A DONOR-ACCEPTOR SUBSTRATE OF THE EXOCELLULAR DD-CARBOXYPEPTIDASE-TRANSPEPTIDASE FROM STREPTOMYCES R61

Allen R. ZEIGER

Department of Biochemistry, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pa 19107, USA

Jean-Marie FRÈRE and Jean-Marie GHUYSEN*

Service de Microbiologie, Faculté de Médecine, Institut de Botanique, Université de Liège, Sart Tilman. 4000 Liège, Belgium

and

Harold R. PERKINS**

National Institute for Medical Research, Mill Hill, London NW7 1AA, UK

Received 22 January 1975

1. Introduction

Peptidoglycan is the rigid structural component of all bacterial cell walls. The basic building block of the nascent peptidoglycan in *Streptomyces* strain R61 consists of a disaccharide of *N*-acetylglucosamine and *N*-acetylmuramic acid which is amidated by the hexapeptide

L-Ala-D-αGln-(L_1)-LL-A_2pm-(L_1)-D-Ala-D-Ala
Gly-(L_2) ——┘ [1]

(A_2pm = diaminopimelic acid). The peptidoglycan itself is a network consisting of chains of both polysaccharide and polypeptide, which are multiply bound to each other [2,3]. In *Streptomyces* R61, the polypeptide chains are produced by transpeptidation between the carboxyl group of the penultimate D-alanine of one hexapeptide and the α-amino group of glycine of another hexapeptide, so that dimers or trimers (or rarely higher polymers) are built up [1]. The location of the physiological transpeptidase appears to be the plasma membrane [4]. An exocellular enzyme has been isolated from *Streptomyces* R61 and shown to have DD-carboxypeptidase and transpeptidase activities [5–8]. The enzyme was shown to catalyse the formation of a D-Ala-Gly peptide bond, such as is found in the native, completed wall peptidoglycan. An example of such a reaction is the transformation of Ac_2-L-Lys-D-Ala-D-Ala and Gly-L-Ala into Ac_2-L-Lys-D-Ala-Gly-L-Ala and D-alanine. In this report, it is shown that the tetrapeptide, N^{α}-[^{14}C]Ac-N^{ϵ}-(Gly)-L-Lys-D-Ala-D-Ala, can serve as both donor and acceptor substrate of this enzyme, yielding several products, including one containing more than one D-Ala-Gly interpeptide linkage.

2. Materials

The *Streptomyces* R61 DD-carboxypeptidase-transpeptidase was purified as described by Frère et al. [6].

* To whom correspondence should be addressed.
** Present address: The University of Liverpool, Department of Microbiology, Life Sciences Building, Liverpool L69 3BX, UK.

