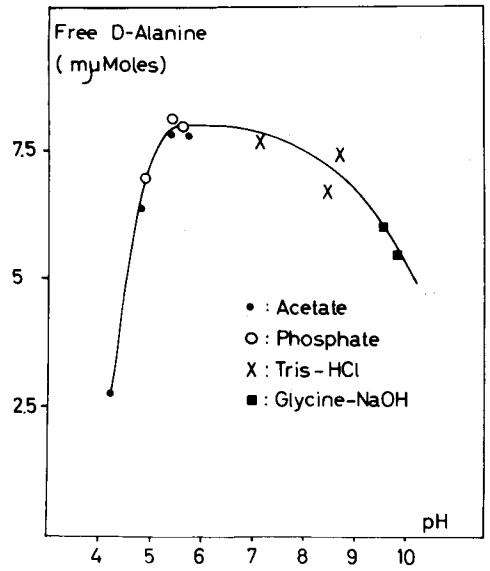


FIGURE 6: pH optimum of DD-carboxypeptidase from *S.* R 61. N^{α},N^{ϵ}-Diacetyl-L-lysyl-D-alanyl-D-alanine (15 nmoles) was incubated at 37°, for 30 min, in the presence of 0.2 μg of enzyme, in a final volume of 35 μl. All the buffers were used at 0.01 ionic strength. Results are expressed in nmoles of D-alanine liberated.

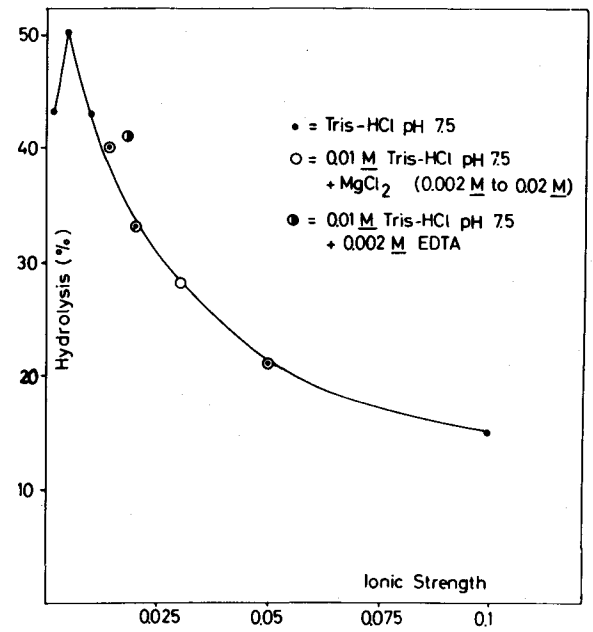


FIGURE 7: Effect on DD-carboxypeptidase from *S.* R 61 of concentration of Tris-HCl pH 7.5 buffer, Mg^{2+} cation, and sodium ethylenediaminetetraacetate. N^{α},N^{ϵ}-Diacetyl-L-lysyl-D-alanyl-D-alanine (15 nmoles) was incubated at 37°, for 30 min, in the presence of 0.2 μg of enzyme, in a final volume of 35 μl. Results are expressed in per cent of hydrolysis. The effects of concentration of acetate and phosphate buffers, pH 5.4 (when expressed in ionic strength), were identical with those observed with the Tris-HCl buffer, pH 7.5.

R_1 residue, by the nature of the penultimate C-terminal R residue, and by the structure of the side chain of the L-R residue, respectively, was examined. The influence of the α substituent of the L-R_3 residue (*i.e.*, the X group) was not studied in detail because previous results with the *S. albus* G DD-carboxypeptidase had shown that it produced no striking effects (Leyh-Bouille *et al.*, 1970b). Table II compares results for the R 61 and *albus* G enzymes. Michaelis constant (K_m) and maximal velocity (V_{max}) were obtained at 37°, in 0.01 M Tris-HCl buffer, pH 7.5, on the basis of initial velocity measurements (Table II). Enzyme "efficiency" defined as the specific activity at enzyme saturation divided by the K_m for each particular substrate (V_{max}/K_m) was also calculated (Table II). This concept of efficiency, introduced by Pollock (1965), expresses the functional efficiency of the enzyme at low substrate concentration. The V_{max} and efficiency values depend upon the purity of the enzyme preparations. Hence the quantitative comparison between the R 61 and *albus* G enzymes with respect to these parameters is valid only if each of the preparations consists of one single protein. Polyacrylamide gel electrophoreses, at least, strongly suggest that both preparations are highly purified (Figure 5).

