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Streptomyces DD-Carboxypeptidases as Transpeptidases

THE SPECIFICITY FOR AMINO COMPOUNDS ACTING AS CARBOXYL ACCEPTORS

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The ability of the water-soluble DD-carboxypeptidases of *Streptomyces* strains *albus* G, R61, K11 and R39 to perform transpeptidation was studied. The donor was diacetyl-L-lysyl-D-alanyl-D-alanine, and a whole range of amino acids, peptides and structurally related amino compounds were tested for acceptor function. No compound tested was an acceptor for the enzyme from strain *albus* G whereas the enzymes from strains R61 and K11 could utilize with varying efficiency a wide range of substances including peptides with *N*-terminal glycine or D-alanine, ω-amino acids, aminohexuronic acids, 6-aminopenicillanic acid and D-cycloserine. Certain peptides, when present in higher concentration, inhibited the transpeptidation observed at lower concentration. The enzyme from strain R39 would not use any dipeptide as an acceptor, but a few compounds that were not glycine or α-amino acids of the D-configuration did function thus. These were D-cycloserine and the lactams of *meso*- or racemic-diaminoadipic acid.

Soluble DD-carboxypeptidases that hydrolyse the *C*-terminal peptide bond of acyl-D-alanyl-D-alanine and analogous peptides have been isolated from certain strains of *Streptomyces* (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1970*b*, 1971, 1972), and it has been proposed that such enzymes might represent a soluble form of the transpeptidases that perform the final peptide cross-linking in peptidoglycan synthesis (Leyh-Bouille *et al.*, 1970*b*). This hypothesis was given considerable support when it was shown that the penicillin-sensitive DD-carboxypeptidases from *Streptomyces* strains R61 and R39 would transfer the penultimate carboxyl group of the synthetic substrate diacetyl-L-lysyl-D-alanyl-D-alanine to the amino group of a suitable acceptor such as D-alanine, glycine or *meso*-diaminopimelic acid (Pollock *et al.*, 1972). The enzyme from strain R61 would also use the dipeptide glycylglycine as an acceptor, thus giving a product that was not a substrate for the enzyme and hence could not possibly be the result of a simple reversal of hydrolysis. This peptide could be considered as an analogue of the glycyl-LL-diaminopimelic acid *N*-terminus that would be expected to be the acceptor for cross-linking during biosynthesis of a peptidoglycan such as that found in *Streptomyces* strain *albus* G (Leyh-Bouille *et al.*, 1970*a*). However, the enzyme from strain R39 did not use glycylglycine as an acceptor and the one from strain *albus* G gave no transpeptidation with any of the acceptors tried.

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These findings stimulated an investigation of the specificity of these enzymes for acceptors in the transpeptidation reaction, the results of which are described in the present paper.

Materials and Methods

Chromatography and electrophoresis

The chromatographic solvent systems were as follows: (1) butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol.); (2) butan-1-ol–acetic acid–water (3:1:1, by vol.). Paper electrophoresis was performed in the following buffer solutions: A, acetic acid–collidine–water (2.65:9.1:1000, by vol.), pH7; B, acetic acid–pyridine–water (1:25:475, by vol.), pH6.5. Whatman no. 3 paper required for preparative purposes was first exhaustively washed by irrigation with 1M-ammonium acetate followed by water.

Peptides

The peptide used as a substrate for carboxypeptidase activity and as the carboxyl donor for transpeptidase activity, diacetyl-L-lysyl-D-alanyl-D-alanine, was prepared as described by Nieto & Perkins (1971). The same peptide radioactively labelled in the acetyl groups was prepared as follows. [1-^{14}C]-Acetic anhydride (50μCi; 26.6μCi/μmol) was condensed at the bottom of the ampoule provided by the suppliers (The Radiochemical Centre, Amersham,

Bucks., U.K.) by cooling it at −80°C and warming up slightly the rest of the ampoule. The top of the ampoule was broken and L-lysyl-D-alanyl-D-alanine (3 μmol) and triethylamine (1 μl) in water–dioxan (1:1, v/v) (40 μl) were added quickly. The frozen solution was melted, mixed thoroughly and kept in an ice bath for 1 h. Then 1 μl of acetic anhydride (non-radioactive) was added and the reaction was allowed to proceed for a further 1 h in the ice bath. The reaction mixture was heated for 1 min at 100°C, concentrated to dryness, redissolved in water and finally purified by paper electrophoresis in buffer A (10 V/cm, 3 h). After radioautography, two main bands were observed corresponding to the [^{14}C]diacetyl-peptide (17 μCi) and the mono[^{14}C]acetylated derivatives.

The following radioactive acceptor peptides were synthesized as described by Nieto *et al.* (1973) for D-alanyl-D-[U-^{14}C]alanine: D-alanyl-L-[U-^{14}C]-alanine, glycyl-L-[U-^{14}C]alanine, glycyl-D-[U-^{14}C] alanine, D-alanyl-[U-^{14}C]glycine and L-alanyl-L-[U-^{14}C]alanine.

N-Glycyl-*N'*-[1-^{14}C]acetyl-LL-diaminopimelic acid was synthesized as follows. Mono[1-^{14}C]acetyl-LL-diaminopimelic acid was obtained by acetylation with radioactive acetic anhydride (50 μCi; 26.6 μCi/μmol) as described above but with a large excess of LL-diaminopimelic acid (1.4 mg) and omitting acetylation in non-radioactive acetic anhydride. By that means 21 μCi of monoacetyl derivative was obtained after purification by paper electrophoresis as described above.

