ucts of DCP action. The two enzymes were not separated by ion-exchange column chromatography on DEAE-cellulose, by gel filtration, or by polyacrylamide gel electrophoresis. Even the activity–pH curves were found to be very similar. Separation of the two enzymes was eventually achieved by chromatography through hydroxyapatite columns. The complementary action of the two enzymes on the carboxyl ends of polypeptide chains represents an alternative mechanism for carboxypeptidase action by which a polypeptide chain is successively degraded from the carboxyl end to amino acids. An interesting regulatory mechanism is indicated by the finding that the dipeptides formed by DCP act as competitive inhibitors of this enzyme and in turn serve as substrates for dipeptidase, the activity of which depends on Mn ions.

Acknowledgment

The financial support granted by the Helena Rubinstein Foundation Inc. is gratefully acknowledged.

[51] Exocellular DD-Carboxypeptidases-Transpeptidases from *Streptomyces*

By JEAN-MARIE FRÉRE, MÉLINA LEYH-BOUILLE, JEAN-MARIE GHUYSEN, MANUEL NIETO, and HAROLD R. PERKINS

Strains and Culture

Strains. Strains R39 and R61 are soil isolates.[1,2] Their designations are arbitrary. In strain R39, the cross-link between the peptide units of the wall peptidoglycan extends from the C-terminal D-alanine of one unit to the amino group at the D-center of *meso*-diaminopimelic acid of another unit[3] (peptidoglycan of chemotype I).[4] The interpeptide bond is in α-position to a free carboxyl group. In strain R61, the cross-link extends from a C-terminal D-alanine of a peptide unit to a glycine residue attached to the ϵ-amino group of LL-diaminopimelic acid of another peptide unit[5] (peptidoglycan of chemotype II).[4] The exocellular DD-

[1] M. Welsch, *Rev. Belge Pathol. Med. Exp.* **18**, Suppl. 2, 1 (1947).
[2] M. Welsch and A. Rutten-Pinckaers, *Bull. Soc. R. Sci. Liege.* **3–4**, 374 (1963).
[3] J.-M. Ghuysen, M. Leyh-Bouille, J. N. Compbell, R. Moreno, J.-M. Frère, C. Duez, M. Nieto, and H. R. Perkins, *Biochemistry* **12**, 1243 (1973).
[4] J.-M. Ghuysen, *Bacteriol. Rev.* 32, 425 (1968).
[5] M. Leyh-Bouille, R. Bonaly, J.-M. Ghuysen, R. Tinelli, and D. J. Tipper, *Biochemistry* **9**, 2944 (1970).

carboxypeptidases-transpeptidases produced by both strains (1) catalyze hydrolysis and transfer reactions according to the general equations R-D-Ala-D-Ala + H_2O → D-Ala + R-D-Ala (DD-carboxypeptidase activity) and R-D-Ala-D-Ala + NH_2-R′ → D-Ala + R-D-Ala-R′ (transpeptidase activity) and (2) react with β-lactam antibiotics (i.e., penicillins and cephalosporins) to form equimolar and inactive antibiotic–enzyme complexes of various half-lives. Both R39 strain and, to a much lesser extent, strain R61 also produce an exocelular β-lactamase (penicillinase, EC 3.5.2.6) which hydrolyzes penicillin to penicilloic acid.

Culture Media. Peptone oxoid medium contains 1% peptone oxoid, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% $NaNO_3$, and 0.005% KCl in water.

Glycerol-casein medium contains 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 contains 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 is previously dissolved at 70° with the help of KOH (0.04 g per gram casein). The solution is then cooled and neutralized.

Agar-APG medium contains per liter of final volume: agar 20 g; asparagine, 0.5 g; peptone oxoid, 0.5 g; glucose, 10 g; and K_2HPO_4, 0.5 g.

Agar-KC medium contains per liter of final volume: agar, 20 g; partially hydrolyzed keratin from white hens' feathers, 2.5 g; partially hydrolyzed casein, 2.5 g; NaCl, 0.5 g; $CaCO_3$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; K_2HPO_4, 1 g; and $FeSO_4 \cdot 7H_2O$, 0.1 g. The final pH is 7.5. Hydrolyzed keratin and casein are prepared as follows: 100 g of dried, white hens' feathers are treated for 1 hr at 100° with 1 liter of 0.125 *N* KOH. After centrifugation, casein (100 g) is added to the supernatant fraction and dissolved by heating at 70°. The pH of the mixture (final volume, 1 liter) is adjusted to 7.5–8.0; 25 ml contain 2.5 g of both partially hydrolyzed keratin and casein.

Maintenance of Strains. Strain R39 is grown at 28° on slants of agar-KC, and strain of R61 on slants of agar-APG. Abundant sporulation occurs after 4–5 days. The strains are then maintained at 4°.

Assay Methods for DD-Carboxypeptidase Activity

Standard Reaction

$$Ac_2\text{-L-Lys-D-Ala-D-Ala} + H_2O \rightarrow \text{D-Ala} + Ac_2\text{-L-Lys-D-Ala} \quad (1)$$

Unit. One unit of DD-carboxypeptidase catalyzes the hydrolysis of 1 μmole of $N^{\alpha},N^{\varepsilon}$-diacetyl-L-lysyl-D-alanyl-D-alanine into D-alanine and

N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanine per minute at 37° under conditions of enzyme saturation by the substrate.

Substrate. Tripeptide Ac_2-L-Lys-D-Ala-D-Ala is prepared as described by Nieto and Perkins.[6]

Standard Incubation Conditions. Streptomyces R39 enzyme: Ac_2-L-Lys-D-Ala-D-Ala (0.25 μmole), is incubated with the enzyme at 37° in 30 μl (final volume) of 0.03 *M* Tris-HCl buffer, pH 7.5, supplemented with 3 m*M* $MgCl_2$. Final substrate concentration is 8 m*M*, i.e., 10 × the K_m value (0.8 m*M*).

Streptomyces R61 enzyme: Ac_2-L-Lys-D-Ala-D-Ala (0.36 μmole) is incubated with the enzyme at 37° in 30 μl of either 5–10 m*M* sodium phosphate buffer pH 7.5 or 10 m*M* Tris-HCl buffer pH 7.5. Final substrate concentration is 12 m*M*, i.e., equivalent to the K_m value. For this enzyme, assays are thus carried out at half the maximum velocity.

The D-Ala released during the reaction is estimated by using one of the following techniques.

Chemical Estimation of Free Alanine

The following is a modification of the technique of Ghuysen *et al.*[7]

FDNB Reagent. Fluorodinitrobenzene, 130 μl in 10 ml of 100% ethanol.

Procedure. Samples containing 10–50 nmoles of alanine are mixed with 10% $K_2B_4O_7$ and water to give a total volume of 100 μl of 1% $K_2B_4O_7$. FDNB reagent (10 μl) is added. The solutions are mixed and incubated at 60° for 30 min. After acidification with 50 μl of 12 *N* HCl, the DNP-alanine is extracted three times with 200 μl of ether. The ether extracts are evaporated in a stream of hot air and dried *in vacuo*. The residues are dissolved in methanol and chromatographed at room temperature on thin-layer plates of silica gel G in chloroform:methanol:acetic acid (220:25:5, v/v/v). DNP-alanine moves faster than DNP-Tris. After drying, the DNP-alanine spots are transferred to 1-ml tubes and eluted by vigorous mixing with 500 μl of water:ethanol:25% (specific gravity 0.91) ammonia (100:100:0.54, v/v/v). After centrifugation, the optical density of the supernatant fractions is measured at 360 nm. The molar extinction coefficient for DNP-alanine is about 15,000.

Enzymic Estimation of L-Alanine

The following modification of the technique of Ghuysen *et al.*[7] permits many simultaneous tests to be carried out in a very short time.

[6] M. Nieto and H. R. Perkins, *Biochem. J.* 123, 789 (1971).

[7] J.-M. Ghuysen, D. J. Tipper, and J. L. Strominger, this series Vol. 8, p. 685.

Reagents

o-Dianisidine (Merck, analytical grade): 10 mg/ml in methanol (freshly prepared)
K pyrophosphate buffer, 0.1 *M*, pH 8.3
FAD (monosodium; Boehringer), 0.3 mg/ml in pyrophosphate buffer
Peroxidase (Boehringer, Reinheitsgrad 1, für analytische Zwecke; suspension 10 mg/ml) to be diluted to 10 μg/ml in H_2O
D-Amino acid oxidase (Boehringer, für analytische Zweke; Kristall-suspension, 5 mg/ml)
Enzymes and coenzyme mixture (freshly prepared): pyrophosphate buffer:FAD solution:diluted peroxidase solution:D-amino acid oxidase suspension (20:10:5:1, v/v/v/v)

Procedure. Samples (30 μl) containing 5–40 nomoles of D-alanine (from the hydrolyzed substrate) are mixed with 5μl of *o*-dianisidine solution and 70 μl of the enzymes–coenzyme mixture. To such solutions incubated for 5 min at 37°, is added 400 μl of methanol–water (v/v). After an additional 2-min incubation at 37°, the optical density at 460 nm is immediately measured. (Coloration of the solution is silghtly labilized after addition of the methanol–water solution.) Blanks consist of the same mixtures as above lacking Ac_2-L-Lys-D-Ala-D-Ala tripeptide. Standards are blanks containing known amounts of D-alanine.

