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Kinetics of Concomitant Transfer and Hydrolysis Reactions Catalysed by the Exocellular DD-Carboxypeptidase–Transpeptidase of *Streptomyces* R61

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When Ac_2-L-Lys-D-Ala-D-Ala and either *meso*-diaminopimelic acid or Gly-L-Ala are exposed to the exocellular DD-carboxypeptidase–transpeptidase of *Streptomyces* R61, transpeptidation reactions yielding Ac_2-L-Lys-D-Ala-(D)-*meso*-diaminopimelic acid and Ac_2-L-Lys-D-Ala-Gly-L-Ala occur concomitantly with the hydrolysis of the tripeptide into Ac_2-L-Lys-D-Ala. The proportion of the enzyme activity which can be channelled in the transpeptidation and the hydrolysis pathways depends upon the pH and the polarity of the environment. Transpeptidation is favoured both by increasing the pH and by decreasing the water content of the reaction mixtures. Kinetics suggest that the reactions proceed through an ordered mechanism in which the acceptor molecule (*meso*-diaminopimelic acid or Gly-L-Ala) binds first to the enzyme. Both acceptors behave as non-competitive inhibitors of the hydrolysis pathway. Transpeptidation is inhibited by high concentrations of Gly-L-Ala but not by high concentrations of *meso*-diaminopimelic acid. The occurrence on the enzyme of an additional inhibitory binding site for Gly-L-Ala is suggested.

Based on the primary structure of the wall peptidoglycan in *Streptomyces* R61 (Ghuysen *et al.*, 1973*a*), the transpeptidation reaction which catalyses peptide cross-linking during the last stages of the wall biosynthesis occurs between hexapeptides L-Ala-D-αGln†-(L_1)[Gly-(L_2)]LL-A_2pm-(L_1)-D-Ala-D-Ala. The carboxyl group of the penultimate D-alanine residue of a peptide donor is transferred to the *N*-terminal glycine residue of a peptide acceptor. Interpeptide bridges (D-Ala-Gly-LL-A_2pm) are formed and equivalent amounts of D-alanine are liberated from the peptide donors. The isolated plasma membrane of *Streptomyces* R61 performs transpeptidase activity (Dusart *et al.*, 1973) when exposed to various donor–acceptor systems. The membrane-bound enzyme catalyses the transfer of Ac_2-L-Lys-D-Ala from the synthetic tripeptide Ac_2-L-Lys-D-Ala-D-Ala to several nucleophilic acceptors (amino acids, peptides and related compounds). This enzyme appears to be the main killing target of the β-lactam antibiotics in *Streptomyces* R61 (Dusart *et al.*, 1973). An exocellular transpeptidase is also produced during growth of *Streptomyces* R61. It was isolated, purified and some of its properties were studied (Leyh-Bouille *et al.*, 1971; Pollock *et al.*, 1972; Dusart *et al.*, 1973; Nieto *et al.*, 1973*a*,*b*; Perkins *et al.*, 1973; Frère *et al.*, 1973). When tripeptide donor and acceptors are exposed to the exocellular R61 enzyme in an aqueous environment, transpeptidation occurs as observed with the membrane-bound enzyme, but, in addition, the tripeptide donor undergoes hydrolysis according to the equations

$$Ac_2\text{-L-Lys-D-Ala-D-Ala} + \text{acceptor} \rightarrow Ac_2\text{-L-Lys-D-Ala-acceptor} + \text{D-Ala}$$

and

$$Ac_2\text{-L-Lys-D-Ala-D-Ala} + H_2O \rightarrow Ac_2\text{-L-Lys-D-Ala} + \text{D-Ala}$$

A theoretical analysis of such enzymic mechanisms, which involve concomitant transfer and hydrolysis