Mono[1-^{14}C]acetyl-LL-diaminopimelic acid (21 μCi) in water–acetonitrile (1:1, v/v) (100 μl) was treated with 1.7 mg of $NaHCO_3$ and 1.5 mg of t-butoxycarbonylglycine *N*-hydroxysuccinimide ester. The reaction was allowed to proceed for 2 h at room temperature. The reaction mixture was then applied to Whatman 3MM paper for ascending paper chromatography in solvent 1 and the product located by radioautography (R_F 0.64, compared with R_F 0.27 for the monoacetyl derivative). After elution of the radioactive band with methanol–0.04 M-NH_3 (1:1, v/v), the dry product was dissolved in trifluoroacetic acid–dichloromethane (1:1, v/v) (400 μl) and kept for 10 min at 0°C followed by 10 min at room temperature. The resulting *N*-glycyl-*N'*-[1-^{14}C]acetyl-LL-diaminopimelic acid trifluoroacetate was concentrated to dryness, redissolved in buffer A and again concentrated. This step was repeated. It was finally applied to washed Whatman no. 3 paper for electrophoretic purification in buffer A (10 V/cm, 3 h). The product moved towards the anode with a mobility relative to glutamic acid of 0.55. Its R_F value by ascending paper chromatography in solvent 1 was 0.22. The product eluted from paper electrophoresis (14.8 μCi) should be of the same specific radioactivity as the acetic anhydride used to label it.

The synthesis of the same peptide not radioactively labelled was carried out similarly on a larger scale. Intermediates and products were identified by their electrophoretic properties compared with those of the parent compounds, their amino acid composition and the Dnp-derivatives obtained after treatment with fluorodinitrobenzene and subsequent hydrolysis in 4 M-HCl at 100°C for 6 h. Also the acetyl dipeptide gave a grey colour with ninhydrin, compared with a normal purple colour for the monoacetyl derivative of diaminopimelic acid.

α-Acetyl-L-lysine was prepared by treating L-lysine monohydrochloride with 1 mol.prop. of acetic anhydride and 4 mol.prop. of $NaHCO_3$. After 2 h at 0°C, the mixture was adjusted to pH 7 with acetic acid and subjected to electrophoresis on washed paper in buffer A (10 V/cm, 3 h). The neutral ninhydrin-positive band was eluted and re-submitted to paper electrophoresis, this time in 0.25 M-formic acid (10 V/cm, 3 h). The main band was α-acetyl-L-lysine (moved 16.4 cm towards the cathode) and there was a minor band of ε-acetyl-L-lysine (moved 11.5 cm). The identities of these bands were confirmed by preparation of the mono-Dnp derivatives, hydrolysis and paper-chromatographic separation in phosphate buffer (Levy, 1954). α-Acetyl-L-[4,5-^{3}H]lysine was prepared by a similar method. L-[4,5-^{3}H]Lysine monohydrochloride (0.2 mCi) was added to unlabelled compound to give a total of 8 nmol, and the sample was dried under vacuum. $NaHCO_3$ (0.125 M; 50 μl) was added, followed by acetic anhydride (0.5 μl). After 30 min at 0°C the mixture was acidified with formic acid and separated by paper electrophoresis in 0.25 M-formic acid as described above. Marker spots were detected with ninhydrin or by the procedure of Rydon & Smith (1952). Measurement of radioactivity in the eluted sample showed the following approximate proportions of the total: unchanged L-lysine, 3%; ε-acetyl-L-lysine, 7%; α-acetyl-L-lysine, 40%; diacetyl-L-lysine, 50%.

ε-Glycyl-α-acetyl-L-[4,5-^{3}H]lysine was then synthesized in a manner similar to that used for the diaminopimelic acid peptide. Chromatographic properties (solvent 1, ascending, Whatman 3MM paper) were as follows: α-acetyl-L-lysine, R_F 0.35, R_{Gly} 1.92, typical purple colour with ninhydrin; ε-(t-butoxycarbonylglycyl)-α-acetyl-L-lysine, R_F 0.85, R_{Gly} 4.6; ε-glycyl-α-acetyl-L-lysine, R_{Gly} 1.35, bright-yellow colour when treated with ninhydrin after chromatography in this solvent.

Other acceptors

2-Aminoglucuronic acid and 2-aminogalacturonic acid were kindly given by Dr. H. Paulsen, Institut für Organische Chemie und Biochemie, Hamburg, Germany, and muramic acid was given by Dr. R. H. Gigg. Diaminoadipic acid isomers and their lactams

were prepared as described by Nieto *et al.* (1973). Homologous ω-aminocarboxylic acids and D-cycloserine (D-4-amino-3-isoxazolidone) were commercial samples.

Enzymes

The solutions of *Streptomyces* DD-carboxypeptidases were those described earlier: *S. albus* G, approx. 20 units/μl (Ghuysen *et al.*, 1970); strain R61, approx. 27 units/μl (Leyh-Bouille *et al.*, 1971); strain R39, approx. 100 units/μl; strain K11, approx. 30 units/μl (Leyh-Bouille *et al.*, 1972). One unit of enzyme releases 1 nmol of D-alanine from diacetyl-L-lysyl-D-alanyl-D-alanine in 1 h at 37°C, when the substrate is present at saturating concentration.

Transpeptidation experiments

The buffer solution used was sodium phosphate, pH 8, 17 mM in phosphate ions, except for the *S. albus* G enzyme, where it was Tris–HCl, pH 7.5, 17 mM in Tris, containing $MgCl_2$, 1.7 mM. The temperature was 37°C, the time of incubation was 1 h, the amount of enzyme solution used was either 5 μl (*S. albus* G, strain R61, strain K11) or 2 μl (strain R39), and the donor peptide, diacetyl-L-lysyl-D-alanyl-D-alanine (50 nmol) was present in a final volume of 30 μl. The concentration of acceptor was varied as indicated in the text.