Assay Method for β-Lactamase

DD-Carboxypeptidases-transpeptidases and β-lactamases react with penicillin. The reaction mechanisms and the reaction products are, however, different (see below). A complete separation between these two classes of enzymes is thus one major goal of the purification procedure of the DD-carboxypeptidases-transpeptidases.

Standard Reaction for β-Lactamase

$$\text{Benzylpenicillin} + H_2O \rightarrow \text{benzylpenicilloic acid} \qquad (2)$$

β-Lactamase Unit. One unit of β-lactamase catalyzes the hydrolysis of 1 μmole of benzylpenicillin per minute at 30° and under conditions of enzyme saturation by the substrate. The K_m value of the β-lactamase from *Streptomyces* R39 is 70 μM.[8] That of the β-lactamase from *Streptomyces* R61 is not known.

[8] K. Johnson, J. Dusart, J. N. Campbell, and J.-M. Ghuysen, *Antimicrob. Agents Chemother.* 3, 289 (1973).

Reagents

Sodium acetate buffer, 1 *M* pH 3.6
Color reagent: equal volumes of a water-soluble starch solution (0.8%, w/v) and of a 240 μM I_2 + 4.8 m*M* KI solution

Procedure. The following is a microscale adaptation of the technique of Novick and Dubnau.[9] Benzylpenicillin (0.3 μmole) is incubated with the enzyme preparation in 30 μl (final volume) of 0.025 *M* sodium phosphate buffer pH 7.0. The benzylpenicillin concentration in the mixture is 10 m*M*; hence, any β-lactamase with a K_m value for this antibiotic equal to or lower than 1 m*M* is saturated. After 10–30 min, 200 μl of 1 *M* acetate buffer and then 200 μl of color reagent are added in sequence to the reaction mixture. After 10 min at 25°, the optical density of the solution is measured at 620 nm. A control consisting of the same mixture lacking enzyme is incubated as above. Acetate buffer, the same amount of enzyme as used in the test, and finally the color reagent are added, and the optical density at 620 nm is determined. A decrease of the optical density of 0.1 corresponds to about 0.37 nmole of benzylpenicilloic acid produced.

Excretion of DD-Carboxypeptidase-Transpeptidase and β-Lactamase by *Streptomyces* R39[10]

Streptomyces R39 is grown for 24 hr at 28° with shaking in peptone medium. After two successive subcultures of increasing size, 100 liters of culture in exponential phase are used to inoculate 400 liters of the same peptone medium contained in a 500-liter tank. This culture is grown at 28° for about 90–100 hr with mechanical stirring (120 rpm), and an airflow rate of 100 liters/min at an air pressure of 1.5×10^5 Pa. Silicone A emulsion (Dow Corning Co., Midland, Michigan; 20 ml) is used as antifoam. After centrifugation, the DD-carboxypeptidase activity in the culture fluid is about 2.5 munits/ml or 1.5 munits/mg of protein, and β-lactamase activity is 50 munits/ml. Similar results are obtained on a smaller scale by growing *Streptomyces* R39 in a New Brunswick Shaker incubator, in 1-liter flasks containing 400 ml of peptone medium. Under both conditions, maximal DD-carboxypeptidase activity and maximal β-lactamase activity occur almost simultaneously. Both activities then disappear progressively and are negligible after 6 days. With time, the two enzyme activities increase and decrease independently of each other.

[9] R. P. Novick, *J. Gen. Microbiol.* 33, 121 (1963).
[10] J.-M. Frère, R. Moreno, J.-M. Ghuysen, H. R. Perkins, L. Dierickx, and L. Delcambe, *Biochem. J.* 143, 233 (1974).

Excretion of DD-Carboxypeptidase–Transpeptidase and β-Lactamase by *Streptomyces* R61[11]

Streptomyces R61 excretes larger amounts of its DD-carboxypeptidase–transpeptidase in glycerol–casein medium than in peptone medium. The strain is grown at 28° in a New Brunswick Shaker incubator in 1-liter flasks containing 500 ml of glycerol–casein medium. Often, the cultures exhibit two peaks of DD-carboxypeptidase activity, the first one occurring after about 100 hr (about 11 mU/ml or 13 mU/mg protein) and the second one after about 300 hr (about 18 mU/ml). No corresponding increase in mycelium production is observed. Large amounts of free amino acids are present in the 100–200 hr cultures, and they subsequently disappear, suggesting that part of the bacterial population resumes growth from autolysis products. Under the above growth conditions, *Streptomyces* R61 is a poor producer of exocellular β-lactamase. Its β-lactamase has not been studied.

Purification of the DD-Carboxypeptidase–Transpeptidase from *Streptomyces* R39 (for 500 Liters of Culture Fluid)[10]

Step 1. The enzyme is adsorbed from 500 liters of culture fluid on 3.7 kg (wet weight) of DEAE-cellulose (MN 2100 DEAE, Macherey, Nagel and Co., D-156 Düren, Germany) equilibrated against 0.1 *M* Tris-HCl, pH 7.5. All subsequent steps are performed at 4°. The enzyme is eluted from the DEAE-cellulose by two subsequent treatments with 5 liters of 0.1 *M* Tris-HCl buffer, pH 7.5, containing 1 m*M* $MgCl_2$ and 0.4 *M* NaCl. The solution is concentrated to 1.5 liter on Carbowax 4000 and solid ammonium sulfate is added to 50% saturation. The precipitate is discarded, and the $(NH_4)_2SO_4$ concentration in the supernatant is increased to 90% saturation. The precipitate is collected by centrifugation, dissolved in 240 ml of 0.1 *M* Tris-HCl buffer, pH 7.7, containing 1 m*M* $MgCl_2$ and the solution is dialyzed against the same buffer.

Step 2. After step 1, the enzyme solution is applied to a 600 ml column of DEAE-cellulose (4 x 48 cm) equilibrated against 0.1 *M* Tris-HCl buffer, pH 7.7, containing 1 m*M* $MgCl_2$ and 0.1 *M* NaCl. Some enzymically inactive proteins are eliminated by washing the column with the same buffer, and others are eluted by increasing the NaCl concentration in the buffer to 0.19 *M*. The resin is then treated with an increasing convex gradient of NaCl (mixing flask: 970 ml of 0.1 *M* Tris-HCl buffer + 1 m*M*

[11] M. Leyh-Bouille, J. Coyette, J.-M. Ghuysen, J. Idczak, H. R. Perkins, and M. Nieto, *Biochemistry* **10**, 2163 (1971).

$MgCl_2$ + 0.19 *M* NaCl; upper flask: 0.1 *M* Tris-HCl buffer + 1 m*M* $MgCl_2$ + 0.28 *M* NaCl). The enzyme is eluted at a NaCl concentration of about 0.24 *M*. The active fractions are pooled and concentrated to 20 ml by ultrafiltration through Amicon UM-10 membranes.

Step 3. After step 2, the concentrated solution is filtered through a 400-ml column of Sephadex G-100 (3 × 45 cm) previously equilibrated against 0.05 *M* cacodylate-HCl buffer, pH 6.0, containing 1 m*M* $MgCl_2$ + 0.3 *M* NaCl. The enzyme is eluted at a K_D value of 0.21 (and well separated from two yellow-brown pigments presenting K_D values of 0–0.02 and 0.72, respectively). The active fractions are pooled (90 ml).

Step 4. After step 3, the solution is applied to a 30-ml (2 × 10 cm) column of DEAE-Sephadex A-50, previously equilibrated against 0.05 *M* cacodylate-HCl buffer, pH 6.0, containing 1 m*M* $MgCl_2$ + 0.3 *M* NaCl. Enzymically inactive proteins are eliminated by washing the column first with 300 ml of the same cacodylate-HCl-$MgCl_2$ buffer containing 0.4 *M* NaCl and then with 300 ml of the same buffer containing 0.47 *M* NaCl. The enzyme is then eluted with a convex gradient of NaCl (mixing flask 500 ml of cacodylate-HCl buffer + 1 m*M* $MgCl_2$ + 0.47 *M* NaCl; upper flask: cacodylate-HCl buffer + 1 m*M* $MgCl_2$ + 0.5 *M* NaCl). A single peak of protein is obtained, which closely correlates with the activity. The active fractions are pooled and concentrated to 20 ml by ultrafiltration. The concentrated solution is filtered through the 400-ml column of Sephadex G-100, in 0.05 *M* cacodylate-HCl buffer, pH 6.0, containing 1 m*M* $MgCl_2$ and 0.4 *M* NaCl. The most active fractions of the eluted peak exhibit the same high specific activity. They are pooled, concentrated by ultrafiltration and dialyzed against 0.1 *M* Tris-HCl buffer, pH 7.7, containing 0.2 *M* NaCl and 0.05 *M* $MgCl_2$ (fraction A, Table I, specific activity: 17.1 units/mg protein). The other fractions of the eluted peak have lower specific activities. They are pooled, concentrated, and dialyzed (fraction B, Table I). Fraction B (specific activity: 13 units/mg protein) may be stored as it is and used for experiments that do not require an enzyme of absolute purity. Fraction A behaves as a homogeneous protein in several analytical tests (see below). However, benzylpenicillin binding occurs at a ratio of 0.9 mole of benzylpenicillin per mole of enzyme, showing that some impurities are still present in the preparation (see below, Fig. 4).