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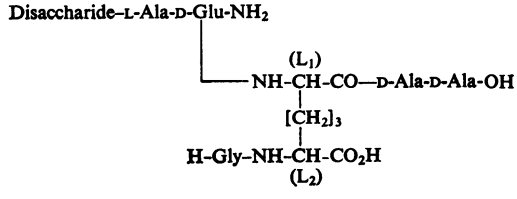
† *Abbreviations:* αGln, α-glutamine; A_2pm, diaminopimelic acid. The symbol (L_1) placed before LL-A_2pm indicates linkage to the amino group of A_2pm in the main peptide chain; (L_2) indicates linkage to the other amino group. Correspondingly, the same symbols placed after LL-A_2pm indicate linkages to the carboxyl group in the main peptide chain (L_1) or the other carboxyl group (L_2). For *meso*-diaminopimelic acid, the symbols (D) and (L) in similar situations have corresponding significance.

reactions, has been presented (Frère, 1973). The goal of this paper is to analyse the kinetics of transfer and hydrolysis catalysed by the exocellular R61 DD-carboxypeptidase–transpeptidase. Ac_2-L-Lys-D-Ala-D-Ala was used as donor and either *meso*-diaminopimelic acid or Gly-L-Ala was used as acceptor. Note that Gly-L-Ala is an analogue of the amino terminal of the natural peptide that undergoes transpeptidation. [Natural peptide: NH_2CH_2CONH-$(R \cdot CH_2)$(L)CH-CO_2H; Gly-L-Ala: NH_2CH_2CONH-(CH_3)(L)CH-CO_2H.]

The peptide undergoing cross-linking *in vivo* has the structure (I)



(I)

Materials and Methods

Materials

Enzyme. The R61 enzyme preparation had a specific activity of 86 units/mg of protein as determined in the carboxypeptidase assay (Ac_2-L-Lys-D-Ala-D-Ala+H_2O → D-Ala+Ac_2-L-Lys-D-Ala) (Frère *et al.*, 1973).

Amino acid and peptides. ^{14}C-labelled Ac_2-L-Lys-D-Ala-D-Ala was prepared as described in Perkins *et al.* (1973). Gly-L-Ala was purchased from Sigma Chemical Co. Solutions of *meso*-diaminopimelic acid dihydrochloride were adjusted to pH7.0 with concentrated NaOH before use.

Estimation of reaction products

Residual donor (Ac_2-L-Lys-D-Ala-D-Ala), hydrolysis product (Ac_2-L-Lys-D-Ala) and transpeptidation products [either Ac_2-L-Lys-D-Ala-(D)-*meso*-A_2pm or Ac_2-L-Lys-D-Ala-Gly-L-Ala] were separated from each other by paper electrophoresis at pH6.5, the radioactive compounds were located on the strips with a Packard radiochromatogram scanner and the amount of radioactivity was measured with a Packard Tri-Carb Liquid-Scintillation Spectrometer. For details and migration of the peptides, see Perkins *et al.* (1973) and Frère *et al.* (1973).

Initial-rate measurements

Symbols. The following symbols are used: [D] = concentration of tripeptide donor; [A] = concentration of acceptor (either *meso*-diaminopimelic acid or Gly-L-Ala); v_T = initial velocity of transpeptidation; v_{Hy} = initial velocity of hydrolysis; $v_P = v_T + v_{Hy}$, i.e. initial velocity of total reaction or of release of D-alanine.

Reactions with Gly-L-Ala as acceptor. Unless otherwise indicated, enzyme (2.1×10^{-3} unit), tripeptide donor (2.75–11mM) and Gly-L-Ala (0–20mM) were incubated at 37°C in 4mM-sodium phosphate buffer, pH7.2 (final volume 35μl).