With some peptides, K_m and V_{max} values were not determined but the rate of hydrolysis was only measured at low substrate concentration and therefore the data (Table III) reflect the relative efficiency of both R 61 and *albus* G enzymes on the selected substrates.

(1) C-TERMINAL RESIDUE (R_1 RESIDUE). The replacement of D-alanine at that position by D-lysine or D-leucine had a more pronounced decreasing effect on the efficiency of R 61 enzyme than of *albus* G enzyme (Table II, peptides 2, 3, 4). These effects of the C-terminal residue were paralleled by the fact that the R 61 enzyme had a poor endopeptidase activity on the disaccharide peptide dimer from *C. poinsettiae* (Table II, peptide 13). This, together with the anionic property of the R 61 enzyme which probably prevents it from combining with the isolated bacterial walls, explains why, unlike the *albus* G enzyme, the R 61 enzyme has no lytic activity upon those walls in which the interpeptide bonds are C-terminal D-alanyl-D linkages. Replacement of D-alanine by glycine at the C-terminal position produced a 10-fold reduction in the efficiency of both enzymes (Table II, peptides 2 and 5). Finally, replacement of D-alanine by L-alanine abolished the activity (Table II, peptides 2 and 6).

(2) PENULTIMATE C-TERMINAL RESIDUE (R_2 RESIDUE). Both enzymes showed considerable specificity for D-alanine residue in this position (Table II, peptides 2, 7, 8, 9).

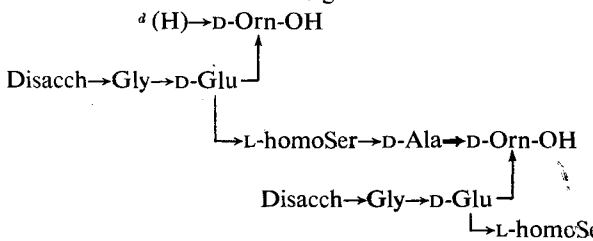
(3) L-R_3 RESIDUE. Increasing the length of the side chains without introduction of charged groups was paralleled by increasing efficiency of both enzymes (Table III). On the other hand, the presence of a free ε-amino group of L-lysine at that position strongly decreased their efficiency (Table II, peptides 2 and 10). The transformation of the ε-amino group to an α-amino group by introduction of a carboxyl group in an α position (*i.e.*, the replacement of L-lysine by diaminopimelic acid) was sufficient to induce a high efficiency of hydrolysis of the C-terminal D-alanyl-D-alanine linkage by the *albus* G enzyme, whereas the low activity of the R 61 enzyme was not improved (Table II, peptides 1, 2, 10, and 11). Strikingly, substitution of the ε-amino group of L-lysine by a pentaglycine sequence restored the efficiency of both enzymes (Table II, peptides 1, 10, and 12). Previous studies carried out on the structure of the wall peptidoglycan of both *Streptomyces albus* G and R 61 (Leyh-Bouille *et al.*, 1970a) had shown that the amino group involved in transpeptidation was a glycyl residue substituting the side chain of LL-diaminopimelic acid residue at the R_3 position (Leyh-Bouille *et al.*, 1970a). It should be noted that among the peptides of Table II, peptide 12 is the only one which contains an N-terminal glycine in an equivalent position.

Inhibition of Enzyme by Antiobiotics. The minimal concen-

TABLE II: Efficiency of Hydrolysis by the DD-Carboxypeptidases from *Streptomyces albus* G and from *Streptomyces* R 61.