Step 5. After step 4, to fraction A (1 ml, 1.6 mg of protein) is added 1 ml of acetone previously cooled to −20°. The mixture is stirred for 30 min at −20°; the precipitate is collected by centrifugation at −5° and redissolved in 1 ml of 0.1 *M* Tris-HCl buffer, pH 7.7, containing 0.2 *M* NaCl and 0.05 *M* $MgCl_2$. At this stage, benzylpenicillin binding occurs at a ratio of 1.04 ± 0.03 mole of benzylpenicillin per mole of enzyme (see Fig. 4). When compared to fraction A, the final preparation has a

TABLE I

PURIFICATION OF THE DD-CARBOXYPEPTIDASES-TRANSPEPTIDASES FROM *Streptomyces* R39 AND R61[a]

Enzyme	Step[b]	Total protein[c] (mg)	Activity (total units)	Yield (%)	Specific activity (units/mg of protein)	Enrichment
R39	Culture supernatant	8.4×10^5	1250	100	0.0015	1
	1	2.9×10^3	1050[d]	84	0.36	240
	2	166	830	66	5.0	3300
	3	65	720	58	11.0	6700
	4A	21.8	375	30	17.1	11400
	4B	11.4	147	12	13.0	8700
	5[e]	1.4			19.8	12700
R61	Culture supernatant	350×10^3	4200	100	0.012	1
	1	7×10^3	2500	60	0.36	30
	2	59	1700	40	29	2400
	3A	11.2	970	23	86	7200
	3B	8	400	9.5	50	4150

[a] From J.-M. Frère, R. Moreno, J.-M. Ghuysen, H. R. Perkins, L. Dierickx, and L. Delcambe, *Biochem. J.* **143,** 233 (1974); and J.-M. Frère, J.-M. Ghuysen, H. R. Perkins, and M. Nieto, *Biochem. J.* **135,** 463 (1973).

[b] The R39 enzyme preparations after steps 4 and 5 and the R61 enzyme preparation after step 3A are devoid of β-lactamase activity.

[c] The protein concentration was determined either by measuring the extinction at 280 and 260 nm and using the formula C (mg/ml) = $1.54\ E_{280} - 0.76\ E_{260}$ or (for the final preparations) by measuring the amount of total amino groups available to fluorodinitrobenzene after 6 M HCl hydrolysis (100°, 20 hr; standard: bovine serum albumin).

[d] The total activity obtained after $(NH_4)_2SO_4$ fractionation by adding up the total units of all fractions (0–50%, 50–90%, and >90%) was always equal to about 130% of the total activity of the original solution. It is possible that an inhibitor was eliminated during this step.

[e] Step 5 was carried out on a fraction of the preparation obtained after step 4A.

specific activity increased by about 11%. The enzyme recovery is about 90%. Table I gives the total recoveries and enrichments in specific activity after each step of the purification procedure.

Purification of the DD-Carboxypeptidase–Transpeptidase from *Streptomyces* R61 (for 400 Liters of Culture Fluid)

The following is an adaptation of the technique of Frère *et al.*[12]

Step 1. The enzyme is adsorbed from 400 liters of culture fluid on 10 kg of Amberlite XE64 H^+ or CG50 H^+ by adjusting the pH to 4.0 with acetic acid. The Amberlite-adsorbed enzyme complex is suspended in 20 liters of cold 0.1 *M* K_2HPO_4, and the pH of the suspension is brought to 8.0 by dropwise addition of concentrated ammonia. The resin is removed by filtration, and the filtrate is clarified by centrifugation. The adsorption of the enzyme on the resin and its elution as well as all subsequent steps are performed at 4°. Solid $(NH_4)_2SO_4$ is added to the eluted enzyme solution and the precipitate obtained at 40% saturation is discarded. Protein (128 g, wet weight) precipitated when the $(NH_4)_2SO_4$ concentration is raised to 60% saturation is redissolved in 1 liter of 0.01 *M* Tris-HCl buffer and dialyzed twice against 50 liters of the same buffer. The dialyzed solution is stirred with 500 g (wet weight) of DEAE-cellulose previously equilibrated against 0.01 *M* Tris-HCl buffer pH 8.0. The enzyme is eluted from the DEAE-cellulose by treating the resin, batchwise, with 1 liter of the same buffer containing 0.1 *M* NaCl. The eluate is dialyzed against water.

Step 2. After step 1, the enzyme is adsorbed on a 600 ml column of DEAE-cellulose (4 × 40 cm) equilibrated against 0.01 *M* Tris-HCl buffer pH 8.0. The column is treated with an increasing convex gradient of NaCl (mixing flask, at constant volume: 800 ml of 0.01 *M* Tris-HCl buffer; solution added: same buffer + 0.13 *M* NaCl). The active fractions are pooled, concentrated to 20 ml by ultrafiltration and filtered through a 425-ml column (3 × 60 cm) of Sephadex G-75 equilibrated against 0.01 *M* cacodylate-HCl buffer pH 6.0. The column is washed with the same cacodylate-HCl buffer and the enzyme is eluted just before the main peak of protein. The active fractions are pooled. At this stage, the solution is still faintly yellow.

Step 3. After step 2, the enzyme is adsorbed on a 30-ml column (2 × 10 cm) of DEAE-Sephadex A-50 previously equilibrated against 0.01 *M*

[12] J.-M. Frère, J.-M. Ghuysen, H. R. Perkins, and M. Nieto, *Biochem. J.* **135**, 463 (1973).

cacodylate-HCl buffer pH 6.0. The enzyme is eluted with an increasing linear gradient of NaCl (0 to 0.15 *M*) in the same buffer. The yellow pigment remains fixed on the top of the column. The active fractions in the center of the eluted peak of protein exhibit a constant high specific activity. They are pooled, concentrated to 10 ml by ultrafiltration, and extensively dialyzed against water (preparation 3A). The other fractions are treated likewise (preparation 3B). Table I gives the total recoveries and enrichments in specific activity after each step of the purification procedure.

Physicochemical Properties of DD-Carboxypeptidases–Transpeptidases from *Streptomyces* R39 and R61

The properties described in the present paragraph (together with the techniques of titration of the *Streptomyces* enzymes by β-lactam antibiotics: see below) constitute the best available criteria of purity. For more details, see Frère *et al.*[10, 12]

Diffusion Constant ($D_{20,w}$), Molecular Weight (MW), and Frictional Ratio (f/f_o)

Buffers. The following buffers are used: 0.1 *M* Tris-HCl, pH 7.7 + 3 m*M* $MgCl_2$ + 0.5 *M* NaCl (density, 1.02) for the R39 enzyme; 0.01 *M* Tris-HCl pH 8.0 + 0.09 *M* NaCl (density, 1.002) for the R61 enzyme.

Procedure. The enzyme solutions (dialyzed against the relevant buffer) are analyzed by equilibrium sedimentation at a speed of 13.000 rpm for 22 hr at 20° and initial protein concentration of 2–3 mg/ml. The rates of diffusion are measured by plotting $A^2/(H^2F^2)$ against time (A = area and H = maximum height of the peak; F = total enlargement used). The apparent molecular weights (M_{app}) at any point x of the solution column (about 2 mm long) are calculated according to the equation

$$M_{app} = [RT/(1 - \bar{v}\rho)\omega^2](1/C_x)(dc/dx)$$

where $\bar{v}$ = partial specific volume (supposed to be equal to 0.75 cm^3 g^{-1}), ρ = density of the solvent (see above); C = protein concentration at point x. The molecular weights are obtained by plotting $1/M_{app}$ against concentration at 0.1-mm intervals of the column. The results are given in Table II.