Reactions with meso-diaminopimelic acid as acceptor. Unless otherwise indicated, enzyme (4.2×10^{-3} unit), tripeptide donor (2.75–11mM) and *meso*-diaminopimelic acid (0–16mM) were incubated at 37°C in 4mM-sodium phosphate buffer, pH7.2, supplemented with NaCl to a final concentration of 32mM (final volume 35μl). The amount of NaCl added to the reaction mixtures was calculated, taking into account that the molar concentration of NaCl in the *meso*-diaminopimelic acid stock solution was twice that of the amino acid. Maximal utilization of the tripeptide donor was 20–25%. Under these conditions, the reaction rates were similar with both acceptors and formation of the products was linear with time. In practice, it was not possible to check the linearity under all conditions, but the following were examined: (*a*) donor, 3mM; acceptor, *meso*-diaminopimelic acid, 12mM. (*b*) Donor, 12mM; acceptor, *meso*-diaminopimelic acid, 8mM. (*c*) Donor, 14mM; acceptor, Gly-L-Ala, 19mM. Furthermore, it was also determined that the addition of D-alanine to the reaction mixture (i.e. the common product of the hydrolysis and transpeptidation reactions) at concentrations up to 2mM did not alter the velocity of both reactions.

Results

Factors influencing hydrolysis and transpeptidation

Influence of pH. The following buffers were used: sodium cacodylate–HCl (pH5–6), sodium phosphate (pH6–8), Tris–HCl (pH8–9) and L-alanine–NaOH (pH9–10). The final ionic strength of the buffers was 0.01. Tripeptide donor (5.5mM), acceptor (8mM) and enzyme (4.2×10^{-3} unit) were incubated for 60min at 37°C in final volumes of 30μl. With both acceptors, hydrolysis was a maximum at low pH values (5–6) whereas transpeptidation was a maximum at high pH values (8–9) (Fig. 1).

Influence of ionic strength. Sodium phosphate buffer, pH7.0, was used for I ranging from 0.02 to 0.22. For I higher than 0.22 (i.e. 0.1M-sodium phosphate), NaCl was added to the reaction mixtures. The ionic strength of the reagents (0.011 for tripeptide donor+acceptor) was taken into account in the final calculations. Tripeptide donor (3mM), *meso*-diaminopimelic acid (4mM) and enzyme (8.4×10^{-3} unit) were incubated for 60min at 37°C in final volumes of

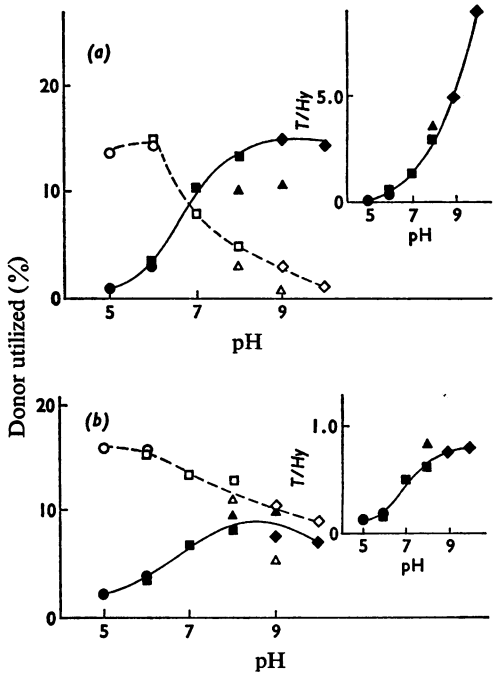


Fig. 1. *Influence of pH upon the rate of hydrolysis and transpeptidation*

Hy, Percentage of tripeptide donor hydrolysed (○, □, △, ◇); *T*, percentage of tripeptide donor transpeptidated (●, ■, ▲, ◆). Acceptor: (*a*) *meso*-diaminopimelic acid; (*b*) Gly-L-Ala. For conditions see the text. ○, ●, Cacodylate; □, ■, phosphate; △, ▲, Tris-HCl; ◇, ◆, L-alanine-NaOH.

