Penicillin-Sensitive DD-Carboxypeptidases from *Streptomyces* Strains R39 and K11†

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ABSTRACT: The two penicillin-sensitive DD-carboxypeptidases from *Streptomyces* R39 and K11 are anionic at pH 8. They specifically recognize a C-terminal L-R_3-D-alanyl-D sequence with a long side chain at the R_3 position. The two enzymes differ from each other with respect to: (1) the effects of ionic strength on activity, (2) the influence exerted on activity by the presence of a free amino group at the end of the L-R_3 side chain, (3) the K_m and V_{max} values. Enzyme K11 has K_m values which are high for both good and poor substrates. The enzyme efficiency reflects itself in V_{max} values which are high for good substrates and low for poor substrates. Enzyme R39 has K_m values which are low for good substrates. The enzyme efficiency toward various substrates reflects itself in the K_m and, to a lesser extent, in the V_{max} values, (4) the effects of penicillin. Kinetically, inhibition of enzyme K11 by penicillin is competitive. On the contrary, inhibition of enzyme R39 by penicillin is noncompetitive and increasing penicillin concentrations cause disproportionate decreases in the catalytic rate. Noncompetitiveness cannot be attributed to an irreversible inactivation of the enzyme by penicillin.

The target of penicillin and related antibiotics is a membrane-bound enzyme which catalyzes the transpeptidation reaction involved in the last step of the bacterial wall peptidoglycan biosynthesis (Tipper and Strominger, 1965; Wise and Park, 1965). No transpeptidase has yet been isolated and characterized. *Streptomyces* sp, however, possess extracellular DD-carboxypeptidases which might be the uncoupled, solubilized transpeptidases. The isolation and the substrate requirements of the penicillin-sensitive DD-carboxypeptidase from *Streptomyces* R61 and of the penicillin-resistant DD-carboxypeptidase from *Streptomyces albus* G have been previously described (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1970a,b; Leyh-Bouille *et al.*, 1971). The present paper deals with the isolation of and the substrate requirements exhibited by two other penicillin-sensitive DD-carboxypeptidases from *Streptomyces* strains R39 and K11.

Materials and Methods

Strains. Streptomyces R39 and K11, and *Streptomyces* R61, which was previously studied (Leyh-Bouille *et al.*, 1971), have similar penicillin sensitivity (LD_{50} on single cell colonies: 0.25 to 2 μg of penicillin G per ml).

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

Analytical polyacrylamide gel electrophoresis was carried out using the Acrylophor apparatus (Pleuger, Antwerp, Belgium). Cylindrical gels (65 × 7 mm) were prepared with 7% acrylamide and 0.18% *N,N'*-methylenebisacrylamide in 0.375 M Tris-HCl buffer, pH 8.5, and polymerized in the presence of 0.07% ammonium persulfate and 0.03% *N,N,N',N'*-tetramethylethylenediamine. The electrolyte was 0.025 M (in Tris) Tris-glycine buffer, pH 8.3. Bromophenol was used as tracing dye. To the enzyme solution (about 25 μg of protein in 0.1 ml), sucrose was added and samples were applied to the top of the gels. A potential of 80 V (4–5 mA per tube) was applied for 2 hr. Proteins were stained with Coomassie Blue. Excess stain was removed by diffusion in 10% acetic acid.

Preparative polyacrylamide gel electrophoresis was carried out using a Shandon preparative apparatus (No. SAE-2782). The gel was polymerized in the presence of 0.14% ammonium persulfate. The Tris-glycine buffer (pH 8.3) was 0.042 M in Tris. Fractions reaching the bottom of the gel were collected by means of a flow of 0.5 M Tris-acetate buffer (pH 8.3).

Walls and Peptides. The walls of *Corynebacterium poinsettiae* and the peptides used in the course of the present study were previously described (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1970a, 1971). The bisdisaccharide peptide dimer isolated from walls of *C. poinsettiae* (see Table II, peptide 13 in Leyh-Bouille *et al.*, 1971) contains two β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramylglycyl-γ-D-glutamyl-L-homoseryl-D-alanine monomers joined together by a D-ornithine residue. D-Ornithine is linked to the C-terminal D-alanine of one peptide by its α-amino group and to the α-COOH of glutamic acid of the other peptide by its δ-amino group. The D-alanyl-D-ornithine bond is thus at a C-terminal position.

Culture Media. Peptone medium (medium A in Leyh-Bouille *et al.*, 1971) was used for strain R39 and glycerol–casein medium (medium B in Leyh-Bouille *et al.*, 1971) was used for strain K11.

DD-Carboxypeptidase Unit. One unit of enzyme catalyzes the hydrolysis of 1 nmole of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine per hr, at 37°, when 5 μmoles (for K11 enzyme) or 0.25 μmole (for R39 enzyme) of peptide is incubated with the relevant enzyme in 30 μl of 0.01 M Tris-HCl buffer (pH 7.5) for K11 enzyme or in 30 μl of 0.03 M Tris-HCl buffer (pH 7.5) supplemented with 0.003 M $MgCl_2$ for R39 enzyme. In both cases, the substrate concentration is about 10 × K_m (*vide infra*).

† From the Service de Microbiologie, Institut de Botanique, Université de Liège, Sart-Tilman, 4000 Liege, Belgium. *Received October 27, 1971.* This research has been supported in part by the Fonds de la Recherche Fondamentale Collective, Brussels, Belgium (Contracts No. 515 and 1000), by the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture, Brussels, Belgium (Contract No. 1699), by a fellowship (to Marlies Nakel) from the Deutsche Forschungsgemeinschaft and by a NATO fellowship (to Kenneth Johnson).

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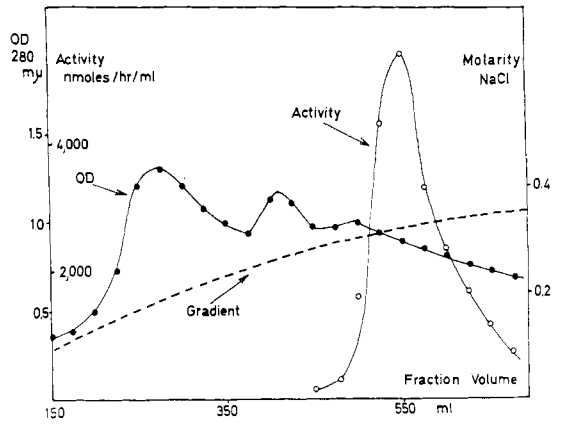

FIGURE 1: Purification of DD-carboxypeptidase from *Streptomyces* R39. Elution from DEAE-cellulose column with increasing gradient of NaCl in 0.1 M Tris-HCl buffer (pH 7.5) (step 2). For conditions, see text. Aliquots (12 μl) were incubated at 37° with 50 nmoles of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine in a final volume of 35 μl for 45 min. Results are expressed in nmoles of peptide hydrolyzed per ml of fractions, per hr. OD = optical density of the effluent fractions at 280 mμ.