TABLE II

MOLECULAR WEIGHT (MW), DIFFUSION CONSTANT ($D_{20,w}$), FRICTIONAL RATIO (f/f_0), AND MOLAR ACTIVITY OF DD-CARBOXYPEPTIDASES-TRANSPEPTIDASES FROM *Streptomyces* R39 AND R61[a]

Enzyme	MW	$D_{20,w}$ ($\times 10^{-3}$ cm^2 sec^{-1})	f/f_0	Molar activity[b] (min^{-1})
R39	53,500	7.88	1.07	1050
R61	37,000	8.45	1.12	3300

[a] From J.-M. Frère, R. Moreno, J. M. Ghuysen, H. R. Perkins, L. Dierickx, and L. Delcambe, *Biochem. J.* **143**, 233 (1974); and J.-M. Frère, J.-M. Ghuysen, H. R. Perkins, and M. Nieto, *Biochem. J.* **135**, 463 (1973).

[b] On Ac_2-L-Lys-D-Ala-D-Ala.

Remarks

1. With a homogeneous protein, the plot of $1/M_{app}$ vs C_r during sedimentation equilibrium gives rise to a line parallel to the abscissa. Figures 1A and 1B show the results obtained with the R61 enzyme after various steps of purification, and Fig. 1C with the purified R39 enzyme.

2. When equilibrium sedimentation of the purified R39 enzyme is carried out in the same Tris–HCl–$MgCl_2$ buffer as above except that the NaCl concentration is 0.2 *M* instead of 0.5 *M*, the plot of $1/M_{app}$ vs concentration gives a line with a slope of -1.1×10^{-4} (with the same coordinates as in Fig. 1). This is because the R39 protein aggregates at low ionic strength.

Sephadex Filtrations. Filtrations are carried out on 1.5×65 cm columns of Sephadex G-100, using dextran blue, ovalbumin, myoglobin, bovine serum albumin, and chymotrypsinogen as molecular weight standards. In 0.01 *M* Tris-HCl buffer, pH 8.0, the apparent molecular weight of the R61 enzyme is about 38,000. The apparent molecular weight of the R39 enzyme decreases as the ionic strength of the buffer increases. Ionic strengths equal to or higher than 0.07 *M* and ionic strengths lower than 0.07 *M* are obtained with 0.1 *M* and 0.01 *M* Tris-HCl buffers, pH 7.7, respectively, supplemented with the appropriate amount of NaCl. The apparent molecular weight is over 100,000 at $I = 0.008$ *M*, 86,000 at $I = 0.025$ *M*, 70,000 at $I = 0.070$ *M*, and about 60,000 at $I > 0.20$ *M*.

Analytical Polyacrylamide Gel Electrophoresis

In the Absence of Sodium Dodecyl Sulfate (SDS). The gels (6×71 mm) are prepared with 7% acrylamide and 0.18% *N,N'*-methylenebisa-

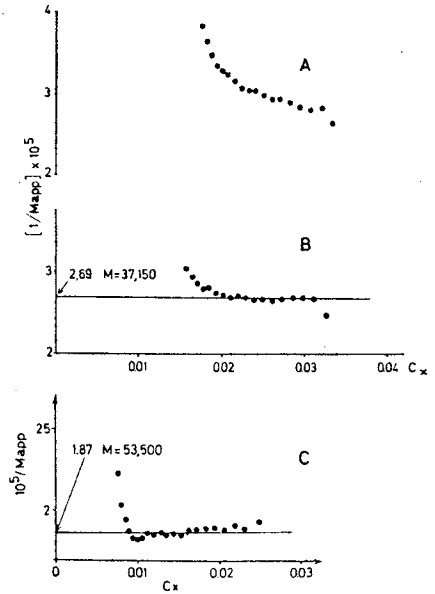


FIG. 1. Reciprocal of the apparent molecular weight ($1/M_{app}$) of *Streptomyces* DD-carboxypeptidases-transpeptidases as a function of the concentration in the column (C_x) during sedimentation equilibrium. (A) R61 enzyme (84% pure); (B) R61 enzyme after step 3A of purification; (C) R39 enzyme after step 5 of purification. Experiments are carried out in 0.01 *M* Tris-HCl buffer, pH 8.0, with ionic strength adjusted to 0.1 with NaCl for the R61 enzyme and in 0.1 *M* Tris-HCl buffer pH 7.7 + 3 m*M* $MgCl_2$ and 0.5 *M* NaCl for the R39 enzyme. For other conditions, see text and the following articles, from which Fig. 1 is reprinted by courtesy of the Biochemical Society. J.-M. Frère, R. Moreno, J.-M. Ghuysen, H. R. Perkins, L. Dierickx, and L. Delcambe, *Biochem. J.* 143, 233 (1974); J.-M. Frère, J.-M. Ghuysen, H. R. Perkins, and M. Nieto, *Biochem. J.* 135, 463 (1973).

crylamide in 0.375 *M* Tris-HCl buffer, pH 8.4, and polymerized in the presence of 0.08% ammonium persulfate and 0.03% *N,N,N′,N′*-tetramethylethylenediamine. The electrolyte is 0.025 *M* (in Tris) Tris-glycine buffer pH 8.4. In some cases, gels are submitted to prior electrophoresis for 3 hr at 4 mA/gel (preruns). Electrophoreses of the samples (25 μg of protein) are performed for 135 min at room temperature and at 3 mA/gel. Quartz tubes are used so that the same gel can be scanned at 280 nm and then removed from the tube and sliced into sections (which are eluted and assayed for DD-carboxypeptidase activity). Parallel gels are stained with Coomassie blue as usual. Irrespective of the procedure used for detection, the enzymes give a single band. On gels that are not prerun, migrations toward the anode are 3.5 cm for the R61 enzyme, 4.4 cm for the R39 enzyme, and 4.4 cm for bromophenol blue. On gels that are prerun, mi-

grations toward the anode are 4.3 cm for the R39 enzyme and 6.4 for bromophenol blue.

In the Presence of SDS. Electrophoreses are performed at room temperature in 0.01 *M* sodium phosphate, pH 7.1, in the presence of 0.1% (w/v) SDS (Weber and Osborn,[13]) with prior incubations of the proteins for 15 hr at 37° in the same phosphate buffer containing 1% (w/v) SDS, with or without the addition of 2-mercaptoethanol (1%, final volume). Each enzyme gives rise to a single band and the mobilities (when compared to that of similarly treated standard proteins) indicate a molecular weight of 56,000 ± 3000 (7 determinations) for the R39 enzyme and of 39,000 ± 1600 (4 determinations) for the R61 enzyme. These values are unaltered when mercaptoethanol is omitted during pretreatment of the proteins with SDS. Each of the R39 and R61 enzymes thus consists of one single polypeptide chain.

Electrofocusing. The gels (6 × 0.5 cm) contain 2.5% carrier ampholytes (pH 3–6), 7% acrylamide, 0.18% *N,N'*-methylenebisacrylamide, and 0.08% ammonium persulfate. For other conditions, see Frère *et al.*[12] The R61 enzyme has an isoelectric point of 4.8 ± 0.14 (4 determinations). Because of the high tendency of the R39 enzyme to aggregate at low ionic strength, its isoelectric point cannot be determined by this technique. In normal gel electrophoresis at pH 8.4, the R39 enzyme is more anionic than the R61 enzyme (see above).

Spectra. All spectra are determined at 25° in 0.1 *M* Tris-HCl buffer, pH 7.7, + 0.05 *M* $MgCl_2$ + 0.2 *M* NaCl for the R39 enzyme and in 1 m*M* Tris-HCl buffer, pH 7.4, for the R61 enzyme.

Ultraviolet Absorption. $E^{1\%}_{1\,cm}$ values at 280 nm: 9.7 for the R39 enzyme and 10.0 for the R61 enzyme.

Fluorescence Emission. Excitation at 285 nm produces an emission maximum at 340 nm with the R39 enzyme[10] and at 320 nm with the R61 enzyme.[14]

Circular Dichroism. Circular dichroism spectrum of the R61 enzyme is given by Nieto *et al.* [14]

Amino Acid Compositions. The compositions are shown in Table III. By summing the residue mole percentages of the hydrophilic amino acids plus one half of the mole percentages of the intermediate class,[15] the polarity index then found is 39.7 for the R39 enzyme and 40.5 for the R61 enzyme.

[13] K. Weber and M. Osborn, *J. Biol. Chem.* **244**, 4406 (1969).
[14] M. Nieto, H. R. Perkins, J.-M. Frère, and J.-M. Ghuysen, *Biochem. J.* **135**, 493 (1973).
[15] R. A. Capaldi and G. Vanderkooi, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 930 (1972).