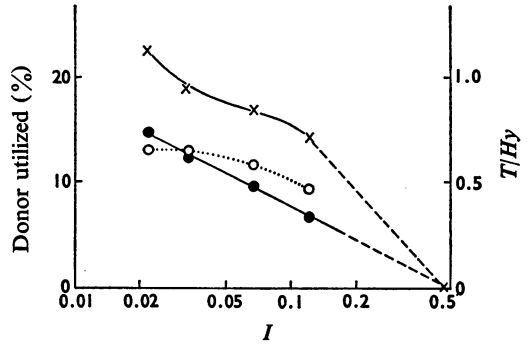


Fig. 2. *Influence of ionic strength upon rate of hydrolysis and transpeptidation*

Hy, Percentage of tripeptide donor hydrolysed (×); *T*, percentage of tripeptide donor transpeptidated (●). ○, *T*/*Hy*. Acceptor: *meso*-diaminopimelic acid. For conditions see the text.

40μl. Both hydrolysis and transpeptidation were maximal at *I* equal to or lower than 0.02 (Fig. 2). At $I = 0.5$, both reactions were completely inhibited. Between $I = 0.02$ and $I = 0.05$, the ratio of transpeptidation to hydrolysis remained constant.

Influence of non-aqueous solvents. Tripeptide donor (2.8mM), *meso*-diaminopimelic acid (4mM) and enzyme were incubated for 60min at 37°C in 4mM-sodium phosphate buffer, pH7 (final vol. 40μl; final *I*, 0.02). Part of the water content of the reaction mixtures (100% in the control assay) was replaced by increasing amounts of ethylene glycol or by a mixture of glycerol and ethylene glycol as indicated in Table 1. In the presence of 75% or 95% ethylene glycol, the enzyme underwent denaturation. Thus when 5×10^{-2} unit of enzyme was incubated at 37°C for 60 and 90min in 75% ethylene glycol but in the absence of donor and acceptor and diluted sixfold before reincubation with donor and acceptor, the enzyme retained only 20% and 5% respectively of its original activity. By contrast, the enzyme was found to be stable for at least 90min at 37°C in water-ethylene glycol-glycerol (6:9:5, by vol.). In this latter medium, the enzyme activities, when compared with those observed in an aqueous environment, were 47% for the transpeptidation reaction and 6% for the hydrolysis reaction, i.e. most of the enzyme activity was channelled through the transpeptidation route, resulting in a 7.5-fold increase of the ratio of transpeptidation to hydrolysis. Radioactive neutral products that could have arisen from formation of esters between donor and glycol or glycerol were not detected.

Stability of the transpeptidation products

Complications in the kinetics of the reactions could be expected if, during the process, the transpeptidation products were reconverted into free acceptors and Ac_2-L-Lys-D-Ala dipeptide. Since the formation of this latter dipeptide is irreversible (Pollock *et al.*, 1972), hydrolysis of the transpeptidation product should result in a decrease with time of the ratio of apparent transpeptidation to hydrolysis. Tripeptide donor (11mM), *meso*-diaminopimelic acid (8mM) and enzyme (2.5×10^{-3} unit) were incubated in 4mM-sodium phosphate, pH7.0, at 37°C (final vol. 40μl). A constant ratio of transpeptidation to hydrolysis of 0.7 was observed throughout the incubation up to 180min, at which time 32% of the tripeptide donor had been utilized, thus demonstrating that the hydrolysis of the D-Ala-(D)-*meso*-A_2pm linkage made by transpeptidation was negligible at least under these conditions. ^{14}C-labelled Ac_2-L-Lys-D-Ala-Gly-L-Ala was prepared from ^{14}C-labelled

Table 1. *Carboxypeptidase and transpeptidase activities of Streptomyces R61 enzyme in solvents with decreased contents of water*

The amounts of enzyme used were: 8.3×10^{-3} units for assays nos. 1, 2, 3 and 6 and 0.033 unit for assays nos. 4 and 5. For other conditions see the text.