After incubation, samples were cooled in ice and then applied under a stream of hot air to Whatman 3 MM paper for electrophoresis either in buffer A (10 V/cm, 2 h) or in a high-voltage apparatus (Electrophorator model D; Gilson Medical Electronics, Middleton, Wis., U.S.A.) with buffer B (80 V/cm, 1 h). In experiments with ^{14}C-labelled compounds the labelled components were located by radioautography and appropriate areas of paper were eluted with water (0.3–0.4 ml). The eluates were placed in vials with scintillation fluid [10 ml of a solution in dioxan (final vol. 1 litre) of naphthalene (180 g), 2,5-diphenyloxazole (4 g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (1 g)], and radioactivity was measured in a Packard Tri-Carb liquid-scintillation counter. The efficiency of counting was 70–73% for ^{14}C and 25% for ^{3}H.

In most experiments high-voltage electrophoresis in buffer B was employed, since this allowed simultaneous measurement of residual donor tripeptide, the dipeptide produced from it by carboxypeptidase action and the transpeptidation product. Relative mobilities are given in Table 1.

Table 1. *Electrophoretic mobility at pH 6.5 of diacetyl-L-lysyl-D-alanyl-D-alanine and its transpeptidation products*

Electrophoresis was performed on Whatman 3MM paper in a high-voltage apparatus, with buffer B (80 V/cm, 1 h).

Compound		Mobility relative to Ac_2-L-Lys-D-Ala-D-Ala
Ac_2-L-Lys-D-Ala		1.14
Ac_2-L-Lys-D-Ala	-*meso*-diaminoadipic acid	0.87
	-*meso*-diaminopimelic acid	0.84
	-Gly-Gly	0.96
	-Gly-Gly-Gly	0.90
	-Gly-Gly-Gly-Gly	0.82
	-Gly-L-Glu	1.60
	-lactam of *meso*-diaminoadipic acid	0.92
	-aminoglucuronic acid	0.89
	-3-aminopropionic acid	0.93
	-4-aminobutyric acid	0.88
	-5-aminovaleric acid	0.79
	-6-aminohexanoic acid	0.75
	-Gly-α′-acetyl-LL-diaminopimelic acid	1.40
	-Gly-Gly-NH_2	0
	-Gly-Gly-L-Leu	0.80
	-Gly-L-Ala	0.92
	-6-aminopenicillanic acid	0.89
	-α-acetyl-L-lysine	0.86
	-D-cycloserine	0.38
bis-(Ac_2-L-Lys-D-Ala)-DD-diaminoadipic acid		1.26

Results

Water-soluble DD-carboxypeptidases from some *Streptomyces* strains, when incubated with diacetyl-L-lysyl-D-alanyl-D-alanine in buffer solution, liberate the *C*-terminal D-alanine residue. When these enzymes were incubated with the same substrate (donor) and certain amino compounds (acceptors), transpeptidation occurred (Pollock *et al.*, 1972) and their specificity for these acceptors has been studied. In initial experiments radioactive acceptors were used and thus only the proportion of acceptor that was converted into transpeptidation product could be measured directly. The proportion of donor converted was then calculated (Table 2).

Enzymes from strains R61 and K11

These enzymes were able to use as acceptor glycine, D-alanine or the D-centre of *meso*-diaminopimelic acid, and in addition a whole range of dipeptides in which the free amino group was that of glycine or D-alanine. With acceptors of the general composition glycyl-R, the highest proportion of transpeptidation occurred when R was L-alanine, glycine or L-lysine linked by its ε-amino group. The presence of a D-asymmetric centre (R = D-alanine) considerably decreased the effectiveness of the acceptor for transpeptidation. A similar decrease occurred when the side chain of the L-amino acid forming the *C*-terminus of the dipeptide was fairly bulky and carried

Table 2. *Transpeptidation by Streptomyces* DD-*carboxypeptidases to radioactively labelled acceptors*

The incubation mixture contained donor (diacetyl-L-lysyl-D-alanyl-D-alanine, 50nmol), buffer solution (sodium phosphate, pH8, final concentration 17mM, except for experiments with enzyme from *S. albus* G when it was Tris–HCl, pH7.5, final concentrations 17mM, Mg^{2+} 1.7mM), enzyme solution 5μl (except for strain R39, 2μl) and acceptor, final volume 30μl. Incubation was for 1h, 37°C. Transpeptidation product was separated from acceptor by paper electrophoresis in buffer A, and their radioactivities were measured. The radioactivity in the product, calculated as a proportion of the donor used, is shown as % transpeptidation. With an acceptor R, the transpeptidation product would be Ac_2-L-Lys-D-Ala-R. Some of the results with amino acids and glycylglycine as acceptors were reported by Pollock *et al.* (1972) and are included here for comparison.