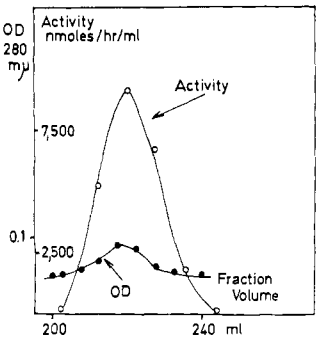

FIGURE 2: Purification of DD-carboxypeptidase from *Streptomyces* R39. Filtration on a 400-ml Sephadex G-75 column (step 4). For enzyme estimation, see Figure 1 except that 9 μl of effluent fractions were incubated with the peptide for 25 min.

Penicillinase Unit. One unit catalyzes the hydrolysis of 1 μmole of benzylpenicillin per hour, at pH 7 and 30°. Penicillinase assays were carried out by the techniques of Novick (1963) modified as follows. Incubations were performed in a final volume of 30 μl containing 5 μl of enzyme preparation, 20 μl of 0.03 M sodium phosphate buffer (pH 7), and 5 μl of 6 mM sodium benzylpenicillin G in the same phosphate buffer. After 10–30 min at 30° the reaction mixtures were added first with 200 μl of 1 M sodium acetate buffer (pH 3.6) and then with 200 μl of color reagent. The color reagent contained equal volumes of a water-soluble starch solution (0.8%; w/v) and of a 240 μM I_2 + 4.8 mM KI solution. After 10 min at 25°, the OD of the reaction mixtures was determined at 620 mμ. For controls, the same mixtures but without enzyme were incubated as above. The reaction was stopped with the pH 3.6 buffer. Color reagent and the same amount of enzyme as in the tests were then added and the OD was measured.

Experimental Section

Excretion of DD-*Carboxypeptidases. Streptomyces* R39 was grown at 28° in a New Brunswick Shaker incubator, in 1-l. flasks containing 400 ml of peptone medium. The extracellular DD-carboxypeptidase activity was estimated by incubating 12 μl of culture filtrate with 50 nmoles of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine in a final volume of 30 μl and in the presence of a 0.03 M (final concentration) Tris-HCl buffer (pH 7.5). Free D-alanine was estimated. Maximal activity occurred after 135 hr of culture. Under these conditions, 1 ml of culture hydrolyzed 600 nmoles of substrate per hr. Penicillinase activity was of 4.1 units/ml.

Similarly, *Streptomyces* K11 was grown in 1-l. flasks containing 500 ml of glycerol–casein medium. Maximal activity occurred after 88 hr of culture. Under the same conditions as above but without addition of Tris buffer, 1 ml of culture hydrolyzed 70 nmoles of substrate per hour. Culture filtrates contained about 3.2 penicillinase units per ml.

Purification of DD-*Carboxypeptidase from Streptomyces R39.* All the following steps were carried out at 4°.

STEP 1. The DD-carboxypeptidase in the culture filtrate (12 l.) was adsorbed on DEAE-cellulose (240 g, wet weight) previously equilibrated against 0.05 M Tris-HCl buffer (pH 7.5). The suspension was stirred for about 1 hr. The resin-adsorbed enzyme complex was collected and resuspended in 700 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 1 M NaCl. After stirring for 1 hr, the resin was filtered and the treatment with Tris-NaCl was repeated. The pooled Tris-NaCl eluates (1400 ml) were concentrated to 120 ml by dialysis against Carbowax 4000 followed by filtration on a Diaflo UM 10 membrane using an Amicon apparatus. The enzyme solution was diluted to 300-ml final volume, with 0.1 M Tris-HCl buffer (pH 7.5) and concentrated, as above, to 200 ml. The enzyme solution was clarified by centrifugation and dialyzed for 4 hr against 0.1 M Tris buffer (pH 7.5) and finally diluted with the same buffer to 1000 ml.

STEP 2. The solution, after step 1, was filtered through a 125-ml column of DEAE-cellulose previously equilibrated against 0.1 M Tris-HCl buffer (pH 7.5). The column was washed with the same buffer until the effluent was almost free of material absorbing at 280 mμ. The enzyme was then eluted from the resin with an increasing gradient of NaCl in Tris-HCl buffer (pH 7.5) (mixing flask, at constant volume: 500 ml of 0.1 M Tris-HCl buffer (pH 7.5); solution added: the same buffer supplemented with 0.5 M NaCl). The active fractions (500–625 ml; Figure 1) were pooled and concentrated (10 ml) by ultrafiltration.

STEP 3. After step 2, the enzyme solution was applied in amounts of 1 ml to a 200-ml Sephadex G-75 column previously equilibrated with 0.1 M Tris-HCl buffer, supplemented with 0.01 M $MgCl_2$, and eluted with the same buffer. The active fractions were pooled and concentrated by ultrafiltration to 10 ml, and the resulting solution was again filtered through a 400-ml Sephadex G-75 column under the same conditions as above. The active fractions (210–270 ml) were pooled and concentrated to 4.5 ml by ultrafiltration.

STEP 4. The enzyme solution after step 3, in amounts of 1 ml, was subjected to preparative polyacrylamide gel electrophoresis. Samples mixed with 0.5 ml of 40% (w/v) sucrose were applied to the top of the gel (4 cm height) under the following conditions: constant current: 40 mA, 3 hr; voltage varied from 300 to 400 V. The enzyme migrated toward the anode as rapidly as Bromophenol Blue. The active fractions were pooled and concentrated by ultrafiltration. The resulting solution (10 ml) was filtered in 0.1 M Tris-HCl buffer (pH 7.5), supplemented with 0.01 M $MgCl_2$, on a 400-ml Sephadex G-75 column (Figure 2). The active fractions (210–230 ml) were pooled and concentrated by ultrafiltration to 4.6 ml. This final solution contained the purified enzyme.

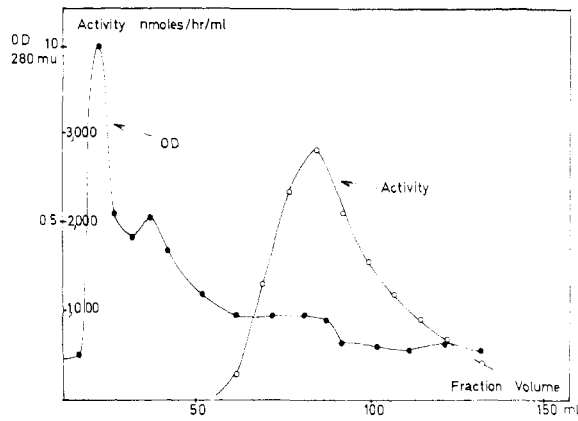

FIGURE 3: Purification of DD-carboxypeptidase from *Streptomyces* K11. Elution from polyacrylamide gel after electrophoresis (height of the gel: 4 cm; for conditions, see text) (step 4). For enzyme estimation, see Figure 1 except that 10 μl of effluent fractions were incubated with the peptide for 90 min.