Assay no.	Solvent mixtures (by vol.) Water	Ethylene glycol	Glycerol	Tripeptide hydrolysed (*Hy*) (%)	Tripeptide transpeptidated (*T*) (%)	Ratio *T*/*Hy*
1	100	0	0	23.8	20.4	0.86
2	75	25	0	11.2	16.8	1.51
3	50	50	0	4.0	13.6	3.35
4	25	75	0	2.3	22.7	10
5	5	95	0	0.18	4.8	26
6	30	45	25	1.45	9.4	6.4

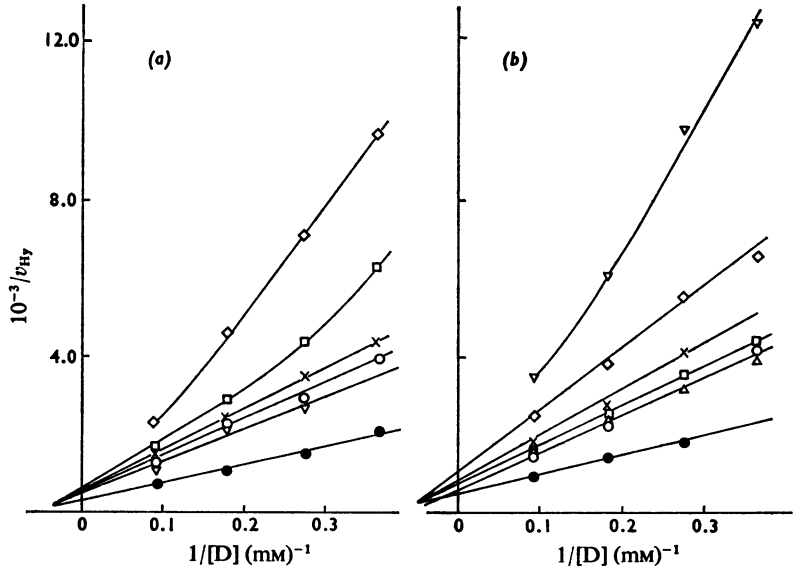


Fig. 3. *Lineweaver–Burk plots* ($1/v_{Hy}$ *versus* 1/[D]) *for various concentrations of acceptor*

For conditions see the Materials and Methods section. (*a*) Concentrations (mM) of *meso*-diaminopimelic acid; 0 (●); 2.7 (▽); 4 (○); 5.3 (×); 8 (□); 16 (◇). (*b*) Concentrations (mM) of Gly-L-Ala: 0 (●); 2.5 (△); 3.3 (○); 5.0 (□): 6.7 (×); 10 (◇); 20 (▽). The $1/v_{Hy}$ values are expressed in $M^{-1} \cdot h$ of tripeptide donor hydrolysed.

Ac_2-L-Lys-D-Ala-D-Ala and Gly-L-Ala by transpeptidation with the R61 enzyme. The labelled tetrapeptide was separated by paper electrophoresis (Materials and Methods section) and further purified by filtration on Sephadex G-15 in water. The tetrapeptide (17nmol) was incubated with 2.1×10^{-3} unit of enzyme in 4mM-phosphate buffer, pH7.0 (final vol. 30μl), and was found to be unaltered after 180min at 37°C. The addition of D-alanine (3.3mM) to the latter reaction mixture was without any effect and, especially, did not cause the formation of detectable amounts of Ac_2-L-Lys-D-Ala-D-Ala, thus demonstrating the irreversibility of the transpeptidation reaction:

$$Ac_2\text{-L-Lys-D-Ala-D-Ala} + \text{Gly-L-Ala} \rightarrow Ac_2\text{-L-Lys-D-Ala-Gly-L-Ala} + \text{D-Ala}$$

In a separate experiment, much more enzyme (3.3×10^{-2} unit) was incubated in 4mM-phosphate buffer, pH7.0, with the tetrapeptide Ac_2-L-Lys-D-