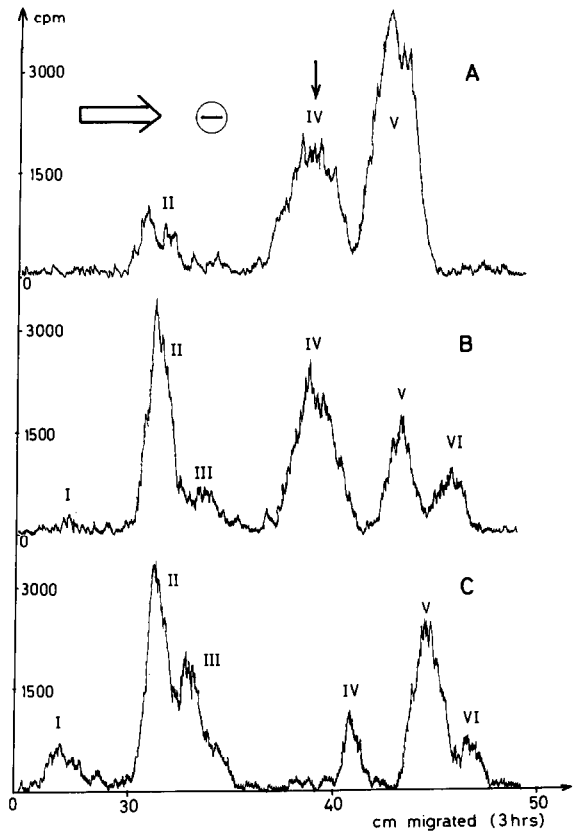


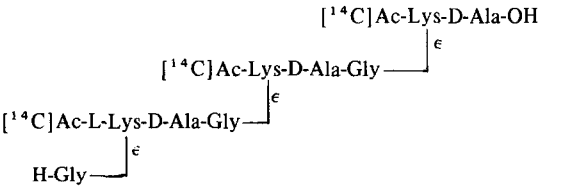
Fig.1. Separation of the enzymic reaction product by paper electrophoresis. Incubation conditions for the enzyme and substrate were as follows: (A) substrate (200 nmol) and enzyme (2 μg) were incubated for 5 min at 37°C in a total volume of 24 μl of an aqueous solution of 3.3 mM sodium phosphate buffer pH 7.5. (B) and (C) substrate (200 nmol) and enzyme (2 μg) were incubated for 5 min (B) and for 40 min (C) in a total volume of 24 μl of 3.3 mM sodium phosphate buffer pH 7.5 in 17% water, 58% ethylene glycol, 25% glycerol (v/v/v). The reaction was stopped by heating the samples for 1 min in a boiling water bath. The samples were spotted on Whatman 3MM paper and submitted to high voltage electrophoresis (Gilson High Voltage Electrophoretor) at 60 V/cm for 3 hr in formic acid/water (20:1,000; v/v) pH 1.8. Radioactive spots were detected by passing the paper in a Packard Radiochromatogram Scanner at a scanning rate of 2 cm/min with a time constant of 10 sec, a slit width of 0.5 cm, and a full scale deflection of 3000 cpm. The vertical arrow refers to the position of the substrate in the absence of enzyme. Peaks were numbered I-VI in order of increasing electrophoretic mobilities.

The substrate (cf. fig.1) was synthesized as follows: to 3.85 mmol of D-alanyl-D-alanine benzyl ester hydrochloride [9] in 150 ml of methylene chloride was added at 4°C 3.46 mmol of α-benzyloxycarbonyl-ε-butoxycarbonyl-L-lysine *p*-nitrophenyl ester and 4.2 mmol of triethylamine. After 16 hr the solvent was removed in vacuo. The residue was dissolved in 150 ml of ethyl acetate and filtered. There was product in the filtered material, but it was impure and not worked up further. The organic layer was extracted with 0.5 M citric acid, water, 1.0 M sodium bicarbonate and water. The ethyl acetate solution was dried over anhydrous sodium sulphate, filtered and concentrated to 30 ml. The product was precipitated with ether. Yield: 0.54 mmol; Mp 160–161°C. Thin-layer chromatography was in 1) methanol : ethyl acetate (1:2; v/v), R_f – 0.88; 2) *n*-butanol : acetic acid : pyridine : water (4:2:1:1; v/v), R_f – 0.84; 3) *n*-butanol : acetic acid : water (4:1:5; v/v), R_f – 0.80. α-*N*-Benzyl-oxycarbonyl-ε-*N*-butoxycarbonyl-L-lysyl-D-alanyl-D-alanine benzyl ester (0.51 mmol) was dissolved in 5 ml of 3.8 N HCl in dioxane. After 15 min, ether was added and the tripeptide hydrochloride was dessicated to dryness. Thin layer chromatography showed only a trace of the starting material. Product R_f in solvent 1) – origin, 2) – 0.63, 3) – 0.48. The residue was dissolved in 15 ml of distilled *N,N*-dimethylformamide. To this solution was added at 4°C 0.57 mmol of triethylamine and 0.60 mmol of butoxycarbonyl-glycine *N*-hydroxysuccinimide ester. After 16 hr, the solvent was removed in vacuo and the residue dissolved in 50 ml of boiling ethyl acetate. The latter layer was washed with 0.5 M citric acid, water, 1.0 M sodium bicarbonate and water as before, dried and crystallized from a small vol. of ethyl acetate. Yield: 0.37 mmol; Mp 168–170°C, R_f 1) – 0.85, 2) – 0.84, 3) – 0.88. In solvent 1, there was a trace at R_f – 0.70. Amino acid analysis showed the following ratios : Lys 0.94 Gly 1.01 Ala 2.05. α-*N*-Benzyloxycarbonyl-ε-*N*-(butoxycarbonylglycyl)-L-lysyl-D-alanyl-D-alanine benzyl ester (63 μmol) was dissolved in 5 ml of methanol containing 50 mg of 10% palladium on charcoal and hydrogenated overnight at 40 psi. The charcoal was centrifuged and the supernatant evaporated to dryness. Yield: 53 μmol. ε-(Butoxycarbonylglycyl)-L-lysyl-D-alanyl-D-alanine (52 μmol) was dissolved in 200 μl water and triethylamine