TABLE I: Isolation and Purification of DD-Carboxypeptidases.

Strain	Step	Relative Specific Activity[a]	Total Activity	Recovery (%)	Total Protein in mg
S. R39	Culture filtrate	430	7,500,000	100	17,400
	1	4,590	6,380,000	85	1,390
	2	36,200	4,050,000	54	111
	3	125,000	1,725,000	23	14
	4	213,000	622,000	8.3	2.9
S. K11	Culture filtrate	50	1,365,000	100	27,300
	1		870,000	65	
	2	100	893,000	66	8,900
	3		250,000	19	
	4	260,000	130,000	10	0.5

[a] N^{α},N^{ϵ}-Diacetyl-L-lysyl-D-alanyl-D-alanine (50 nmoles) was incubated with the enzyme preparation, at 37°, in 35 μl (final volume) of 0.03 M Tris-HCl buffer (pH 8) + 0.003 M $MgCl_2$ (R39 enzyme) or 0.01 M Tris-HCl buffer (pH 8) (K11 enzyme). The activities were estimated under the conditions required to liberate 25% of the D-alanine that is liberated at completion of the reaction. Specific activity is expressed in nanoequivalents of D-alanyl-D-alanine linkage hydrolyzed per hr, per mg of protein. The final preparations after step 4 exhibited, under conditions of peptide concentrations close to enzyme saturation, absolute specific activities of 330,000 units/mg of protein for enzyme R39 (K_m: 0.8 M, *vide infra*) and of 2,000,000 units/mg of protein for enzyme K11 (K_m: 11 mM, *vide infra*). Protein concentration was estimated for R39 enzyme by using Lowry's technique (Lowry *et al.*, 1951) and for K11 enzyme, by measuring the amount of total amino groups available to fluorodinitrobenzene after 6 N HCl hydrolysis (100°, 20 hr).

Table I gives the recoveries and increases in specific activities as determined with N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (concentration: 1.5 mM). The final preparation contained 330,000 DD-carboxypeptidase units per mg of protein (using a peptide concentration of 7.5 mM; *i.e.*, 10 × K_m). Although only one protein band could be detected by polyacrylamide gel electrophoresis (*vide infra*), some purified enzyme preparations exhibited a weak penicillinase activity of 0.03 unit, or less, per mg of protein. When necessary, the last traces of penicillinase were removed by additional chromatography on DEAE-cellulose (under the same conditions as those described above: see step 2) and finally on Sephadex G-75.

Purification of DD-*Carboxypeptidase from Streptomyces K11.* All the steps described below were carried out at 4°.

STEP 1. The enzyme in the culture filtrate (20 l.) was added to 600 g (dry weight) of Amberlite CG50 H^+ and the pH value adjusted to 4 with acetic acid. The resin with the adsorbed enzymes was filtered and resuspended in 4 l. of 0.1 M K_2HPO_4 and the pH was brought to 8 with ammonia. The resin was again removed by filtration.

STEP 2. Solid $(NH_4)_2SO_4$ (183 g/l. of solution) was added to the enzyme solution after step 1. The precipitate, containing most of the penicillinase present in the culture filtrate, was collected by centrifugation and discarded. Solid $(NH_4)_2SO_4$ (366 g/l. of solution) was added to the supernatant. The precipitate containing most of the DD-carboxypeptidase activity was collected by centrifugation and solubilized in 750 ml of water, and the salting out was repeated. The precipitate was collected by centrifugation and dissolved in 150 ml of water. This solution was clarified by centrifugation.

STEP 3. The enzyme solution after step 2 (150 ml) was filtered on a 1-l. column of Sephadex G-75 in 0.05 M Tris-HCl buffer (pH 8). The active fractions (480–750 ml) were partially separated from a black ink-like pigment. They were pooled and the resulting solution was filtered through a 200-ml column of DEAE-cellulose previously equilibrated against 0.05 M Tris-HCl buffer (pH 8). The column was washed with the same buffer containing 0.05 M NaCl until the effluent contained only traces of material absorbing at 280 mμ. The enzyme was eluted from the resin with an increasing gradient of Tris-NaCl buffer (pH 8) (mixing flask at constant volume: 300 ml of 0.05 M Tris-HCl buffer (pH 8); solution added: 0.05 M Tris-HCl buffer and 1 M NaCl). The majority of the enzyme was eluted at about 0.4 M. Some enzyme, however, was eluted at higher molarity, together with the pigment. The active fractions (50 ml to 250 ml) were pooled, concentrated by ultrafiltration to 4 ml, and dialyzed against 0.05 M Tris-HCl buffer (pH 8).

STEP 4. The enzyme solution after step 3 was subjected to preparative polyacrylamide gel electrophoresis (4 hr) under the same conditions described for the enzyme R39 (height of the gel column: 4 cm) (Figure 3). Enzyme K11 migrated toward the anode less rapidly than Bromophenol Blue. The enzyme was separated from most of the pigment which was much more anionic at that pH. The active fractions (65–125 ml) were pooled, concentrated by ultrafiltration, dialyzed against 0.05 M Tris-HCl buffer (pH 8), and filtered on a 150-ml column of Sephadex G-75 in the same buffer. The active fractions, pooled and concentrated by ultrafiltration, were again submitted to polyacrylamide gel electrophoresis (6 hr; height of the column: 6 cm) under the same conditions as above. The active fractions were pooled, concentrated by ultrafiltration (5 ml), and dialyzed against 0.01 M Tris-HCl buffer (pH 8). The final solution was completely free of pigment and contained the purified enzyme.

Table I gives the recoveries and increases in specific activities with N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine as sub-

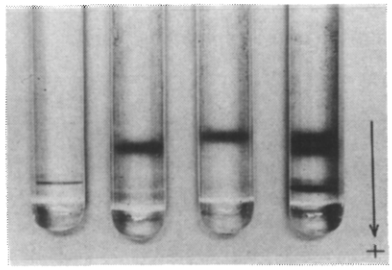

FIGURE 4: Analytical polyacrylamide gel electrophoresis of DD-carboxypeptidases from *Streptomyces* strains R39, R61, and K11. Samples, *i.e.*, each of the three purified enzymes either separately or mixed together, were applied directly to the tops of the gels. A potential of 80 V was applied for 2 hr. For conditions, see text. Prints show half-gels stained for protein with Coomassie Blue. The three enzymes ran toward the anode at pH 8.5. In all cases, activity upon N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine was found associated with the protein bands. From left to right: enzyme R39, enzyme R61, enzyme K11, and a mixture of three enzymes.