TABLE III

AMINO ACID COMPOSITION OF THE DD-CARBOXYPEPTIDASES-TRANSPEPTIDASES FROM *Streptomyces* R39 AND R61

	Strain R39			Strain R61		
Residue	Residues/ enzyme molecule (MW = 53,300)	Total mass	% (in number)	Residues/ enzyme molecule (MW = 38,000)	Total mass	% (in number)
Asp	50	5750	9.5	38	4370	10.9
Thr	34	3434	6.4	38	3838	10.9
Ser	38	3306	7.2	29	2523	8.3
Glu	57	7353	10.8	28	3612	8.0
Pro	25	2425	4.7	11	1067	3.1
Gly	66	3762	12.5	32	1824	9.1
Ala	82	5822	15.5	34	2414	9.7
Cys[a]	2	204	0.37	3	306	0.86
Val	51	5049	9.7	30	2970	8.6
Met	3	393	0.57	6	786	1.71
Ile	10	1130	1.9	9	1017	2.6
Leu	50	5650	9.5	33	3729	9.4
Tyr	10	1630	1.9	13	2119	3.7
Phe	11	1617	2.1	12	1764	3.4
His	9	1233	1.7	8	1096	2.3
Lys	5	640	0.95	8	1024	2.3
Arg	19	2945	3.6	14	2170	4.0
Trp[a]	6	1122	1.1	4	748	1.14
	528	53483[b]		350	37395[b]	

[a] Half-cystine is measured as cysteic acid after performic acid oxidation of 200 μg of protein. Tryptophan is measured from the UV spectrum in alkali.

[b] Taking into account a mass of 18 for H_2O.

Interaction between DD-Carboxypeptidases–Transpeptidases from *Streptomyces* R39 and R61 and β-Lactam Antibiotics[16–18]

The reactions and properties that are described below are relevant to experimental conditions where the enzymes, by themselves, are perfectly stable, i.e., in 0.1 *M* Tris-HCl buffer, pH 7.7, + 0.05 *M* $MgCl_2$ +

[16] J.-M. Frère, J.-M. Ghuysen, P. E. Reynolds, R. Moreno, and H. R. Perkins, *Biochem. J.* **143**, 241 (1974).

[17] J.-M. Frère, M. Leyh-Bouille, J.-M. Ghuysen, and H. R. Perkins, *Eur. J. Biochem.* **50**, 203 (1974).

[18] J.-M. Frère, J.-M. Ghuysen, and M. Iwatsubo, *Eur. J. Biochem.* **57**, 343 (1975).

0.2 *M* NaCl for the R39 enzyme and in 5–10 m*M* sodium phosphate buffer, pH 7.0, for the R61 enzyme.

Reaction. At room temperature, the R39 and R61 enzymes react readily with β-lactam antibiotics to form equimolar and inactive antibiotic–enzyme complexes. When maintained at 37°, these complexes undergo spontaneous breakdown during which the enzyme is reactivated whereas the antibiotic molecule is released in a chemically altered form. The reactions occur according one of the two following possible mechanisms:

$$E + P \overset{K}{\rightleftharpoons} EP \overset{k_3}{\rightarrow} EP^* \overset{k_4}{\rightarrow} E + X \qquad (3)$$

or

$$E + P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EP \overset{k_3}{\rightarrow} EP^* \overset{k_4}{\rightarrow} E + X \qquad (4)$$

E = active enzyme; P = intact antibiotic molecule; EP = inactive antibiotic–enzyme complex; EP* = inactive complex after isomerization of the antibiotic molecule; X = released and chemically altered antibiotic molecule. Mechanism (3) is the simplest one that accounts for all the experimental facts so far accumulated. Table IV gives the values of the various constants (K = dissociation constant of complex EP) for the reactions between the R61 enzyme and various β-lactam antibiotics. Table IV also gives the half-lives of various EP* complexes formed with both R61 and R39 enzymes. For more details, see Frère *et al.*[18]

Effects on β-Lactam Antibiotics. Reaction of benzylpenicillin with both R39 and R61 enzymes[16,17] yields complexes that subsequently release a compound X that is neither benzylpenicillin nor benzylpenicilloic acid (nor a product arising from a spontaneous degradation of free benzylpenicillin). Unlike benzylpenicilloic acid, compound X cannot be titrated with the iodine reagent (see assay method for β-lactamase).

Reaction of cephaloridine with the R39 enzyme[16] causes a 70% decrease of the absorbance at 250 nm, as observed when the β-lactam ring of cephaloridine is hydrolyzed by β-lactamase. Reaction of cephalosporin 87-312 (see below) with the R39 enzyme[16] causes a shift of the absorption maximum of the antibiotic molecule from 386 to 482 nm. The absorption spectrum of the R39 enzyme–cephalosporin 87-312 complex is identical to that of cephalosporin 87-312 hydrolyzed by β-lactamase (Fig. 2). In both cases, the ratio $\epsilon_{482}/\epsilon_{386}$ is equal to 2.40.

The R61 enzyme–cephalosporin 87-312 complex, once formed, has a ratio $\epsilon_{482}/\epsilon_{386}$ equal to 1.20.[17] This value is considerably lower than normally expected if the β-lactam ring were hydrolyzed as with β-lactamase. The difference spectrum between the R61 enzyme–cephalosporin 87-312

TABLE IV

VALUES OF THE CONSTANTS FOR THE REACTION BETWEEN THE R61 ENZYME AND VARIOUS β-LACTAM ANTIBIOTICS

$$(E + P \overset{K}{\rightleftharpoons} EP \overset{k_3}{\rightarrow} EP^* \overset{k_4}{\rightarrow} E + X)$$

AND k_4 VALUES FOR THE BREAKDOWN OF EP* COMPLEXES FORMED WITH THE R39 ENZYME[a]

	R61				R39
Antibiotic	K (mM)	k_3 (sec^{-1})	k_3/K (M^{-1} sec^{-1})	k_4 (sec^{-1})	k_4 (sec^{-1})
Benzylpenicillin	13 (25°)	179 (25°)	1.20×10^4 (25°)	0.21×10^{-4} (25°) 1.4×10^{-4}	3×10^{-6}
Carbenicillin	0.109	0.091	830	1.4×10^{-4}	5×10^{-6}
Ampicillin	7.2	0.77	107	1.4×10^{-4}	$>4.4 \times 10^{-6}$
Penicillin V	>1	>1	1,500	2.8×10^{-4}	ND
Cephalosporin C	>1	>1	1,150	1×10^{-6}	$>0.3 \times 10^{-6}$
Cephaloglycine	0.4	8.5×10^{-3}	21	3×10^{-6}	$>0.8 \times 10^{-6}$
Cephalosporin 87/312	0.2(10°)	>0.1(10°)	460(10°)	3×10^{-4}	1.5×10^{-6}
Cephaloridin	ND	ND	ND	ND	0.6×10^{-6}

[a] At 37° unless otherwise indicated; ND, not done.

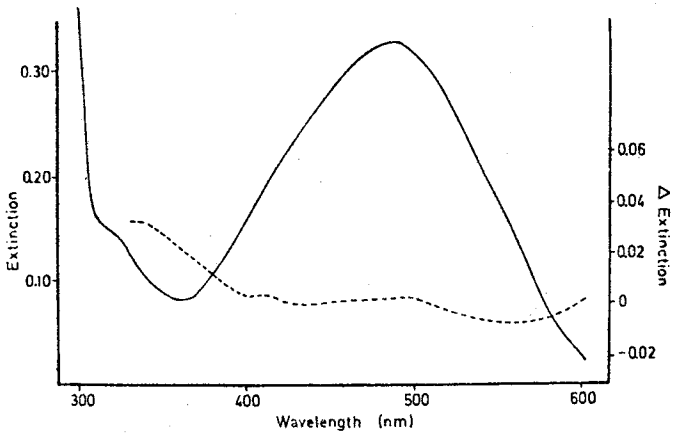


FIG. 2. Absorption spectrum of cephalosporin 87-312 after treatment with the *Streptomyces* R39 enzyme and with β-lactamase. ——, Solution containing 15.5 nmoles of *Streptomyces* R39 protein in 0.4 ml of Tris-NaCl-$MgCl_2$ buffer was mixed with 0.2 ml of a 67.7 μM solution of cephalosporin 87-312. The mixture was incubated for 5 min at room temperature before the absorption spectrum was determined. ---, Difference spectrum between cephalosporin treated with *Streptomyces* R39 enzyme and cephalosporin treated with β-lactamase. Note the change of scale. Solution containing 6 units of β-lactamase in 0.4 ml of Tris-NaCl-$MgCl_2$ buffer was mixed with 0.2 ml of the same solution of cephalosporin 87-312 as above. The mixture was incubated at 37° until the absorbance at 482 nm was a maximum (less than 5 min). Reprinted by courtesy of the Biochemical Society from J.-M. Frère, J.-M. Ghuysen, P. E. Reynolds, R. Moreno, and H. R. Perkins, *Biochem. J.* **143**, 241 (1974).

complex, once formed, and the same concentration of cephalosporin 87-312 hydrolyzed by β-lactamase shows extrema at 390 and 525 nm (Fig. 3). Incubation at 37° of the R61 enzyme–cephalosporin 87-312 complex results in the reactivation of the enzyme and in a parallel decrease of the two extrema in the difference spectrum (Fig. 3).