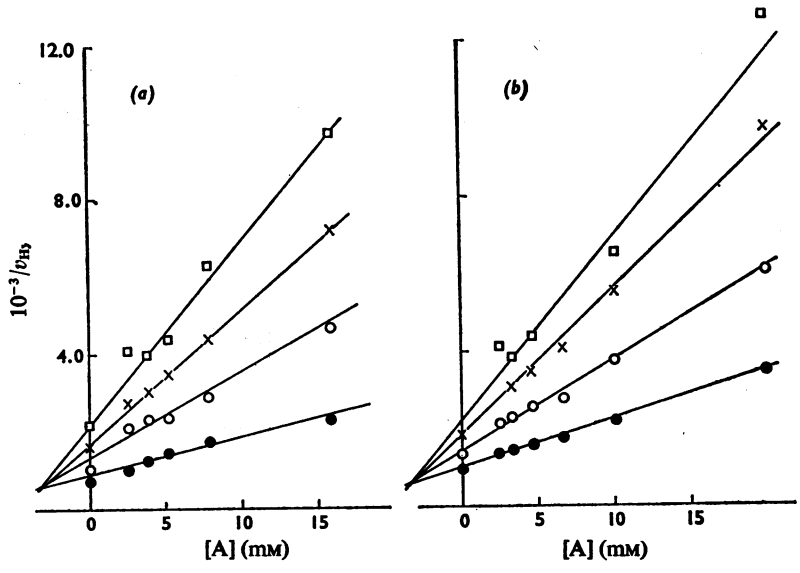


Fig. 4. *Dixon plots* ($1/v_{Hy}$ *versus* [A]) *for various concentrations of tripeptide donor*

For conditions see the Materials and Methods section. Acceptor: (*a*) *meso*-diaminopimelic acid; (*b*) Gly-L-Ala. Concentrations (mM) of donor are: 2.8 (□); 3.7 (×); 5.5 (○); 11 (●). The $1/v_{Hy}$ values are expressed in $M^{-1} \cdot h$ of tripeptide donor hydrolysed.

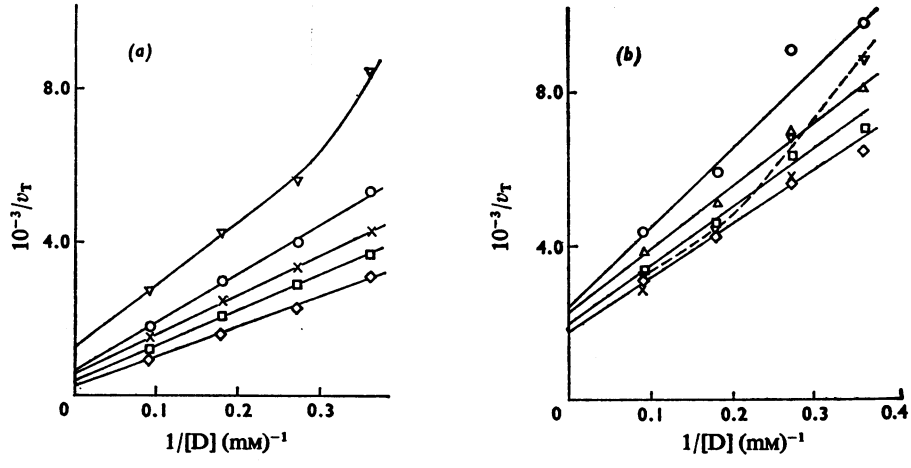


Fig. 5. *Lineweaver–Burk plots* ($1/v_T$ *versus* $1/[D]$) *for various concentrations of acceptor*

For conditions see the Materials and Methods section. (*a*) Concentrations (mM) of *meso*-diaminopimelic acid: 2.7 (▽); 4 (○); 5.3 (×); 8 (□); 16 (◇). (*b*) Concentrations (mM) of Gly-L-Ala: 2.5 (○); 3.3 (△) 5; (□); 6.7 (×); 10 (◇); 20 (▽). The $1/v_T$ values are expressed in $M^{-1} \cdot h$ of tripeptide donor transpeptidated.