	Acceptor/donor molar ratio	Enzyme from strain ...	Transpeptidation (%)			
			R61	K11	R39	*S. albus* G
α-Amino acids						
[^{14}C]Glycine	1:1		13.7			
	10:1		48	45	30	0
D-[^{14}C]Alanine	1:1		7.5			
	10:1		50		35	0
L-[^{14}C]Alanine	10:1		0		0	
meso-[^{3}H]Diaminopimelic acid*	1:1		16.0	18.2	7.4	
	10:1			48	45	0
Mono[^{14}C]acetyl-LL-diaminopimelic acid	1:1		0			
Dipeptides						
1-[^{14}C]Glycylglycine	1:1		18.4			0
	10:1				0	0
Glycyl-D-[^{14}C]alanine	1:1		3.6	4.0	0	
Glycyl-L-[^{14}C]alanine	1:1		25.5	24.6	0	
ε-Glycyl-α-acetyl-L-[^{3}H]lysine	1:1		18.2		0	0
α-Glycyl-α′-[^{14}C]acetyl-LL-diaminopimelic acid	0.56:1		4.8		0	0
	4.1:1		2.7		0	0
D-Alanyl[^{14}C]glycine	1:1		3.6	3.4	0	
D-Alanyl-L-[^{14}C]alanine	1:1		2.6	2.1	0	
D-Alanyl-D-[^{14}C]alanine	1:1		0.4		0	
L-Alanyl-L-[^{14}C]alanine	1:1		0.2	0.2	0	

* It was shown by Pollock *et al.* (1972) that the D-centre of *meso*-diaminopimelic acid was the one involved in transpeptidation.

a free carboxyl group (R = monoacetyl-LL-diaminopimelic acid). This peptide was synthesized as an analogue of the side chain that would be the natural acceptor for a transpeptidase cross-linking peptidoglycan in the cell walls of strains *S. albus* G, R61 and K11 (Leyh-Bouille *et al.*, 1970*a*). It is noteworthy that with this particular peptide a tenfold increase in acceptor/donor ratio produced a decrease in the amount of donor undergoing transpeptidation. This observation will be discussed further below.

Dipeptides with D-alanine at the *N*-terminus were much poorer acceptors than the corresponding glycyl peptides. Indeed, D-alanyl-D-alanine was hardly an acceptor at all, whereas glycyl-D-alanine was reasonably good. Since glycyl-L-alanine was such a good acceptor it was theoretically possible that L-alanyl-L-alanine might be an acceptor, even though L-alanine itself was not. However, the L-alanine dipeptide hardly functioned as an acceptor, showing that an asymmetric centre of the L-configuration at the amino end of the dipeptide effectively prevents transpeptidation.

The enzyme from strain K11 closely resembles that from strain R61 in many respects (Leyh-Bouille *et al.*, 1972), and it was therefore hardly surprising that it behaved similarly in transpeptidation of the few acceptors examined.

Enzyme from strain R39

This enzyme, when functioning as a carboxypeptidase, has a fairly similar substrate specificity to that observed with the enzymes from strains *albus* G, R61 and K11 (Leyh-Bouille *et al.*, 1970*b*, 1971, 1972). The chief difference is that a peptide with a free ϵ-amino group as in α-acetyl-L-lysyl-D-alanyl-D-alanine was a particularly good substrate, whereas it was a very poor one for the other enzymes. However, in the transpeptidation reaction with diacetyl-L-lysyl-D-alanyl-D-alanine as donor, only glycine and the D-centres of α-amino acids were accepted (Table 2). It seemed possible that with this enzyme the nature of the donor might affect the efficiency of transpeptidation, and so a parallel experiment was set up in which the donor was α-acetyl-L-lysyl-D-alanyl-D-alanine (a particularly good substrate for carboxypeptidase action) and the acceptors, at a molar ratio of 10:1, were glycine, glycylglycine or glycyl-L-alanine. Once more only glycine served as acceptor (53% of the donor was converted into transpeptidation product). Hence, with either donor, none of the dipeptides studied served as acceptor, and this was particularly surprising for the α-glycyl-α'-acetyl-LL-diaminopimelic acid, which was known to be the 'natural' acceptor for the peptidoglycan cross-linking reaction in other strains of *Streptomyces* (Leyh-Bouille *et al.*, 1970*a*).

Enzyme from S. albus G

This enzyme would not effect transpeptidation with any of the acceptors tried. In other respects it is also exceptional (Pollock *et al.*, 1972) and it may well be that the true transpeptidase of this particular organism is not the same as the isolated carboxypeptidase.

Simultaneous measurement of donor hydrolysis and transpeptidation

Previous results showed that with D-alanine as acceptor, transpeptidation took place faster than hydrolysis of the donor peptide (Pollock *et al.*, 1972), and Table 2 showed that increased concentrations of α-glycyl-α'-acetyl-LL-diaminopimelic acid decreased the amount of transpeptidation. To facilitate the simultaneous study of hydrolysis and transpeptidation and the use of a greater range of acceptors, we prepared di[^{14}C]acetyl-L-lysyl-D-alanyl-D-alanine for use as donor. This compound was incubated with enzymes and acceptors and components of the reaction mixture were separated by paper electrophoresis at high voltage (pH 6.5, buffer B); compounds were located by radioautography and their radioactivity was measured.

The effect of increasing the acceptor concentration was studied with glycine and with glycyl-L-glutamic acid, a commercially available analogue (in the sense that it has a free ω-carboxyl group) of α-glycyl-α'-acetyl-LL-diaminopimelic acid, and which is also closely related to glycyl-L-alanine, previously shown to be a very good acceptor for the enzyme from strain R61 (Table 2). The enzyme was incubated with radioactive donor and increasing concentrations of acceptors, and the amounts of transpeptidation product and residual donor were measured (Table 3). The results show that, as might be expected, increasing the concentration of glycine at fixed donor concentration increased the proportion of transpeptidation relative to hydrolysis. The amount of donor remaining unattacked after incubation for 1 h decreased slightly as more acceptor was added. However, with glycyl-L-glutamic acid as acceptor the result was different. Although the ratio of transpeptidation to hydrolysis again increased with acceptor concentration, the proportion of residual donor rose steadily from 18.5% in the absence of acceptor to 55% in the presence of 50 mM-glycyl-L-glutamic acid. A somewhat similar result was observed with α-glycyl-α'-acetyl-LL-diaminopimelic acid, although in this case overall breakdown of donor decreased even more in the presence of the acceptor. An analogous peptide, identical save for the carboxyl group α to the amino group bearing the glycine residue, was ϵ-glycyl-α-acetyl-L-lysine. Increasing the concentration of this peptide also decreased the overall breakdown of the donor (Table