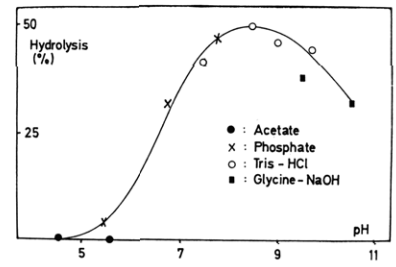

FIGURE 5: pH optimum of DD-carboxypeptidase from *Streptomyces* R39. All the buffers were used at 0.02 M. N^{α},N^{ϵ}-Diacetyl-L-lysyl-D-alanyl-D-alanine (40 nmoles) was incubated at 37° for 1 hr in the presence of 0.65 μl of the enzyme solution after step 2, in a final volume of 35 μl. Results are expressed in per cent of hydrolysis.

strate (concentration: 1.5 mM). The final preparation contained 2×10^6 DD-carboxypeptidase units per mg of protein (at a peptide concentration close to enzyme saturation). Analytical polyacrylamide gel electrophoresis revealed one protein band (*vide infra*). No penicillinase activity could be detected in the preparation.

Comparative Polyacrylamide Gel Electrophoresis of DD-*Carboxypeptidases from S. R39, S. K11, and S. R61.* The procedure used (Materials and Methods) was essentially that described for the R61 enzyme in Leyh-Bouille *et al.* (1971). After electrophoresis, the gels were divided in two lengthwise. One half was stained for protein and the other half was cut into 2-mm slices at right angles to the length of the gel. Each slice was eluted overnight with water (100 μl) and aliquots (10 μl) of the eluates were tested upon N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine. As shown in Figure 4, each of the final enzyme preparations contained only one visible protein band. Enzyme activity was always associated with the protein band. Enzymes R39 was the most anionic protein. Enzymes K11 and R61 exhibited very similar electrophoretic properties but enzyme R61 was always slightly more anionic than enzyme K11.

pH and Salt Requirements of DD-*Carboxypeptidases from S. R39 and S. K11.* For enzyme R39, the pH optimum of the reaction was between 7 and 10, in the presence of phosphate, Tris-HCl, or glycine–NaOH buffers (Figure 5). Optimum ionic strength for Tris-HCl buffer was 0.03 M or higher (Figure 6). The reaction was not inhibited at 0.2 M. Cations had a small activating effect. Maximal activity was observed when Mg^{2+}, Ca^{2+}, or Zn^{2+} (0.002–0.01 M) was added to Tris-HCl buffer (pH 8). Sodium ethylenediaminetetraacetate (10^{-3} M) did not inhibit the reaction. The pH and salt requirements of the K11 enzyme were found to be identical with those of the R61 enzyme (Leyh-Bouille *et al.*, 1971). The pH optimum was between 5 and 9 and the optimum ionic strength was between 0.002 and 0.01 for Tris-HCl buffers. Mg^{2+} did not activate the reaction and sodium ethylenediaminetetraacetate caused no inhibition.

Substrate Requirements of DD-*Carboxypeptidases from S. R39 and S. K11.* These were studied on peptides presenting the general structure X → L-R_3 → R_2 → R_1 (OH). The Michaelis constant (K_m) and maximal velocity (V_{max}) were obtained (Table II) on the basis of initial velocity measurements at 37°, in 0.03 M Tris-HCl buffer (pH 8) + 3 mM $MgCl_2$ for enzyme R39 and in 0.01 M Tris-HCl buffer (pH 7.5) for enzyme K11. Enzyme efficiency (V_{max}/K_m) was also calculated. With some peptides (Table III), the rate of hydrolysis was only measured at low substrate concentrations. Tables II and III compare results for the R39 and K11 enzymes. K_m and V_{max} values for the action of enzymes R61 (Leyh-Bouille *et al.*, 1971) and *albus* G (Leyh-Bouille *et al.*, 1970b) upon the same peptides, have been previously presented.

With enzyme K11, the influences exerted by the C-terminal R_1 residue (Table II, peptides **1–5**), by the penultimate C-terminal R_2 residue (Table II, peptides **6–8**), by the length of an uncharged side chain on the preceding L-R_3 residue (Table II, peptide **9** and Table III), and by the introduction of charged groups at the end of the L-R_3 side chain (Table II, peptides **10–12**) were virtually identical with those observed with the R61 enzyme. Both K11 and R61 enzymes showed a considerable specificity for a C-terminal D-alanyl-D sequence, for a L-R_3 residue with a long side chain, and for the absence of any charged groups at this latter position. A N^{ϵ}-pentaglycyl-L-lysine group, however, is compatible with a high enzyme efficiency (Table II, peptide **12**). The enzymes R61 and K11, as they were prepared, only differ from each other in that the specific activity of the K11 enzyme (2×10^6 units/mg) is over twice as high as that of the R61 enzyme (0.89×10^6 units/mg).

The R39 enzyme also showed a considerable specificity for a C-terminal L-R_3-D-alanyl-D sequence with a long side chain at the L-R_3 position (Tables II and III). Strikingly, however, and in marked contrast with enzymes K11 and R61, the presence of a free amino group at the end of the L-R_3 side chain always enhanced considerably the efficiency of the R39 enzyme (Table II, peptides **10–12**). This was observed when

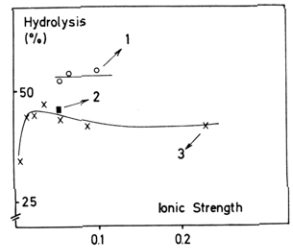

FIGURE 6: Effect on DD-carboxypeptidase from *Streptomyces* R39 of concentration of Tris-HCl buffer (pH 8), Mg^{2+}, Ca^{2+}, and Zn^{2+} cations, and sodium ethylenediaminetetraacetate. For conditions, see Figure 5. (1) -O-O-: Effect of Mg^{2+} (or Ca^{2+} or Zn^{2+}) at 2×10^{-3}–2×10^{-2} M concentrations in 0.05 M Tris-HCl buffer (pH 8). (2) Effect of 1×10^{-3} M EDTA in 0.05 M Tris-HCl buffer (pH 8). (3) -X-X-: Effect of increasing molarity of Tris-HCl buffer pH 8.