Molar Activity on Benzylpenicillin. On the basis of the k_4 values for the breakdown of the EP* complexes (Table IV), the specific activity (in equivalents of β-lactamase unit and at 37°) of the R61 enzyme is 2.2×10^{-4} unit per milligram of protein and that of the R39 enzyme is 0.35×10^{-6} unit per milligram of protein. Molar activity (in min^{-1}) is 8.4×10^{-3} for the R61 enzyme and 2.1×10^{-4} for the R39 enzyme. These values are to be compared with molar activities on Ac_2-L-Lys-D-Ala of 3300 and 1050, respectively.

Effects on Circular Dichroism (CD) and Fluorescence of Streptomyces Enzymes. The circular dichroism and fluorescence spectra of the R39 enzyme are not modified by benzylpenicillin.

The near UV CD spectrum of the R61 enzyme is affected by benzylpenicillin whereas the peptide region of the CD spectrum in the far UV

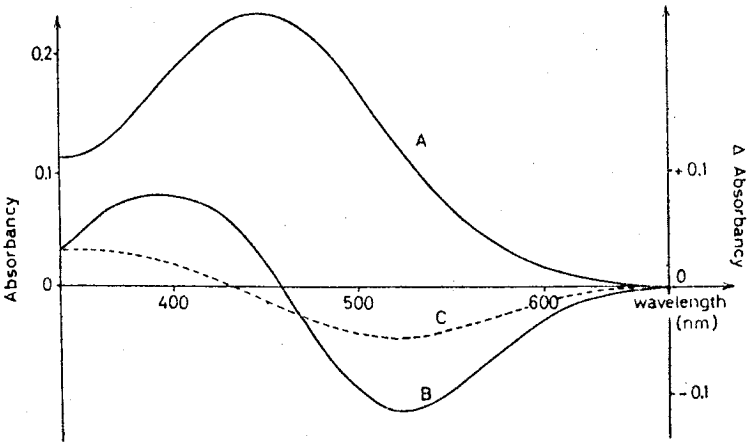


FIG. 3. Absorption spectrum of cephalosporin 87-312 after treatment with the *Streptomyces* R61 enzyme and with β-lactamase. The spectra were recorded with 0.5 ml cuvettes and an optical pathway of 1.0 cm. Buffer: 5 m*M* sodium phosphate pH 7.0. For other conditions, see text. Curve A: Absorption spectrum of a freshly prepared R61 enzyme-cephalosporin 87-312 complex. Concentration of the complex, 15 μ*M*. Curve B: Difference spectrum between the freshly prepared R61 enzyme-cephalosporin 87-312 complex and an equivalent amount of cephalosporin 87-312 hydrolyzed by penicillinase. The solutions contained 7.5 nanoequivalents of cephalosporin 87-312 (either combined with the enzyme or hydrolyzed by penicillinase) in a final volume of 500 μl. Curve C: Difference spectrum between the same complex as in B, maintained for 60 min at 37°, and an equivalent amount of cephalosporin 87-312 hydrolyzed by penicillinase. Note that after 60 min at 37°, 65% of the initially inhibited activity had recovered and, parallel to this, the intensities of the two extrema in the difference spectrum had decreased to 40% of the original values. Reprinted by courtesy of the Federation of European Biochemical Societies from J.-M. Frère, M. Leyh-Bouille, J.-M. Ghuysen, and H. R. Perkins, *Eur. J. Biochem.* **50**, 203 (1974).

remains unchanged.[14] The overall change in CD is too small, and the concentration of enzyme too high, to be convenient for quantitative work.

Saturating concentrations of benzylpenicillin decrease the fluorescence emission at 320 nm of the R61 enzyme by 25–30%.[14] Quenching of the fluorescence of the enzyme (concentration, 0.76 μ*M*) in the presence of an equimolar amount of benzylpenicillin is not immediate and is maximal after 5–10 min.[14]

Titration of DD-Carboxypeptidases-Transpeptidases from *Streptomyces* R39 and R61 by β-Lactam Antibiotics

Buffers. Tris-HCl buffer, 0.1 *M*, pH 7.7 + 0.2 *M* NaCl + 0.05 *M* $MgCl_2$ (Tris-NaCl-$MgCl_2$ buffer) is used for the R39 enzyme, and 5–10 m*M* sodium phosphate buffer is used for the R61 enzyme.

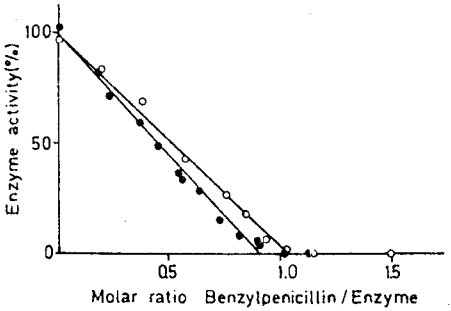


FIG. 4. Titration of the *Streptomyces* R39 protein with benzylpenicillin, based on the inhibition of enzyme activity. ●——●, Enzyme after step 4A of purification; ○——○, enzyme after step 5 of purification. For conditions, see text. Reprinted by courtesy of the Biochemical Society from J.-M. Frère, J.-M. Ghuysen, P. E. Reynolds, R. Moreno, and H. R. Perkins, *Biochem. J.* **143**, 241 (1974).

Antibiotics. Benzylpenicillin and cephaloridine solutions are made fresh, by weight, in the appropriate buffer.

Cephalosporin S7-312 [i.e., 3-(2,4-dinitrostyryl)-(6*R*-7*R*)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer][19] solutions (usually 0.1 m*M*) are made by dissolving the antibiotic in 1 ml of dimethylformamide, and the volume of the solution is adjusted to 250 ml with the appropriate buffer. The final concentration is estimated by measuring the extinction at 386 nm and by using a molar coefficient $\epsilon_{386}^{1\,cm}$ of 17,500.

Titration of the R39 and R61 Enzymes with Benzylpenicillin Based on the Disappearance of Enzyme Activity.[16] Samples of a 5 μM benzylpenicillin solution (either in Tris-NaCl-$MgCl_2$ or phosphate buffer) are added stepwise to 100 μl of a solution containing 0.3 nmole of either R39 or R61 enzyme in the relevant buffer. After each addition, the solution is mixed and maintained at room temperature for 5 min; a sample (2–10 μl) is removed and used for the measurement of the residual DD-carboxypeptidase activity (by incubating the sample with Ac_2-L-Lys-D-Ala-D-Ala for 10 min at 37°). After correction for the decrease in the amount of enzyme owing to the removal of samples and, in the case of the R61 enzyme, after correction of the estimated residual enzyme activity (see below), the end points of the titration occur at a molar ratio of benzylpenicillin to enzyme of 1:1 (Fig. 4).

Because of the short half-life of the R61 enzyme–benzylpenicillin complex (Table IV), breakdown of the complex and reactivation of the

[19] C. O'Callaghan, A. Morris, S. A. Kirby, and A. H. Shingler, *Antimicrob. Agents Chemother.* **1**, 283 (1972).

enzyme occur during estimation of residual enzyme activity at 37°. The exact residual activity[17] (as percent, after addition of x moles of antibiotic) is equal to

$$\frac{(A_m/A_0) + [(1 - e^{-k_4 t})/k_4 t] - 1}{(1 - e^{-k_4 t})/k_4 t}$$

where A_m is the amount of hydrolyzed tripeptide measured in each case, A_0 the amount of hydrolyzed tripeptide obtained with the same amount of uninhibited enzyme, and t the duration of the incubation with the tripeptide (i.e., 600 sec). For the k_4 value, see Table IV.

Breakdown of the R61 enzyme–antibiotic complex formed at the beginning of the titration also occurs during the subsequent steps of the titration. Since the titration is carried out at 22°, however, the error thus introduced is negligible.

Titration of the R61 Enzyme with Benzylpenicillin Based on Fluoresence Quenching.[14] Binding of benzylpenicillin to the R61 enzyme can be followed by the fluorescence quenching at 320 nm (ΔF_{320}). Samples of a 0.232 m*M* solution of benzylpenicillin in 10 m*M* sodium phosphate buffer pH 7.0 are added stepwise at 25° to 2 ml of a solution containing 1.4 nmoles of R61 enzyme in the same buffer. Readings are taken 10 min after each addition to allow time for completion of the reaction. Excitation is at 273 nm. Extrapolation of the two lines of the curve intersect at a point where the molar ratio of benzylpenicillin to enzyme is 1:1 (Fig. 5).