No.[a]	Substrates[c]	*S. albus* G[b] K_m	*S. albus* G[b] V_{max}	*S. albus* G[b] Efficiency	*S.* R 61[b] K_m	*S.* R 61[b] V_{max}	*S.* R 61[b] Efficiency
A-1	Acetyl→L-Ala→γ-D-Glu→L-Lys(ε)→D-Ala→D-Ala	1.80	100	56	10	215	21
2	Acetyl→L-Lys(ε)→D-Ala→D-Ala	0.33	100	300	12	890	72
3	Acetyl→L-Lys(ε)→D-Ala→D-Lys	0.80	85	106	13	90	7
4	Acetyl→L-Lys(ε)→D-Ala→D-Leu	0.33	33	100	10	50	5
5	Acetyl→L-Lys(ε)→D-Ala→Gly	2.50	60	24	36	200	6
6	Acetyl→L-Lys(ε)→D-Ala→L-Ala		No hydrolysis			Virtually no hydrolysis	
B-7	Acetyl→L-Lys(ε)→Gly→D-Ala	15.0	107	7	15.5	1.7	0.1
8	Acetyl→L-Lys(ε)→D-Leu→D-Ala		No hydrolysis		10	10	1
9	Acetyl→L-Lys(ε)→L-Ala→D-Ala		No hydrolysis			Virtually no hydrolysis	
C-10	Acetyl→L-Lys(ε)→D-Ala→D-Ala	6.0	20	3	15	4	0.3
11	UDP-MurNAc→L-Ala→γ-D-Glu→(L)-*meso*-DAP-(L)→D-Ala→D-Ala	0.4	10	25	11	3	0.3
12	N^{α}-[Disacch→L-Ala→γ-D-Glu(NH_2)]→L-Lys(ε)→D-Ala→D-Ala	0.28	9	32	14	800	57
D-13	*C. poinsettiae* bisdisaccharide–peptide dimer[d]	1.50	78	52		*e*	

[a] A and B, influence exerted by the C-terminal dipeptide sequence; C, influence exerted by the preceding residue at the R_3 position; D, "endopeptidase" activity. [b] K_m values are expressed in mM; V_{max} values in μmoles, per mg of enzyme per hr; Efficiency in V_{max}/K_m. These values were obtained at 37° in 0.01 M Tris-HCl buffer, pH 7.5 (R-61 enzyme), or in 0.02 M Tris-HCl buffer, pH 7.5, supplemented with 0.002 M $MgCl_2$ (*albus* G enzyme) (Leyh-Bouille *et al.*, 1970b). [c] Disacch = β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid. Heavy arrows show the sensitive linkage.

[d]


The peptide preparation is a mixture of two dimers with either D-alanine or L-homoserine at the C-terminal position (Leyh-Bouille *et al.*, 1970b). [e] First order kinetics were observed with substrate concentrations up to 16 mM. Under this latter condition, V_0 was equal to 20 μmoles of dimer hydrolyzed per mg of enzyme, per hr. At low substrate concentrations (0.4 mM) the functional efficiency of the *S. albus* G enzyme was 15 times higher than that of the R 61 enzyme.

TABLE III: Influence of the Length of the Side Chain of the Residue That Precedes C-Terminal D-Alanyl-D-alanine on Carboxypeptidase Activity.

Substrate	Specificity Activity[a] of Enzyme from *S. albus* G	Specificity Activity[a] of Enzyme from *S.* R 61
Acetyl→L-Ala→D-Ala⇒D-Ala	300	650
UDP-MurNAc→Gly→D-Glu	2,000	1,400
↳L-homoSer→D-Ala⇒D-Ala		
Acetyl→L-DAB→D-Ala⇒D-Ala (↑γ Acetyl)	22,000	4,000
Acetyl→L-Orn→D-Ala⇒D-Ala (↑δ Acetyl)	22,000	21,000
Acetyl→L-Lys→D-Ala⇒D-Ala (↑ε Acetyl)	40,000	47,000

[a] Expressed in nanoequivalents of D-Ala–D-Ala linkage hydrolyzed per mg of enzyme, per hr. Conditions of incubation were: 15 nmoles of peptide was incubated at 37° in the presence of enzyme in a final volume of 35 μl of 0.01 M Tris-HCl buffer, pH 7.5 (enzyme from *S.* R 61), or of 0.02 M Tris-HCl buffer, pH 7.5, supplemented with 0.002 M $MgCl_2$ (enzyme from *S. albus* G) (Leyh-Bouille *et al.*, 1970b). Activities were always calculated on an extent of hydrolysis of 50% or less. DAB = diaminobutyric acid. MurNAc = *N*-acetylmuramic acid. Heavy arrows show the sensitive linkage.