TABLE II: Efficiency of Hydrolysis of the DD-Carboxypeptidases from *Streptomyces* R39 and *Streptomyces* K11.[a]

No.	Substrate	K11 K_m	K11 V_{max}	K11 Efficiency	R39 K_m	R39 V_{max}	R39 Efficiency
1	R_1 → L-Lys → D-Ala → D-Ala (ε: acetyl)	8.3	740	90	0.8	143	180
2	Acetyl → L-Lys → D-Ala → D-Ala (ε: acetyl)	11	2000	180	0.8	330	410
3	→ D-Ala → D-Leu	10	160	16	0.7	230	320
4	→ D-Ala → Gly	12	220	18	2.5	100	40
5	→ D-Ala → L-Ala	Virtually no hydrolysis				No hydrolysis	
6	→ Gly → D-Ala	14	10	0.7		No hydrolysis	
7	→ D-Leu → D-Ala	13	38	2.9		No hydrolysis	
8	→ L-Ala → D-Ala	Virtually no hydrolysis				No hydrolysis	
9	Acetyl → L-DAB → D-Ala → D-Ala (γ: acetyl)				2.35	400	170
10	Acetyl → L-Lys → (ε: H)	30	9	0.3	0.20	600	3000
11	R_2 → (L) DAP (H)–(OH) (D) →	17	18	1	0.25	400	1600
12	R_3 → L-Lys → (ε: (H)-Gly_5)	11	1050	95	0.30	420	1400

[a] K_m values are expressed in mM; V_{max} in μmoles per mg of enzyme per hr; Efficiency in V_{max}/K_m. The values were obtained at 37° in 0.01 M Tris-HCl buffer (pH 7.5) (K11 enzyme) or in 0.03 M Tris-HCl buffer (pH 8) + 0.003 M $MgCl_2$ (R39 enzyme). R_1: Acetyl-L-Ala-D-Glu-γ; R_2: UDP-MurNAc-L-Ala-D-Glu-γ; R_3: N^{α}-(GlN_{AC}-$MurN_{AC}$-L-Ala-D-Glu-NH_2-γ) MurNAC = *N*-acetylmuramic acid; GlNAC = *N*-acetylglucosamine; DAP = diaminopimelic acid. Vertical arrow indicates the sensitive linkage.

N^{ϵ}-acetyl-L-lysine at that position was replaced either by an N^{ϵ}-unsubstituted-L-lysine or by *meso*-diaminopimelic acid or by N^{ϵ}-pentaglycyl-L-lysine.

Survey of the Ability of Streptomyces sp to Excrete DD-*Carboxypeptidases.* The above studies carried out with the K11 and R39 enzymes showed that the presence of an amino group occurring on the L-R_3 side chain can differently influence the efficiency of the DD-carboxypeptidases from *Streptomyces* sp. A previous survey of the ability of *Streptomyces* sp to excrete DD-carboxypeptidases, carried out with the help of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine, had led to the conclusion that, seemingly, few strains were able to excrete the enzyme. A new survey carried out with the help of N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl); N^{ϵ}-(pentaglycyl)-L-lysyl-D-alanyl-D-alanine (peptide **12** in Table II) showed that, on the contrary, most if not all of the strains, produced DD-carboxypeptidases. Strains K11, R61, *albus* G, and to a lesser extent, strain R39, are thus rather exceptional in that they do not require the presence of a free amino group at the L-R_3 position for enzyme activity. Another conclusion from these studies is that the presence of an N-terminal glycine at the end of a long side chain at the L-R_3 position (as it occurs in the above disaccharide pentapeptide pentaglycine and in the natural peptide which undergoes transpeptidation in *Streptomyces* sp) is a feature that is probably common to the specificity profiles of all *Streptomyces* DD-carboxypeptidases.

Endopeptidase Activity of DD-*Carboxypeptidases from S. R39 and S. K11.* The activities of the enzymes K11 and R39 were also tested upon isolated walls of *C. poinsettiae* and upon the bisdisaccharide peptide dimer isolated from these walls (Materials and Methods; see also peptide **13** in Table II Leyh-Bouille *et al.*, 1971). Like the R61 enzyme, neither the K11 enzyme or the R39 enzyme were lytic upon walls. When tested upon the isolated bisdisaccharide peptide dimer, both K11 and R39 enzymes exhibited a weak activity comparable to that of the R61 enzyme (Leyh-Bouille *et al.*, 1971). At a peptide concentration of 0.7 mM, the enzyme activity expressed in nanoequivalents of N^{δ}-(D-Ala-D-Orn) linkage hydrolyzed per milligram of enzyme, per hour, was 130 for K11 enzyme and 570 for R39 enzyme (to be compared with a value of 90,000 when N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine is used as substrate; see Table III).

Inhibition of DD-*Carboxypeptidases from S. R39 and S. K11*

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

Substrate	Specific Activity[a] of *S.* K11	Enzyme from *S.* R39
Ac → L-Ala → ↓ D-Ala → D-Ala	1,100	200
R_1 → L-homoSer →	1,600	6,000
N^{α},N^{γ}-diacetyl → L-DAB →	6,500	14,000
N^{α},N^{δ}-diacetyl → L-Orn →	50,000	75,000
N^{α},N^{ϵ}-diacetyl → L-Lys →	90,000	90,000

[a] Expressed in nanoequivalents of D-Ala-D-Ala linkage hydrolyzed per mg of enzyme, per hr. Conditions of incubation were as in Table II; substrate concentrations: 0.45 mM. Activities were always calculated on an extent of hydrolysis of 50% or less. DAB = diaminobutyric acid. R_1 = UDP-*N*-acetylmuraminyl-Gly-D-Glu-γ. Vertical arrow indicates the sensitive linkage.

by Penicillin G. Double reciprocal plots of $1/v$ *vs.* $1/s$ in the presence of different concentrations of penicillin G and plots of $1/v$ *vs.* penicillin concentration in the presence of different concentrations of substrate are shown in Figures 7 and 8 for enzyme R39 and in Figures 9 and 10 for enzyme K11. N^{α},-N^{ϵ}-Diacetyl-L-lysyl-D-alanyl-D-alanine was used at concentrations below and above K_m values, as indicated in the relevant figures. Determinations were carried out in 0.02 M sodium phosphate buffer (pH 8) for enzyme R39 and in 0.01 M Tris-HCl buffer (pH 7.5) for enzyme K11. All reaction mixtures were incubated at 37° for 20 min. The concentration of enzyme K11 (6 units per μl final) and that of enzyme R39 (1 unit per μl final) were such that the extent of hydrolysis ranged from 5 to 25% depending upon the concentrations in substrate.

With K11 enzyme and as previously observed with R61 enzyme, plots of $1/v$ *vs.* $1/s$ gave straight lines intersecting at one point located on the $1/v$ axis (Figure 9). Dixon plots (Figure 10) also gave straight lines meeting at a point from which a K_i value of 6×10^{-8} M was calculated (a K_i value of 7.5×10^{-8} M had previously been found for R61 enzyme: Leyh-Bouille *et al.*, 1971).

With R39 enzyme, plots of $1/v$ *vs.* $1/s$ (Figure 7) in the presence of penicillin, gave straight lines intersecting at one point located to the left of the origin and above the $1/s$ axis, kinetics diagnostic of a noncompetitive inhibition. Dixon plots (Figure 8) did not give straight lines. Upward concavity of the curves strongly suggests that the penicillin molecules cooperate in their inhibiting action upon the catalytic rate of the reaction. Extrapolation of the values obtained at low concentrations of penicillin gave an apparent K_i value of 1×10^{-8} M. Effects on the affinity (*i.e.*, increase in K_m values) were found to be proportional to penicillin concentration.