Titration of the R39 Enzyme with Cephaloridine.[16] Samples of 0.5 m*M* cephaloridine in Tris-NaCl-$MgCl_2$ buffer are added stepwise, at room temperature, to 0.4 ml of a solution containing 6.5 nmoles of R39 enzyme in the same buffer. After each addition, the extinction of the solution is measured at 250 nm and the DD-carboxypeptidase is measured in a 2-μl sample. A plot of the increased extinction of the solution at 250 nm as a function of the amount of cephaloridine added yields two lines intersecting at a point. On the basis of these data and the determination of enzyme activity, the end points of the reaction occur at a molar ratio of cephaloridine to enzyme of 1:1. (For illustration, see Frère *et al.*[16])

Titration of the R39 and R61 Enzymes with Cephalosporin 87-312. Samples of a solution of cephalosporin 87-312 in either Tris-NaCl-$MgCl_2$ or phosphate buffer, are added stepwise at room temperature to 0.4 ml of a solution of either R39 or R61 enzyme in the relevant buffer. (For concentration of enzymes and cephalosporin 87-312, see legend of Fig. 6.) After each addition, the mixture is maintained at room temperature for 5 min, after which time the absorbances of the solutions are measured both at 386 nm (intact cephalosporin) and at 482 nm (modified cephalo-

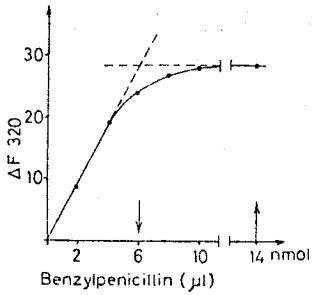


FIG. 5. Titration of the *Streptomyces* R61 protein with benzylpenicillin, based on fluorescence quenching at 320 nm (ΔF_{320}). R61 enzyme (2 ml of 26.5 μg/ml) is dissolved in 10 m*M* sodium phosphate buffer, pH 7.0, at 25°. Excitation is at 273 nm and the fluorimeter settings are: sensitivity 20, meter multiplier 0.01. Fluorescence intensity (F) is expressed in arbitrary units, the maximum emission being taken as 100. The sodium benzylpenicillin solution (0.232 m*M*) is in 10 m*M* sodium phosphate buffer, pH 7.0. Readings are taken 10 min after each addition to allow time for equilibration. After 10 μl of penicillin solution has been added, 2.32 m*M* penicillin (5 μl) is added to ensure saturation (↑). The arrow ↓ indicates the titration end point. Reprinted by courtesy of the Biochemical Society from M. Nieto, H. R. Perkins, J.-M. Frère, and J.-M. Ghuysen, *Biochem. J.* 135, 493 (1973).

sporin), and the DD-carboxypeptidase activity is measured in 2-μl samples. With the R61 enzyme, the residual enzyme activity after each addition of x moles of cephalosporin 87-312 is corrected as indicated above (see titration with benzylpenicillin). As revealed by the three procedures, the end points of the titration of both enzymes occur at a molar ratio of cephalosporin 87-312 to enzyme of 1.25:1 (Fig. 6). This high ratio might be due to the fact that the amount of cephalosporin 87-312 used is estimated on the basis of a ϵ_{386}^{cm} value of 17,500. An underestimation of this coefficient would result in an overestimation of the amount of antibiotic required to estimate the enzyme.

In the case of the R39 enzyme, the extinction values at 386 and 482 nm can be transformed into concentrations, and from these into nanomoles, of intact and modified cephalosporin 87-312 from the following equations:[16]

$$\epsilon_{386} = 17{,}500\ [\text{intact cephalosporin}] + 7700\ [\text{modified cephalosporin}]$$
$$\epsilon_{482} = 2600\ [\text{intact cephalosporin}] + 16{,}700\ [\text{modified cephalosporin}]$$

where 17,500 and 2600 = molar extinction coefficients of intact cephalosporin and 7700 and 16700 = molar extinction coefficients of cephalosporin hydrolyzed by β-lactamase, at the relevant wavelengths.

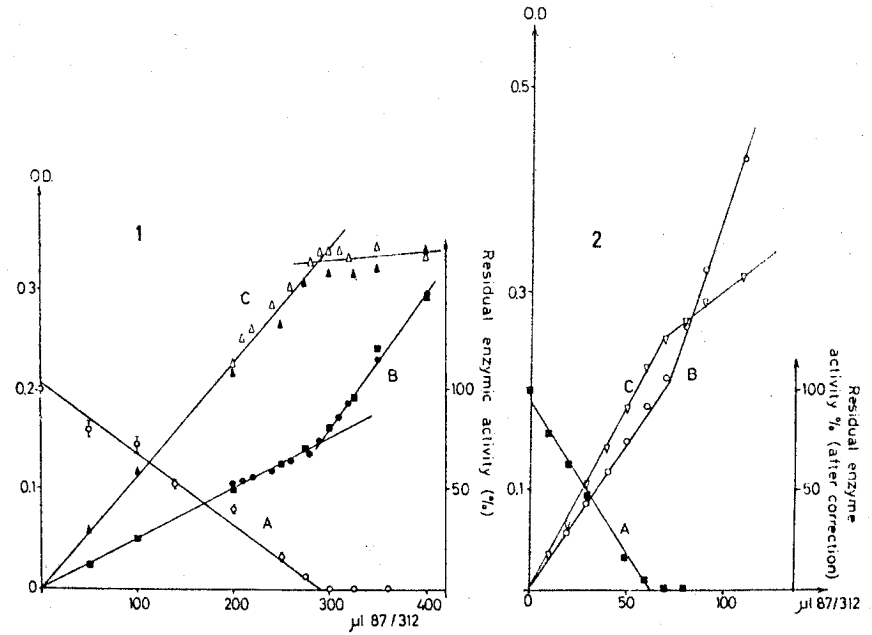


FIG. 6. Titrations of *Streptomyces* R39 enzyme (1) and *Streptomyces* R61 enzyme (2) with cephalosporin 87-312 based (A) on the inhibition of enzyme activity, (B) on the increase of the absorbance of the solution at 386 nm, and (C) on the increase of the absorbance of the solution at 482 nm. The R39 enzyme (13.3 nanomoles) is titrated with a 58.8 *M* cephalosporin 87-312 solution. The R61 enzyme (7.8 nanomoles) is titrated with a 0.15 m*M* cephalosporin 87-312 solution. After each addition of cephalosporin 87-312, the optical densities of the solutions are normalized for final volumes of 800 µl (R39 enzyme) and 510 µl (R61 enzyme). For other conditions, see text. Reprinted by courtesy of the Federation of European Biochemical Societies from J.-M. Frère, M. Leyh-Bouille, J.-M. Ghuysen, and H. R. Perkins, *Eur. J. Biochem.* **50**, 203 (1974).

Hydrolysis Reactions Catalyzed by the DD-Carboxypeptidases–Transpeptidases from *Streptomyces* R39 and R61

General Reaction

$$\text{X-L-R}_3\text{-R}_2\text{-R}_1\text{(OH)} + \text{H}_2\text{O} \rightarrow \text{X-L-R}_3\text{-R}_2\text{(OH)} + \text{R}_1 \qquad (5)$$

Standard reaction (1) is an example.

Procedure. The liberated R_1 residue can be estimated by the fluorodinitrobenzene as indicated above for alanine. Samples containing known amounts of R_1 residue are treated similarly.

Substrate Requirements. These were studied with peptides presenting the above general sequence by measuring the amount of C-terminal R_1

residue released.[11,20] With each enzyme, the C-terminal sequence giving the highest activity is D-Ala-D-Ala, but the preceding L-R_3 residue also has a large effect, both enzymes exhibiting a considerable specificity for the occurrence of a long aliphatic side chain at the R_3 position. Typical Michaelis–Menten kinetics are observed over a wide range of substrate concentrations.

Effects of Peptide Analogs. Peptides that are close analogs of the substrate donor inhibit the activity of the R61 enzyme.[21] Ac-D-Ala-D-Asp behaves as a competitive inhibitor with a K_i value of 3.2 mM (substrate: Ac_2-L-Lys-D-Ala-D-Ala). It has been suggested that the two D-Ala-D-Ala C-terminal residues of the substrates and inhibitors are mainly responsible for the initial binding whereas the side chain of the L-R_3 residue is critical in inducing catalytic activity.

None of the peptide inhibitors of the R61 enzyme have any effect on the carboxypeptidase activity of the R39 enzyme. Some of them are good substrates for this enzyme.[21]

Concomitant Hydrolysis and Transfer Reactions Involving Distinct Donor and Acceptor Peptides, Catalyzed by the DD-Carboxypeptidases–Transpeptidases from *Streptomyces* R39 and R61

General Reaction

$$[^{14}C]Ac_2\text{-L-Lys-D-Ala-D-Ala} \xrightarrow{H_2O} [^{14}C]Ac_2\text{-L-Lys-D-Ala} + \text{D-Ala} \quad \text{(a)} \tag{6}$$

$$[^{14}C]Ac_2\text{-L-Lys-D-Ala-D-Ala} \xrightarrow{NH_2\text{-R}} \text{D-Ala} + [^{14}C]Ac_2\text{-L-Lys-D-Ala-R} \quad \text{(b)}$$

(a) = hydrolysis pathway; (b) = transfer pathway. D-Ala is the reaction product common to both pathways.