tration of antibiotics that produced a visible zone of growth inhibition (under those conditions described in Leyh-Bouille *et al.*, 1970c) of *S.* R 61 was 10 mg/ml of penicillin G and vancomycin, 20 mg/ml of ristocetin, and 100 mg/ml of oxacillin and cephalothin. Dixon plots of enzyme inhibition carried out in 0.01 M Tris-HCl buffer pH 7.5 with concentrations of peptide 2 (Table II) from 2.13×10^{-3} M to 6.6×10^{-3} M, in the presence of either penicillin G (0.5 to 3×10^{-7} M) or cephalothin (2×10^{-7} to 15×10^{-7} M), gave straight lines meeting in a point from which K_i values and V_{max} values were calculated. The K_i values were 7.5×10^{-8} M for penicillin G and 1.06×10^{-6} M for cephalothin. V_{max} values were those reported in Table II.

Discussion

The specificity profile of the R 61 enzyme closely resembles that of the *albus* G enzyme in that both the structure of the C-terminal DD dipeptide and that of the preceding L-R_3 residue are essential for its action. The results obtained with the R 61 enzyme are consistent with the hypothesis previously proposed (Leyh-Bouille *et al.*, 1970b) that the DD-carboxypeptidase may be the membrane-bound transpeptidase that has undergone solubilization. Lawrence and Strominger (1970), discussing their work with a particulate carboxypeptidase from *Bacillus subtilis*, have made a similar proposal. The two DD-carboxypeptidases of *S. albus* G and *S.* R 61, however, exhibit different catalytic properties. (1) In contrast with the *albus* G enzymes, the R 61 enzyme has high K_m values with all the peptides which were used (Table II), but it is more effective than the *albus* G enzyme in promoting the hydrolysis of at least some of them (high V_{max} values, Table II). (2) *S. albus* G was resistant to penicillin and so, too, was its DD-carboxypeptidase (Leyh-Bouille *et al.*, 1970c). By contrast, *S.* R 61 is a strain sensitive to penicillin and related antibiotics, and, so too, is its DD-carboxypeptidase.

The R 61 DD-carboxypeptidase is competitively inhibited by penicillin G and cephalothin, and in this respect it behaves as the DD-carboxypeptidase previously isolated from *Escherichia coli* by Izaki and Strominger (1968). These kinetics suggest that these antibiotics resemble the substrate sufficiently to be bound in its stead on the catalytically active site and thus, they give support to the idea that penicillins are structural analogs of C-terminal acyl-D-alanyl-D-alanine (Tipper and Strominger, 1965). Following this hypothesis and in order to explain the penicillin resistance of the DD-carboxypeptidase from *S. albus* G, it has been proposed (Leyh-Bouille *et al.*, 1970c) that the specific conformation in the C-terminal L-R_3-D-alanyl-D-alanine specifically recognized by both the *E. coli* and *S.* R 61 enzymes would be different from that recognized by the *S. albus* G enzyme, and that penicillin would be structurally analogous only to the conformation recognized by the two former penicillin-sensitive enzymes. An alternative explanation, however, is suggested by the data reported in Table II. As can be seen from this table, peptides which are poor substrates for the penicillin-sensitive R 61 enzyme have K_m values as high as the good substrates but the former have much lower V_{max} values. The interpretation could be that this penicillin-sensitive enzyme has a binding surface which is not very specific, being able to bind even molecules that are only remotely analogous to the substrate; however, the alignment of the catalytic groups induced on binding would be incorrect or unfavorable for poor substrates, thus decreasing very much the V_{max} or even preventing enzyme action. By analogy

with the mechanism of action of penicillinases (Citri, 1971), this model involving conformational responses of the enzyme toward substrates and effectors has the advantage of stressing the independence of binding and hydrolysis and it allows the possibility that molecules such as penicillin which are not obvious substrate analogs could be bound by some DD-carboxypeptidases more strongly than the natural substrate. The competitive type kinetics exhibited by these penicillin-sensitive DD-carboxypeptidases in the presence of penicillin would then reflect a decrease in the binding constant of the enzyme for the substrate due to a change in the conformational state of the protein. Much remains to be done in order to establish this new idea. Study of other bacterial DD-carboxypeptidases that differ from those of *S. albus* G and *S.* R 61 by their capabilities of recognizing the penicillin molecule is in progress. It is hoped that the comparative biochemistry of these enzymes will contribute to the elucidation of the precise molecular basis of the antibacterial action of penicillin.

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