Table 3. *Effect of acceptor concentration on transpeptidation and hydrolysis of donor by the enzyme from strain R61*

The experimental conditions were as in Table 2, except that the donor was di-[^{14}C]acetyl-L-lysyl-D-alanyl-D-alanine. The products were separated by high-voltage electrophoresis in buffer B. Since in this system diacetyl-L-lysyl-D-alanylglycine did not separate from unchanged donor, the appropriate area was eluted and these two components were separated by chromatography on silica-gel loaded paper (Whatman S.G.81) in solvent 2 (Pollock *et al.*, 1972). Before use acceptor solutions were adjusted to pH8 by addition of ammonia. 'Hydrolysis product' refers to di[^{14}C]acetyl-L-lysyl-D-alanine.

Acceptor	Acceptor concentration (mM)	Acceptor/donor molar ratio	Percentage of total radioactivity in: Transpeptidation product	Hydrolysis product	Residual donor	Ratio of transpeptidation to hydrolysis
Glycine	0	0	0	81.1	18.9	0
	3.33	2	16.6	68.0	15.4	0.24
	16.7	10	45.7	40.5	13.8	1.13
	50	30	63.4	24.4	12.2	2.60
Glycyl-L-glutamic acid	0	0	0	81.5	18.5	0
	3.33	2	13.8	63.4	22.9	0.22
	16.7	10	34.0	33.0	33.0	1.03
	50	30	29.7	14.8	55.5	2.01
α-Glycyl-α′-acetyl-LL-diaminopimelic acid	0	0	0	80.4	19.6	0
	0.93	0.56	4.8	69.9	25.3	0.07
	6.8	4.1	2.7	26.9	70.5	0.10
ε-Glycyl-α-acetyl-L-lysine*	0	0	0	67.1	32.9	0
	2.0	1.2	18.6	42.7	38.7	0.44
	16.7	10	29.1	26.4	44.5	1.10
	33.3	20	10.2	27.8	62.1	0.37

* In this experiment a different enzyme solution was used, hence the difference in the amount of hydrolysis in the control sample.

3). At the highest concentration used, however, the proportion of donor hydrolysed to dipeptide hardly changed, whereas the amount of transpeptidation product diminished greatly. This contrasts with the results obtained with glycyl-L-glutamic acid, where the hydrolysis was much the more affected, or with α-glycyl-α'-acetyl-LL-diaminopimelic acid, where the two processes were affected more nearly to the same extent.

To see whether the side-chain carboxyl group was essential for the inhibitory effect, the relative proportion of transpeptidation and hydrolysis was compared with glycyl-L-alanine and glycyl-L-glutamic acid as acceptors, each at a concentration of 16.7mM (acceptor/donor ratio 10:1). With increasing time of incubation the decrease in residual donor remained exactly parallel for both acceptors, each of which decreased overall donor breakdown (Fig. 1). At any time the proportion of transpeptidation relative to hydrolysis was higher for glycyl-L-alanine than for glycyl-L-glutamic acid.

The effect of the concentration of glycyl-L-alanine on transpeptidation was further examined under initial velocity conditions (a maximum of 17% of available donor was consumed). Acceptor concentrations ranged from 2.5 to 20mM. A plot of $1/v$ against 1/[acceptor] (Fig. 2) shows that, starting at a concentration below 10mM, glycyl-L-alanine exercised considerable substrate inhibition on the transpeptidation reaction.

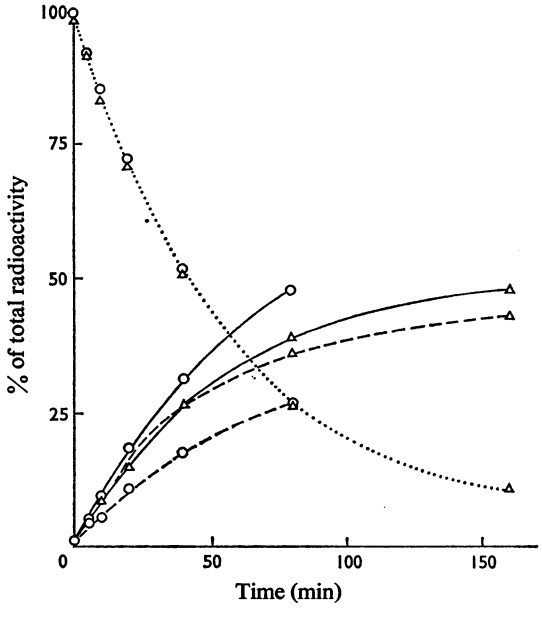

Fig. 1. *Relative transpeptidation and hydrolysis of donor by enzyme from strain R61 in the presence of glycyl-L-alanine or glycyl-L-glutamic acid*

The incubation mixture (90μl) contained di[^{14}C]-acetyl-L-lysyl-D-alanyl-D-alanine (150nmol), acceptor (1.5μmol), phosphate buffer, pH8 (17mM), and enzyme from strain R61 (15μl). The temperature was 37°C. At intervals samples were taken and the components separated by high-voltage paper electrophoresis as described in the Methods section. Transpeptidation product, ——; hydrolysis product, – – –; residual donor, ·····; ○, acceptor, glycyl-L-alanine; △, acceptor, glycyl-L-glutamic acid. Under the same conditions in the absence of acceptor, the residual donor after 60min had decreased to only 18% of the initial value.