Another possible explanation of the observed kinetics would be that penicillin became irreversibly bound to the enzyme *e.g.*, by penicilloylation, as proposed for the membrane-bound enzyme of *Bacillus subtilis* (Lawrence and Strominger, 1970a,b). Although such inhibition should not show the particular type of concentration dependence observed with R39 enzyme, the following experiments were carried out to eliminate the possibility of irreversible binding.

Reversibility of the Inhibition of DD-*Carboxypeptidase from S. R39 by Penicillin G.* (1) COMPETITION FOR PENICILLIN BETWEEN DD-CARBOXYPEPTIDASE AND PENICILLINASE. N^{α},N^{ϵ}-Diacetyl-L-lysyl-D-alanyl-D-alanine (0.7×10^{-3} M) was incubated at 37° for 20 min in 0.02 M phosphate buffer (pH 8) with the R39 DD-carboxypeptidase (1 unit/μl) in the absence of penicillin G and in the presence of three different concentrations of antibiotic (0.66, 1.32, and 2×10^{-8} M, respectively). Final volumes were 150 μl. The same experiments were simultaneously carried out in the presence of 0.02×10^{-3} unit/μl and 0.27×10^{-3} unit/μl of a purified penicillinase isolated from the same *Streptomyces* R39 strain (unpublished results; for penicillinase unit see Materials and Methods). As shown in Figure 11, there was virtually no inhibition of the carboxypeptidase activity by penicillin when 0.27×10^{-3} unit of penicillinase per μl was present in the reaction mixture. The K_m value of the R39 penicillinase for penicillin is 6.5×10^{-5} M (unpublished data) whereas the

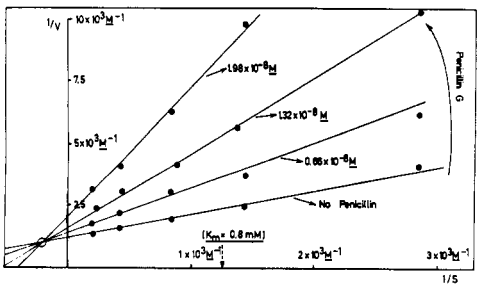

Figure 7: Effect of penicillin G on DD-carboxypeptidase from *Streptomyces* R39. Plot of $1/v$ *vs.* $1/s$ in the presence of different concentrations of penicillin. For conditions, see text. The $1/v$ values are calculated for 1-hr incubation.

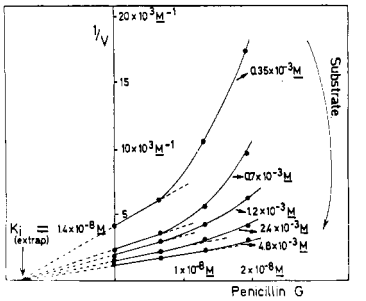

FIGURE 8: Effect of penicillin G on DD-carboxypeptidase from *Streptomyces* R39. Plot of $1/v$ *vs.* penicillin in the presence of different concentrations of substrate. For conditions, see text. The $1/v$ values are calculated for 1-hr incubation.

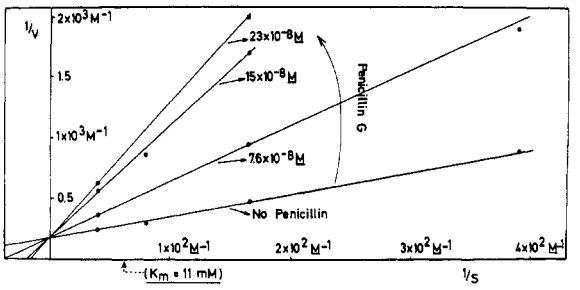

FIGURE 9: Effect of penicillin G on DD-carboxypeptidase from *Streptomyces* K11. Plot of $1/v$ *vs.* $1/s$ in the presence of different concentrations of penicillin. For conditions, see text. The $1/v$ values are calculated for 1-hr incubation.

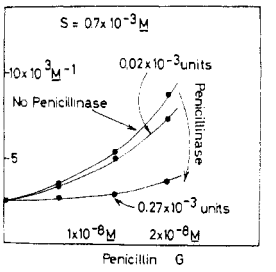

FIGURE 11: Competition between DD-carboxypeptidase from *S.* R39 and penicillinase from *S.* R39, for penicillin G. The upper curve in this figure (No penicillinase) represents data which were obtained under identical conditions as those given for the second upper curve of Figure 8. The $1/v$ values are calculated for 1-hr incubation.

extrapolated K_i value of the R39 carboxypeptidase for the antibiotic is 1.4×10^{-8} M (Figure 8). The fact that under these conditions, the penicillinase successfully competes with the carboxypeptidase for penicillin so that the carboxypeptidase activity is virtually unaltered, strongly suggests that the inhibition of the carboxypeptidase by penicillin (in the absence of penicillinase) is not carried out through a penicilloylation reaction or indeed any other type of irreversible binding.

(2) DILUTION OF CARBOXYPEPTIDASE–PENICILLIN MIXTURE SOLUTIONS. In a second set of experiments, more concentrated enzyme was preincubated with penicillin in an attempt to favor irreversible inactivation. The solutions were then diluted and enzyme activity was measured as before. The R39 carboxypeptidase (15 units/μl) was maintained at 37°, in 0.1 M Tris-HCl buffer (pH 7.5), for 0, 10, 30, and 90 min, in the absence of penicillin (assays 1), and in the presence of antibiotic at the two following concentrations 2×10^{-8} M (assays 2) and 1×10^{-7} M (assays 3). The reaction mixtures were then diluted 15-fold with a solution of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine in 0.02 M phosphate buffer (pH 8). The final volumes were 150 μl and the final concentrations were 0.7×10^{-3} M for the peptide, 1 unit/μl for the enzyme, and 0 (assays 1), 0.13×10^{-8} M (assays 2), and 0.66×10^{-8} M (assays 3) for penicillin. The reaction mixtures were then incubated at 37° for 20 min and free D-alanine was measured. The results of assays 1 showed that a preincubation of the carboxypeptidase at 37°, in Tris buffer, and in the absence of penicillin had no effect on the enzyme activity. In the cases where the carboxypeptidase was pretreated with 2×10^{-8} M penicillin (assays 2), the 15-fold dilutions of the carboxypeptidase–penicillin mixtures were found to be fully active on the peptide substrate. As shown in Figure 8, virtually no inhibition of the carboxypeptidase activity was observed when the peptide (0.7×10^{-3} M) was directly incubated for 20 min at 37° in the presence of the enzyme (1 unit/μl) and of penicillin (0.13×10^{-8} M). In the cases where the carboxypeptidase was pretreated with 1×10^{-7} M penicillin (assays 3), the 15-fold dilutions of the enzyme–penicillin mixtures were found to have a reduced carboxypeptidase activity. Irrespective of the conditions of the pretreatment (from 0 to 90 min), however, the inhibition of the carboxypeptidase was identical with that observed (Figure 8) when the peptide (0.7×10^{-3} M) was directly incubated for 20 min at 37° in the presence of enzyme (1 unit/μl) and of penicillin (0.66×10^{-8} M). These results thus indicate that no detectable irreversible inactivation of the carboxypeptidase occurs when the enzyme is treated in Tris buffer (pH 7.5) at 37°, for 0 to 90 min, with penicillin at concentrations equal to or less than 1×10^{-7} M.