Donor Substrate. The tripeptide Ac_2-L-Lys-D-Ala-D-Ala radioactively labeled with ^{14}C in both acetyl groups (specific activity, 10,000 dpm/nanomole) is prepared as described by Perkins *et al.*[22]

Assay Method (with meso-Diaminopimelic Acid as Acceptor). R39 enzyme: [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (5 mM) and *meso*-diaminopimelic

[20] M. Leyh-Bouille, M. Nakel, J.-M. Frère, K. Johnson, J.-M. Ghuysen, M. Nieto, and H. R. Perkins, *Biochemistry* 11, 1290 (1972).

[21] M. Nieto, H. R. Perkins, M. Leyh-Bouille, J.-M. Frère, and J.-M. Ghuysen, *Biochem. J.* 131, 163 (1973).

[22] H. R. Perkins, M. Nieto, J.-M. Frère, M. Leyh-Bouille, and J.-M. Ghuysen, *Biochem. J.* 131, 707 (1973).

acid (8 m*M*) are incubated at 37° with 2.2 pmoles (0.12 μg) of enzyme in 30 μl of 0.03 *M* Tris-HCl buffer, pH 7.5, containing 0.1 *M* NaCl and 0.02 *M* $MgCl_2$. After 30 min, about 18 and 12.5% of the tripeptide donor are converted into [^{14}C]Ac_2-L-Lys-D-Ala and [^{14}C]Ac_2-L-Lys-D-Ala-(D)-*meso*-diaminopimelic acid, respectively.

R61 enzyme: [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala (5.5 m*M*) and *meso*-diaminopimelic acid (8 m*M*) are incubated at 37° with 1.35 pmoles (0.05 μg) of enzyme in 30 μl of 5 m*M* sodium phosphate pH 7.0. After 60 min, about 10% of the tripeptide donor is converted into [^{14}C]Ac_2-L-Lys-D-Ala and the same amount into [^{14}C]Ac_2-L-Lys-D-Ala-(D)-*meso*-diaminopimelic acid.

Estimation of [^{14}*C*]*Ac*$_2$-L-*Lys*-D-*Ala*-D-*Ala* (*Residual Donor*), [^{14}*C*]*Ac*$_2$-L-*Lys*-D-*Ala* (*Hydrolysis Product*), *and* [^{14}*C*]*Ac*$_2$-L-*Lys*-D-*Ala*-*R* (*Transpeptidation Product*)

Reagents

Collidine:acetic acid:water (9.1:2.65:1000, v/v/v), buffer, pH 6.4
Liquid scintillation: 2,2-*p*-phenylenebis (5-phenyloxazole) (POPOP), 100 mg; 2,5-diphenyloxazole (PPO), 4 g; toluene, 1 liter

Procedure. Samples of the reaction mixtures (30 μl) containing 10,000–20,000 dpm are diluted with 40 μl of water. These are spotted as bands, 30 cm from the cathode on 4 cm × 1.5 m strips of Whatman 3 MM paper and subjected to electrophoresis at pH 6.4 for 4 hr at 60 V/cm, under a Sol.T Shell. A Gilson high voltage, 10,000 V, Electrophoretor Model DW equipped with a cooling device is used as power source. Residual [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala and the hydrolysis product [^{14}C]Ac_2-L-Lys-D-Ala move about 65 and 75 cm, respectively, toward the anode. Transpeptidation products [^{14}C]Ac_2-L-Lys-D-Ala-R move differently depending upon the nature of the R residue (about 55 cm where R = *meso*-diaminopimelic acid). The radioactive compounds are located on the dried strips with a Packard Radiochromatogram Scanner Model 7201. Cuts of the radioactive spots (10 mm section) are placed in vials, to each of which is added 0.75 ml of the scintillation liquid. Counting is performed in a Packard Tri-Carb liquid scintillation spectrometer.

Specificity Profiles for Acceptors. The range of substrates that function as acceptors reflects the type of cross-linking that exists in the peptidoglycan of the organism which produces the exocellular enzyme (see

the above section: Strains). Correspondingly, glycine and many peptides with an N-terminal glycine residue act as acceptors in transpeptidation reactions catalyzed by the R61 enzyme (although other amino compounds also function).[22] In marked contrast, suitable transpeptidation acceptors for the R39 enzyme must have an amino group in α-position to the carboxyl group of a D-amino acid (or glycine).[3,22,23]

Kinetics. The kinetics are necessarily complex. For a theoretical analysis, see Frère.[24] For application to the R61 enzyme (with either Gly-L-Ala or *meso*-diaminopimelic acid as acceptor) see Frère *et al.*[25]

The proportion of the enzyme activity channeled in either transpeptidation or hydrolysis depends upon the environmental conditions.[3,23,25] Transpeptidation is increased and hydrolysis decreased by raising the pH of the reaction mixture and the concentration of acceptor within it (this latter behaves as a noncompetitive inhibitor of the hydrolysis pathway). Replacement of part of the water of the reaction mixture by a solvent of low polarity preferentially decreases the hydrolytic activity of the enzyme so that transpeptidation then largely supersedes hydrolysis. In the case of the R39 enzyme, transpeptidase activity is increased at high ionic strengths.[3]

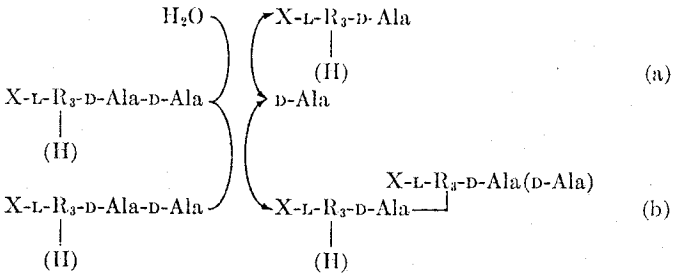
With some peptide acceptors, transpeptidation itself is inhibited at high acceptor concentrations.[3,23,25] For instance, when increasing concentrations of tetrapeptide L-Ala-D-αGln-(L)-*meso*-A_2pm-(L)-D-Ala are provided as acceptor to the R39 enzyme (with 0.27 m*M* Ac_2-L-Lys-D-Ala-D-Ala as donor), transpeptidation rises to a maximum at an acceptor concentration of about 0.8 m*M*, and at higher concentrations both transpeptidation and hydrolysis reactions are progressively inhibited until eventually the tripeptide donor remains unused. In this example, this phenomenon is dependent on the α-amido group on the D-glutamic acid residue of the tetrapeptide acceptor.[3]

For extensive studies on the functioning of the R39 enzyme as a transpeptidase in relation to the degree of saturation of its donor site, see Ghuysen *et al.*[23] Under conditions where the donor site of the enzyme is saturated, the rate of the total reaction (hydrolysis + transpeptidation) is the same as the rate of hydrolysis alone when no acceptor is added, i.e., the enzyme has the same turnover number for D-alanine release whether or not acceptor is present.

[23] J.-M. Ghuysen, P. E. Reynolds, H. R. Perkins, J.-M. Frère, and R. Moreno, *Biochemistry* **13**, 2539 (1974).
[24] J.-M. Frère, *Biochem. J.* **135**, 469 (1973).
[25] J.-M. Frère, J.-M. Ghuysen, H. R. Perkins, and M. Nieto, *Biochem. J.* **135**, 483 (1973).

Concomitant Hydrolysis and Transfer Reactions Catalyzed by the DD-Carboxypeptidases–Transpeptidases from *Streptomyces* R39 and R61 and in Which the Same Peptide Acts as Donor and Acceptor

General Reaction



(7)

(a) hydrolysis pathway; (b) transfer pathway.

The C-terminal D-Ala-D-Ala sequence of the dimer formed can also be hydrolyzed through the carboxypeptidase activity of the enzyme.

Kinetics. Examples of such studies can be found in Ghuysen *et al.*[23] for the R39 enzyme substrate:

L-Ala-D-Glu—(L)-*meso*-A_2pm-(L)-D-[^{14}C]Ala-D-[^{14}C]Ala

in which the amino group on the D center of *meso*-diaminopimelic acid functions as acceptor, and in Zeiger *et al.*[26] for the R61 enzyme substrate:

[^{14}C]Ac-L-Lys-D-Ala-D-Ala
Gly

Inhibition of DD-Carboxypeptidases–Transpeptidases from *Streptomyces* R39 and R61 by β-Lactam Antibiotics in the Presence of Substrates

Procedure. Ideally, the experiments are carried out under conditions where the enzyme concentration (10–20 n*M*) is considerably lower than the concentrations of antibiotics (0.3 μ*M* and higher). Substrate(s), antibiotic (when present), enzyme and buffer are precooled and mixed together at 0°. The solutions are incubated at 37° and after a given time (usually 60 min) the reaction products are estimated. For more details, see Frère *et al.*[25]

[26] A. R. Zeiger, J.-M. Frère, J.-M. Ghuysen, and H. R. Perkins, *FEBS Lett.* **52**, 221 (1975).