(10 μl) and dioxan (400 μl) were added. This was followed by a benzene solution of 1-[^{14}C]acetic anhydride (20 mCi/mmol; 20 μl containing 40 μCi) and the miscible mixture was kept in an ice-bath for 3 hr. Then unlabelled acetic anhydride (10 μl) and more triethylamine (20 μl) were added and the reaction was left at 2°C overnight. The mixture was then dried on a rotary evaporator and twice more taken to dryness after addition of 0.3 ml of 0.1 M acetic acid. The product was then transferred as a streak to washed Whatman No. 3 filter paper and subjected to electrophoresis at pH 6.5, pyridine acetate buffer 90 V/cm, 30 min. The product was detected by radioautography (it moved about 13 cm towards the anode) and eluted. The protecting BOC-group was then removed from the dried samply by treatment with anhydrous trifluoroacetic acid at 0°C for 15 min [10]. The spec. act. of the substrate was 0.18 mCi/mmol.

3. Results and discussion

Substrate and the enzyme were incubated in either a fully aqueous solution or a water, ethylene glycol, glycerol medium (17:58:25; v/v/v). Since the enzyme catalyses both the synthesis of D-Ala-Gly linkages (by transpeptidase action) and the simple hydrolysis of C-terminal D-alanyl-D-alanine linkages (by DD-carboxypeptidase action), the following compounds were anticipated to occur in the reaction mixtures : residual substrate monomer (tetrapeptide), hydrolysed monomer (tripeptide N^{α}-Ac-N^{ϵ}-(Gly)-L-Lys-D-Ala), dimers and oligomers either complete (in which the C-terminal peptide is the original tetrapeptide) or hydrolysed (in which the C-terminal peptide is a tripeptide). The reaction mixtures were submitted to high voltage electrophoresis (fig.1). Radioactive compounds were detected by passing the electrophoretogram through a radiochromatogram scanner. The corresponding areas were cut out, eluted with water, and further characterized by amino acid analysis and chromatography on Sephadex G-15 (table 1). The results are consistent with the following assignments : compound I, hydrolysed trimer,



compound II, dimer; compound III, hydrolysed dimer; compound IV, residual substrate monomer; compound V, hydrolysed monomer; compound VI, unknown. The relationship between the electrophoretic mobilities or the chromatographic retention constants obtained on Sephadex G-15 and the logarithm of the assigned mol. wts is illustrated in fig.2. It has been previously shown that the electrophoretic mobilities of peptides having the same electrical charge is linearly related to the logarithm of the mol. wt of the peptide [11].

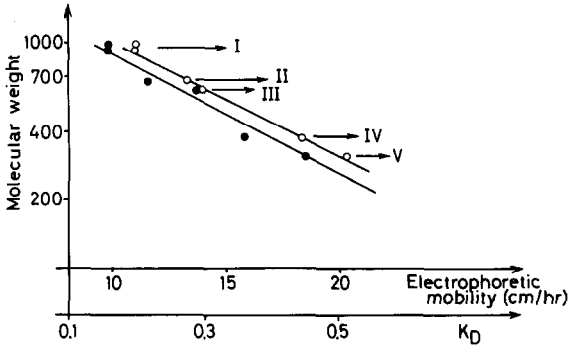


Fig.2. Comparison of electrophoretic mobilities (-o-) and chromatographic retention constants (K_D -•-) of compounds I-V with the logarithms of their assigned mol. wts. Data were taken from fig.1 and table 1. For compound I, the mol. wt of the trimer was included with that of the hydrolysed trimer for comparison.

The identification of compound I as the hydrolysed trimer was confirmed by reaction with dinitrofluorobenzene, followed by amino acid analysis (see footnote d of table 1). Data of table 1 show that upon dinitrophenylation, there is a 33% decrease in glycine relative to alanine and a 36% decrease in glycine relative to lysine, or approximately one glycine lost for every three glycine residues. These results are in agreement with the hypothesis that one glycine in three occupies an amino terminal position in compound I. They are the first indication that a degree of polymerisation higher than that of a dimer can be obtained with the R61 enzyme.