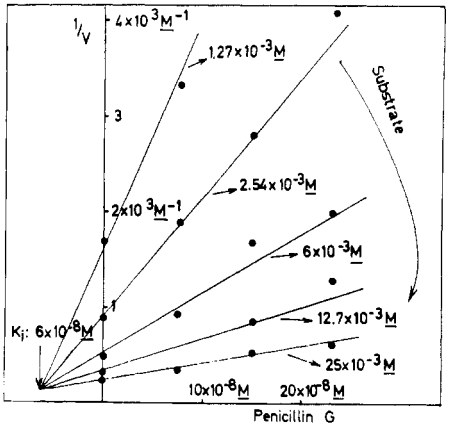

FIGURE 10: Effect of penicillin G on DD-carboxypeptidase from *Streptomyces* K11. Plot $1/v$ *vs.* penicillin in the presence of different concentrations of substrate. For conditions, see text. The $1/v$ values are calculated for 1-hr incubation.

(3) ULTRAFILTRATION OF CARBOXYPEPTIDASE–PENICILLIN MIXTURE SOLUTIONS. In the third type of experiment, concentrated enzyme was incubated with penicillin, and after 25-fold dilution the solution was reconcentrated by ultrafiltration. Under these conditions irreversibly bound penicillin would be expected to remain with the protein, whereas unbound penicillin in the dilute solution would pass into the filtrate. Conditions were chosen so that if completely retained, the penicillin would be sufficient to inhibit the enzyme by about 80%. The carboxypeptidase R39 (12,800 units) was dissolved in 2 ml of 0.05 M Tris-HCl buffer (pH 7.5) supplemented with 2×10^{-3} M $MgCl_2$. The solution was maintained at 37° for 1 hr, diluted to 50 ml with the same Tris Mg^{2+} buffer, and finally reconcentrated to the original volume (2 ml) by ultrafiltration at 4° on an UM-10 Diaflo membrane (assays 1). In another assay, the R39 enzyme was treated as above except that the 1-hr incubation at 37° was carried out in the presence of 2×10^{-6} M penicillin (assay 2). In a third assay, the enzyme R39 was incubated for 1 hr, at 37°, in the presence of penicillin (2×10^{-6} M). The enzyme–penicillin mixture, however, was not diluted but held at 4° for a period of time equal to that used for the ultrafiltrations in the previous assays (assay 3). Identical aliquots (25 μl) of the three enzyme assays were then added to 45 nmoles of diacetyl-L-lysyl-D-alanyl-D-alanine dissolved in 5 μl of water. The final concentrations were 5 units/μl for the enzyme and 1.35×10^{-3} M for the peptide. The solutions were incubated for 1 hr at 37°. The amounts of D-alanine liberated under these conditions

were 39 nmoles in assay 1, 27 nmoles in assay 2, and 8 nmoles in assay 3. Again, the substantial recovery of the carboxypeptidase activity which was observed under the conditions of assay 2 strongly suggests that penicillin does not irreversibly inactivate the DD-carboxypeptidase.

Discussion

Except that the K11 enzyme has a higher specific activity and is slightly less anionic at pH 8.5, the properties of the R61 and K11 enzymes are very similar. Both enzymes show competitive type inhibition by penicillin. On the other hand, the R39 enzyme is more anionic than the foregoing enzymes and markedly differs from them with respect to the K_m values, the influence exerted by an amino group at the L-R_3 position of the substrate, the effects of ionic strength and noncompetitive inhibition by penicillin. From an integration of the studies reported here and of those carried out on the DD-carboxypeptidase from *S. albus* G (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1970ab) and from *S.* R61 (Leyh-Bouille *et al.*, 1971) the following conclusions can be drawn.

Diversity of DD-*Carboxypeptidases.* All the *Streptomyces* DD-carboxypeptidases have in common the property of hydrolyzing the C-terminal D-alanyl-D linkage in acyl-L-R_3-D-alanyl-D peptides when a free N-terminal glycine group occurs at the end of a long side chain at the L-R_3 position. The disaccharide peptide N^{α}-(β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl); N^{ϵ}-(pentaglycyl)-L-lysyl-D-alanyl-D-alanine seems to be a substrate for all *Streptomyces* DD-carboxypeptidases. It also appears that the nature of the acyl group (*i.e.*, the β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl sequence in the above disaccharide peptide) which substitutes the L-R_3-D-alanyl-D sequence is not at least of prime importance for the enzyme activity. Individual DD-carboxypeptidases, however, differ from each other with respect to several properties. (1) Some, and perhaps most of them, are anionic at pH 8 but at least that of *S. albus* G is cationic at that pH. (2) The four enzymes so far studied (*albus* G, R61, K11, and R39) are able to hydrolyze C-terminal N^{α}-(D-alanyl)-D-diamino acid linkages that serve as interpeptide bonds in some wall peptide oligomers and hence, seemingly, behave as endopeptidases. Endopeptidase activity, however, does not necessarily confer upon the enzymes the ability to lyse walls. It may be significant that the *albus* G enzyme which is the only one to exhibit lytic activity, is also the only one that is cationic. (3) The length of the side chain of the L-R_3 group is critical for all *Streptomyces* DD-carboxypeptidases but the presence of a free amino group at that position is not required by all of them. (4) Depending upon the enzymes, the K_m values for good substrates may be high or low and the relative enzyme efficiency upon different substrates may be related either to V_{max} or to K_m values. With both R61 and K11 enzymes, peptides which are good substrates have K_m values (10–15 mM) as high as the poor substrates but the good substrates have much higher V_{max} values (V_{max} values vary from 2 to 900 μmoles per mg of protein per hr for R61 and from 9 to 2000 for K11 enzyme). As already discussed (Leyh-Bouille *et al.*, 1971), these properties indicate that the binding surfaces of these enzymes are not very specific in terms of K_m values and suggest a correlation between the structure of the substrate and its effect on the enzyme conformation and on the catalytic activity. In contrast with the R61 and K11 enzymes, the R39 enzyme appears to have a much better fit with the substrate and a more specific binding surface. The K_m values are much lower and vary from 0.2 mM for good substrates up to 2.5 mM for poor substrates. The V_{max} values are relatively little affected and range, depending upon the substrates, from 100 to 600 μmoles per mg of protein per hr. The *albus* G enzyme previously studied (Leyh-Bouille *et al.*, 1970b) resembles the R39 enzyme in this respect. (5) Penicillin is a powerful inhibitor of some DD-carboxypeptidases (R61, K11, and R39 enzymes) but not of all of them (*albus* G enzyme).