Acceptors other than simple amino acids and dipeptides

Although the enzyme from strain R39 appeared to require glycine or a free D-amino acid as acceptor, the enzyme from strain R61 was evidently far less specific. We therefore studied a range of amino compounds as acceptors to discover the effect of the length of the peptide chain, whether amino groups

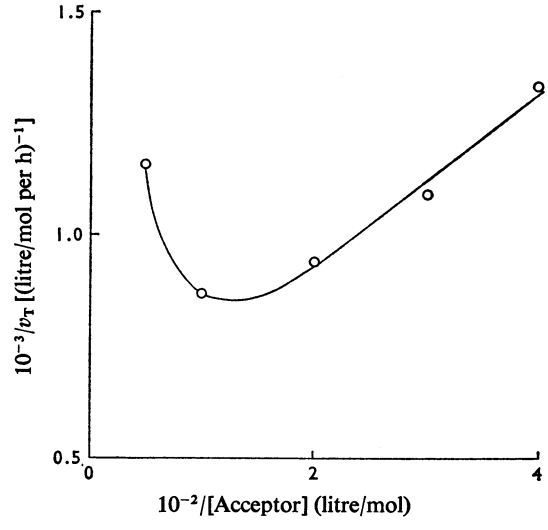

Fig. 2. *Kinetics of transpeptidation with glycyl-L-alanine as acceptor*

The incubation mixture (35μl) contained di[^{14}C]-acetyl-L-lysyl-D-alanyl-D-alanine (87.5nmol), enzyme from strain R61 (25ng), phosphate buffer, pH7.0 (5mM), and various concentrations of glycyl-L-alanine. Incubation was at 37°C for periods of up to 80min. The product was separated and measured as described in the Methods section. v_T is the velocity of the transpeptidation reaction.

Table 4. *Function of various peptides and amides as acceptors for transpeptidation by the enzyme from strain R61*

The experimental conditions were as in Table 2, modified as in Table 3.

Acceptor	Acceptor concentration (mM)	Acceptor/donor molar ratio	Percentage of total radioactivity in: Transpeptidation product	Hydrolysis product	Residual donor	Ratio of transpeptidation to hydrolysis
Peptides						
Gly-Gly	1.67	1	18.4			
	16.7	10	44.5	43.0	12.5	1.03
Gly-Gly-Gly	1.67	1	8.5	77.5	14.0	0.11
	16.7	10	29.8	54.0	16.2	0.55
Gly-Gly-Gly-Gly	1.67	1	5.0	80.3	14.7	0.06
	16.7	10	16.9	60.3	22.8	0.28
Gly-Gly-L-Leu	1.67	1	11.6	68.9	19.5	0.17
	16.7	10	31.3	43.6	25.1	0.72
Amides						
Glycine amide	1.67	1	1.9	78.1	20.0	0.02
	16.7	10	12.0	65.5	22.5	0.18
Gly-Gly-NH_2	1.67	1	1.2	81.7	17.1	0.01
	16.7	10	5.2	80.8	14.0	0.06

Table 5. *Function of compounds other than peptides as acceptors for transpeptidation*

The experimental conditions were as in Table 2, modified as in Table 3.

Acceptor	Acceptor concentration (mM)	Acceptor/donor molar ratio	Enzyme source (strain)	Percentage of total radioactivity in: Transpeptidation product	Hydrolysis product	Residual donor	Ratio of transpeptidation to hydrolysis
Diaminoadipic acids							
Lactam of *meso*-diaminoadipic acid	9.3	2.8*	R61	12.3	70.5	17.2	0.17
			R39	7.2	86.5	6.3	0.08
meso-Diaminoadipic acid	6.7	4	R61	2.5	76.8	20.7	0.03
			R39	1.1	91.5	7.4	0.01
Lactam of racemic (DD+LL) diaminoadipic acid	13.4	4*	R61	15.2	64.7	20.1	0.23
			R39	5.6	87.3	7.1	0.06
Racemic (DD+LL) diaminoadipic acid	8.3	2.5*	R61	54.4†	12.0	30.3	4.8
			R39	63.0†	17.2	18.3	3.8

Amino sugars							
2-Amino-2-deoxy-D-glucuronic acid	8.3	5	R61	8.1	70.9	21.0	0.11
			R39				
2-Amino-2-deoxy-D-galacturonic acid	8.3	5	R61	18.5	48.5	33.0	0.38
			R39	0			
2-Amino-2-deoxy-D-glucose	8.3	5	R61	0			
			R39	0			
2-Amino-3-*O*-(D-1′-carboxy-ethyl)-2-deoxy-D-glucose (muramic acid)	8.3	5	R61	0			
			R39	0			
ω-Amino acids							
3-Aminopropionic acid	8.3	5	R61	6.1	77.5	16.4	0.08
			R39	0			
4-Aminobutyric acid	8.3	5	R61	3.7	74.6	21.7	0.05
			R39	0			
5-Aminovaleric acid	8.3	5	R61	2.2	76.9	20.9	0.03
			R39	0			
6-Aminohexanoic acid	8.3	5	R61	5.4	73.0	21.6	0.07
			R39	0			
6-Amino-2-acetamido-hexanoic acid (α-acetyl-L-lysine)	8.3	5	R61	2.4	77.0	20.6	0.03
			R39	0			
Antibiotics							
6-Aminopenicillanic acid	0	0	R61	0		18.0	
	1.67	1		1.3	59.1	39.6	0.02
	8.3	5		2.5	26.2	71.3	0.10
	0	0	R39	0		9.0	
	1.67	1		0		71.0	
	8.3	5		0		99.2	
D-Cycloserine	0	0	R61	0		20.0	
	1.67	1		28.0	56.2	15.8	0.50
	8.3	5		65.5	22.0	12.5	2.98
	16.7	10		75.9	11.4	12.7	6.7
	16.7	10	R39	16.7	70.7	12.6	0.24

* Calculated for the proportion of the compound that has a free amino group on an asymmetric D-centre, on the assumption that transpeptidation will not occur at an L-centre.