It is apparent from table 1 that reducing the

Table 1
Properties of compounds corresponding to electrophoretic peaks I-VI

Compound	Yield %			Sephadex G-15[b] chromatography		Amino acid analyses[c] (Lys = 1)	
	Exp A	Exp B	Exp[a] C	Elution Vol (ml)	K_D	Ala	Gly
I	0	2.2	9	39.7	0.16	1.16 (1.09)[d]	1.10 (0.78)[d]
II	12	32	31	41.4	0.22	1.44	0.96
III	0	6	18	43.4	0.29	0.91	1.04
IV	30	36	8	45.3	0.36	2.02	1.06
V	58	16	28	47.9	0.445	0.97	0.83
VI	0	8	6	49	0.48		

[a] Details of these experiments are given in fig.1.

[b] The areas corresponding to electrophoretic peaks I–VI were cut out and eluted with water (1 ml). The five solutions were then separately passed through a column (0.7 × 65 cm) of G-15 Sephadex equilibrated against 10 mM NaCl. The elution volumes (V_e) and retention constants (K_D) were recorded. The void vol was 35 ml and the salt vol (NaCl) was 64 ml.

[c] Amino acid analyses were done as follows: samples (20–50 nmol) were hydrolysed for 24 hr in 6 N HCl, dessicated to dryness and applied to a Bio-Cal 2000 Automatic Analyzer. Ratios were calculated assuming a lysine value of one. Compound VI was obtained in insufficient quantity to perform amino acid analysis.

[d] Amino acid analysis of compound I previously submitted to reaction with dinitrofluorobenzene. This experiment was done as follows: an amount of compound I corresponding to 20 nmol of [^{14}C]Ac-Lys was reacted with 30 mmol of dinitrofluorobenzene in an ethanol–triethylamine–water solution (39:1:60; v/v/v) at 60°C. After 30 min, the solution was acidified to 4 N with HCl and extracted with 300 µl of ether. The aqueous phase was evaporated, redissolved in 300 µl 6 N HCl and hydrolysed overnight at 105°C. After evaporation, the residue was submitted to a new AA analysis. The ratio Lys/Ala/Gly was found to be 1/1.09/0.78.

aqueous content of the incubation mixture favors the transfer reaction over the hydrolysis reaction. This observation is consistent with expectations for a membrane-bound enzyme. The ability of the R61 DD-carboxypeptidase-transpeptidase enzyme to produce dimers and a trimer from tetrapeptide monomeric units by catalysing the synthesis of interpeptide linkages which closely resemble those synthesized in vivo also indicates that we may be dealing with a soluble form of the membrane-bound, physiological transpeptidase.

Acknowledgements

The work was supported in part by the Fonds de la Recherche Fondamentale Collective, Brussels, Belgium (contract n° 1000). J. M. F. is Chargé de recherches du Fonds National de la Recherche Scientifique, Brussels, Belgium.

References

[1] Leyh-Bouille, M., Bonaly, R., Ghuysen, J. M., Tinelli, R. and Tipper, D. J. (1970) Biochemistry 9, 2944.
[2] Ghuysen, J. M. (1968) Bacteriol. Rev. 32, 425.
[3] Schleifer, K. H. and Kandler, O. (1972) Bacteriol. Rev. 36, 407.
[4] Dusart, J., Marquet, A., Ghuysen, J. M., Frère, J. M., Moreno, R., Leyh-Bouille, M., Johnson, K., Lucchi, C., Perkins, H. R. and Nieto, M. (1973) Antimicrob. Ag. Chemother. 3, 181.
[5] Leyh-Bouille, M., Coyette, J., Ghuysen, J. M., Idczak, J., Perkins, H. R. and Nieto, M. (1970) Biochemistry 10, 2163.

[6] Frère, J. M., Ghuysen, J. M., Perkins, H. R. and Nieto, M. (1973) Biochem. J. 135, 463.
[7] Frère, J. M., Ghuysen, J. M., Perkins, H. R. and Nieto, M. (1973) Biochem. J. 135, 483.
[8] Perkins, H. R., Nieto, M., Frère, J. M., Leyh-Bouille, M. and Ghuysen, J. M. (1973) Biochem. J. 131, 707.
[9] Zeiger, A. R. and Maurer, P. H. (1973) Biochemistry 12, 3387.
[10] Nieto, M. and Perkins, H. R. (1971) Biochem. J. 123, 789.
[11] Offord, R. E. (1966) Nature 211, 591.