Mechanism of Action of Penicillin. Kinetically, the inhibition of the R39 DD-carboxypeptidase by penicillin is noncompetitive. Since the inhibition is reversible, these kinetics cannot result from a two-stage process in which a competitive inhibition of the enzyme would be followed by its permanent inactivation, for example, through acylation, as it has been reported for a particulate DD-carboxypeptidase from *B. subtilis* (Lawrence and Strominger, 1970a,b). On the contrary, the kinetics suggest that penicillin combines with the enzyme at a second site not identical with the substrate binding site, so that the presence of penicillin on the enzyme decreases its affinity for the substrate and that the penicillin–enzyme–substrate complex is either nonproductive or is converted to products at a much reduced rate. Increasing penicillin concentrations causes disproportionate decreases in the catalytic rate. This could mean the occurrence of two penicillin-binding sites or might indicate conformational responses of the enzyme toward the inhibitor. Conformational responses of the enzyme toward substrates had been previously invoked to explain the enzymatic properties of the R61 enzyme (Leyh-Bouille *et al.*, 1971).

The kinetics of inhibition of both R61 and K11 enzymes by penicillin are apparently competitive. Competitive type kinetics are more difficult to interpret than noncompetitive ones in that the former do not necessarily reflect direct competition between substrate and inhibitor for the same site on the free enzyme. A possible explanation would be that binding of penicillin to the enzyme could alter the enzyme conformation in such a way that penicillin–enzyme–substrate complex could not be formed. If this idea is accepted, it is no longer necessary to postulate a structural analogy between penicillin and the D-alanyl-D-alanine sequence in the nascent peptidoglycan (Tipper and Strominger, 1965; Izaki *et al.*, 1968). The strongest argument that can be raised against the structural analogy hypothesis is as follows. As a first approximation, K_m values may be taken as dissociation constants for the formation of the enzyme–substrate complex. If this is accepted, then the more specific enzymes, so far as recognition of substrate and binding are concerned, are those from *S. albus* G and *S.* R39. Neither of these enzymes, however, recognizes penicillin as a substrate analog, since the former is unaffected by the antibiotic and the latter shows noncompetitive inhibition kinetics. The insensitivity to penicillin of the *albus* G enzyme is not easy to interpret on the basis of the structural analogy hypothesis. On the other hand, if one assumes the occurrence of penicillin binding sites that are not identical with the substrate binding sites, then one can visualize a change in the former site leading to penicillin resistance, without a concomitant change in the DD-carboxypeptidase activity.

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Further Studies on Ribulose 1,5-Diphosphate Carboxylase from *Chromatium* Strain D†

T. Akazawa,* H. Kondo, Tetsuko Shimazue, M. Nishimura, and T. Sugiyama

ABSTRACT: Ribulose 1,5-diphosphate carboxylase was isolated from autotrophically grown cells of purple sulfur photosynthetic bacterium, *Chromatium* strain D, and purified to a homogeneous state on the basis of ultracentrifugation and polyacrylamide gel disc electrophoresis at pH 7.5. Two bands detected in the sodium dodecyl sulfate–polyacrylamide gel disc electrophoresis indicated that the bacterial ribulose 1,5-diphosphate carboxylase consisted of two nonidentical polypeptide chains (subunits). The molecular weight of native enzyme was estimated to be 5.5×10^5, and that of each monomeric subunit (A and B) being 5.7×10^4 and 1.2×10^4, similar to that of spinach and *Chlorella* enzymes. Polyacrylamide gel electrophoresis at pH 8.9 indicated dissociation of the enzyme molecule as shown by the formation of a fast-moving band in addition to a slowly moving major band. These bands were considered to be A_m and B, respectively, where A_mB_n (A_8B_8 or A_8B_6) represents compositional subunit organization of this bacterial enzyme signifying that the enzyme molecule consists of *m* molecules of subunit A and *n* molecules of subunit B. Dissociation of the carboxylase molecule at the alkaline pH and its reassociation into the original molecule upon neutralization as accompanied by restoration of the enzyme activity were further ascertained by sucrose density gradient centrifugation.

In the series of our work on structure–function relationships of ribulose 1,5-diphosphate (RuP_2)[1] carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] from lower to higher photosynthetic organisms, the enzyme of the purple sulfur bacterium, *Chromatium* strain D, was of special interest. The *Chromatium* RuP_2 carboxylase was similar to the enzymes from spinach and a green alga, *Chlorella ellipsoidea*, in its large molecular size (Anderson *et al.*, 1968; Kieras and Haselkorn, 1968; Gibson and Hart, 1969; Akazawa *et al.*, 1970a), some enzymic properties (Akazawa *et al.*, 1970a), and immunological specificities (Kieras and Haselkorn, 1968; Akazawa *et al.*, 1970a), but was distinguishable from those of purple nonsulfur photosynthetic bacteria, *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* (Akazawa *et al.*, 1969; Akazawa *et al.*, 1970b). Previously we reported that the RuP_2 carboxylase isolated from either spinach or *Chlorella* consisted of two nonidentical polypeptide chains (Sugiyama and Akazawa, 1970; Sugiyama *et al.*, 1971). The amino acid composition of the large subunit (A) was similar, whereas that of the smaller subunit (B) was very different between the two plant species. Determination of the carboxyl-terminal amino acid of each subunit further revealed structural differences between the two enzymes. In this paper we report a rigorous purification of RuP_2 carboxylase from *Chromatium* cells and the studies made on the structure–activity relationships of the homogeneous enzyme preparation thus obtained. It is from these studies that we found that the *Chromatium* RuP_2 carboxylase consists of two nonidentical subunits, and that the native enzyme molecule is reversibly dissociated into subunits under an alkaline condition.

Materials and Methods

Bacterial Culture. Chromatium strain D was grown in the culture medium of Newton (1962) as described previously (Akazawa *et al.*, 1970a). The bacterial cells growing photo-

† From the Research Institute for Biochemical Regulation, Nagoya University, School of Agriculture, Chikusa, Nagoya, Japan. *Received October 25, 1971.* This investigation was supported in part by research grants from the Ministry of Education of Japan (10792), the Asahi Press (Tokyo), and the Matsunaga Science Foundation (Tokyo). This is paper XVI in the series Structure and Function of Chloroplast Proteins.

[1] Abbreviations used are: RuP_2 carboxylase, ribulose 1,5-diphosphate carboxylase; TM buffer, 0.05 M Tris-HCl buffer (pH 7.0) containing 5 mM freshly distilled β-mercaptoethanol.