† A portion of this product was hydrolysed in acid (6M-HCl, 105°C, 18h) and it was shown chromatographically (Nieto *et al.*, 1973) that only the DD-isomer of diaminoadipic acid was present. Hence the LL-isomer had not functioned as an acceptor. There was an extra spot of transpeptidation product, which from its position on paper electrophoresis at pH6.5 (Table 1) was taken to be *NN′*-bis(diacetyl-L-lysyl-D-alanyl)-DD-diaminoadipic acid. With the enzyme from strain R61 it represented 3.5% of the total radioactivity, and with that from strain R39, 1.6%.

other than α-amino groups could undergo transpeptidation and if a terminal carboxyl group was also required. The effectiveness of various peptides and amides as acceptors is shown in Table 4. Increase in chain length of polyglycines beyond the dipeptide caused a decrease in transpeptidation, but introducing a bulky side chain on the *C*-terminal residue of the tripeptide (L-leucine replacing glycine) improved acceptor capacity somewhat. Amidation of the *C*-terminal carboxyl group of glycine or glycylglycine greatly decreased their function as acceptors. In each case the introduction of an amide group resulted in a poorer acceptor than if another glycine residue had been introduced. Thus to achieve the same decrease in acceptor function produced by amidation of glycylglycine, it was necessary to add another two glycine residues. There was a distinction here between the enzymes from strains R61 and R39. Although the enzyme from strain R39 would not use any of the dipeptides tried as acceptors (Table 2), it gave weak transpeptidation with glycineamide (0.5% at an acceptor/donor ratio of 1:1 and 2.7% at a ratio of 10:1). Thus for acceptor function by this enzyme, amidation of the α-carboxyl group was not so damaging as the introduction of a second amino acid residue.

D-Alanyl-L-alanine was an acceptor for the enzyme from strain R61 (Table 2). For other purposes we synthesized an analogue of this peptide, namely the lactam of *meso*-diaminoadipic acid, in which the two methyl side chains were joined by a covalent bond, thus forcing a *cis* conformation on the peptide link (Nieto *et al.*, 1973), and we therefore tested this compound as a transpeptidation acceptor. It functioned quite well for the enzyme from strain R61 (Table 5), being probably a better acceptor than D-alanyl-L-alanine (Table 2). Thus the change in conformation enforced by cyclization may favour acceptor function. Surprisingly, the enzyme from strain R39 could also use the *meso*-diaminoadipic acid lactam as a transpeptidation acceptor. Apart from glycine amide, this was the only substance so far found, other than simple α-amino acids, that would serve this function. For comparison, Table 5 also shows the proportion of transpeptidation that occurred with the lactam of racemic diaminoadipic acid, and the uncyclized *meso*- and DD-isomers. The values for the straight-chain *meso*-diaminoadipic acid may be somewhat low, as this compound is rather insoluble and gave a slightly turbid solution at the concentration used. The DD-isomer of diaminoadipic acid was a particularly good acceptor for both enzymes, and it is noteworthy that both were able to perform transpeptidation at each end of the same acceptor molecule, leading to a disubstituted acceptor. A similar result was also observed with the enzyme from strain R61 when DD-diaminopimelic acid was the acceptor.

Since the lactams of diaminoadipic acid are six-membered rings with an amino and a carboxyl group attached diametrically, it seemed possible that other molecules of the same general pattern might also function as acceptors. The 2-amino-2-deoxyhexuronic acids are such molecules, and Table 5 shows that the two tested, with the D-*gluco* and D-*galacto* configurations, were both quite good acceptors for the enzyme from strain R61, but not at all for the one from strain R39. Thus the arrangement of carboxyl and amino groups was clearly acceptable to the former enzyme, even with the large number of hydroxyl groups present, but the latter could no longer perform transpeptidation, either because the relative distance or arrangement of charged groups was incorrect, or because of steric hindrance by the hydroxyl groups.

To check that the amino sugar configuration alone was not enough to make a compound an acceptor for the enzyme from strain R61, glucosamine and muramic acid were examined. Like 2-amino-2-deoxy-D-glucuronic acid, both have the D-glucose configuration, but the former has no carboxyl group and the latter has a carboxyl group in a different situation. Neither compound was an acceptor for either enzyme (Table 5).

The lactams of the diaminoadipic acid isomers and the aminohexuronic acids each have a carboxyl group separated from an amino group by an approximately similar distance. Atomic models suggested that this distance would be about the same as in the extended form of 4-aminobutyric acid or 5-aminovaleric acid. These ω-amino acids, and their next lower and higher homologues, were therefore tested as transpeptidation acceptors (Table 5). None was an acceptor for the enzyme from strain R39, and contrary to the above suggestion, the four-carbon and five-carbon acids were poorer acceptors for the enzyme from strain R61 than their three- and six-carbon homologues. The most striking difference was between glycine, with its α-amino group, for which the percentage transpeptidation at an acceptor/donor ratio of 5:1 was calculated to be about 33% (interpolation on a semi-logarithmic plot of the results given in Table 3), and 3-aminopropionic acid, with a value of only 6%. Similarly, 5-aminovaleric acid was a much poorer acceptor than glycylglycine, which has about the same separation between its free carboxyl and amino groups. Clearly this distance alone is not the major factor governing the effectiveness of a compound as a transpeptidation acceptor.

The introduction of an acetamido group α to the carboxyl group of 6-aminohexanoic acid (α-acetyl-L-lysine) decreased its acceptor function considerably (Table 5). This contrasts with the effect of a bulky side chain at the *C*-terminus of triglycine (glycylglycyl-L-leucine), which somewhat improved acceptor function (Table 4). Other cyclic compounds that to

some extent resemble the lactams of diaminoadipic acid isomers are 6-aminopenicillanic acid and D-cycloserine. The former is rather a poor antibiotic of the penicillin type and the second is regarded as an analogue of D-alanine, normally supposed to function by interfering with alanine racemase and D-alanyl-D-alanine synthetase (Strominger *et al.*, 1960; Neuhaus & Lynch, 1964). 6-Aminopenicillanic acid functioned as a poor acceptor only for the enzyme from strain R61 (Table 5). In the same experiment carboxypeptidase action was greatly inhibited. Thus 6-aminopenicillanic acid was able to occupy the acceptor site of the enzyme from strain R61, but this does not necessarily mean that this site was the seat of inhibitory action. It could mean, however, that in some organisms the action of 6-aminopenicillanic acid, as distinct from its acyl derivatives the penicillins, might be complicated by its ability to function as a transpeptidase acceptor. D-Cycloserine, however, was an excellent acceptor for the enzyme from strain R61 (better than glycine, for instance: compare Tables 5 and 3) and quite a good one even for the enzyme from strain R39. It had no effect on the overall breakdown of the donor. Thus in this respect, as in others, D-cycloserine serves as an excellent analogue of D-alanine, which is also a good acceptor (Pollock *et al.*, 1972). Some of the transpeptidation product, di[^{14}C]acetyl-L-lysyl-D-alanyl-D-cycloserine, was isolated after paper electrophoresis and tested as a substrate and inhibitor for the carboxypeptidases from strains *albus* G, R61 and R39. It functioned as an inhibitor for the former two enzymes, but was also a substrate (Nieto *et al.*, 1973).

Discussion

The observation that the soluble DD-carboxypeptidases from strains R61 and R39, but not strain *albus* G, will function *in vitro* as transpeptidases (Pollock *et al.*, 1972) has been greatly extended. The enzyme from strain R61, which as a carboxypeptidase has high K_m values for a whole range of substrates (Leyh-Bouille *et al.*, 1971), would transpeptidate a large number of acceptors, including glycine, D-amino acids, peptides with D-alanine or glycine *N*-terminal, and certain cyclic compounds with suitably arranged amino and carboxyl groups. In general, the presence of a free carboxyl group seemed to be highly desirable for acceptor function, and amidation greatly decreased it. The only effective acceptor studied that had no free carboxyl or simple amide group was D-cycloserine. However, this antibiotic behaves as a zwitterion with pK_a values of 4.4 and 7.4, and it has been proposed that the carbonyl oxygen atom α to the amino group bears an effective negative charge (Kuehl *et al.*, 1955). Similarly, at pH 6.5 the transpeptidation product diacetyl-L-lysyl-D-alanyl-D-cycloserine behaved as an anion on paper electrophoresis (Table 1). Thus at pH 8, the value used for the transpeptidase assay, the charge properties would be similar to those of an α-amino acid, and its function as an acceptor would be explicable.

The results show that certain acceptors such as glycyl-L-alanine, glycyl-L-glutamic acid and α-glycyl-α'-acetyl-LL-diaminopimelic acid, when present at higher concentrations, caused a decrease in overall breakdown of the donor to an extent independent of their function as acceptors. Thus glycyl-L-alanine inhibited overall breakdown of donor to exactly the same extent as glycyl-L-glutamic acid, and yet at acceptor/donor ratios of 10:1 transpeptidation/hydrolysis ratios were 1.80 and 1.08 respectively (Fig. 1). Similarly α-glycyl-α'-acetyl-LL-diaminopimelic acid was a relatively poor acceptor and yet greatly decreased the overall breakdown of donor. Nevertheless, the effect of this peptide on the relative proportion of transpeptidation and hydrolysis was different from that of the analogous ϵ-glycyl-α-acetyl-L-lysine (Table 3).

One mechanism by which an acceptor might decrease the overall breakdown of the donor is as follows. Alanine may be released by two pathways, either by direct hydrolysis or, in the presence of acceptor, by the competing process of transpeptidation. If binding of the acceptor to the enzyme were good, but the transpeptidation reaction were slow relative to hydrolysis, then the presence of acceptor would lead to a decrease in the rate of release of alanine. If, however, excess of acceptor led to a decrease in the amount of transpeptidation, then this inhibitory effect might well be exercised separately from acceptor function, perhaps by binding at a different site on the enzyme. Further work with the enzyme from strain R39 and acceptors derived from peptidoglycans has produced results strongly supporting this suggestion (Ghuysen *et al.*, 1973).

The limited range of compounds that would function as acceptors for the enzyme from strain R39 was surprising, particularly since α-glycyl-α'-acetyl-LL-diaminopimelic acid, an analogue of the transpeptidase acceptor site in some *Streptomyces* peptidoglycans, was not an acceptor. However, further work has shown that strain R39 has *meso*-diaminopimelic acid and no bridging amino acid in its cross-linked peptidoglycan, and that 'natural' acceptors will function perfectly well *in vitro* with the enzyme from the same strain (Ghuysen *et al.*, 1973).

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