Ala-Gly-L-Ala (17nmol). After 80min 30% of the substrate was hydrolysed to dipeptide Ac_2-L-Lys-D-Ala. The enzyme preparation (degree of purity 95%; Frère *et al.*, 1973) thus exhibits some endopeptidase activity. This latter activity, however, is much too low to affect the kinetics of the transpeptidation and hydrolysis reactions under the conditions used throughout the present study.

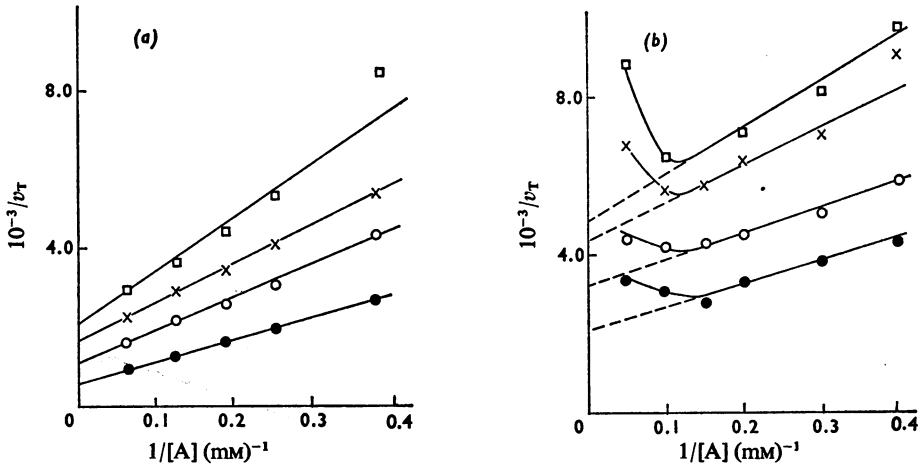


Fig. 6. *Lineweaver–Burk plots* ($1/v_T$ *versus* 1/[A]) *for various concentrations of donor*

For conditions see the Materials and Methods section. Acceptor: (*a*) *meso*-diaminopimelic acid; (*b*) Gly-L-Ala. Concentrations (mM) of donor: 2.8 (□); 3.7 (×); 5.5 (○); 11 (●). The $1/v_T$ values are expressed in $M^{-1}\cdot h$ of tripeptide donor transpeptidated.

Kinetics of hydrolysis and transpeptidation

The conditions were those described in the Materials and Methods section (see 'Initial-rate measurements'). Incubation times were 80 min.

Hydrolysis. Fig. 3 and Fig. 4 show the double-reciprocal plots $1/v_{Hy}$ versus 1/[D] for various concentrations of A and the Dixon plots $1/v_{Hy}$ versus [A] for various concentrations of D. Both *meso*-diaminopimelic acid and Gly-L-Ala acceptors behaved as non-competitive inhibitors of the hydrolysis reaction.

Transpeptidation. Fig. 5 and Fig. 6 show the double-reciprocal plots $1/v_T$ versus 1/[D] for various concentrations of A and $1/v_T$ versus 1/[A] for various concentrations of D. Gly-L-Ala at high concentrations inhibited the transpeptidation reaction. Such an inhibitory effect was not observed with *meso*-diaminopimelic acid. With both *meso*-diaminopimelic acid and Gly-L-Ala as acceptors (assuming that inhibition would not occur at high concentrations of Gly-L-Ala), plotting $1/v_T$ obtained by extrapolation to $1/[D] = 0$ versus 1/[A], and $1/v_T$ obtained by extrapolation to $1/[A] = 0$ versus 1/[D], gave rise to straight lines (Fig. 7). The 'affinity' of the enzyme, at saturating concentration of D, was greater for Gly-L-Ala than for *meso*-diaminopimelic acid, and the 'affinity' of the enzyme for the tripeptide donor was greater at saturating concentration of Gly-L-Ala than at saturating concentration of *meso*-diaminopimelic acid. Finally, the $v_{T(max.)}$ value (at saturating concentrations of both donor and acceptor) was lower with Gly-L-Ala than with *meso*-diaminopimelic acid.

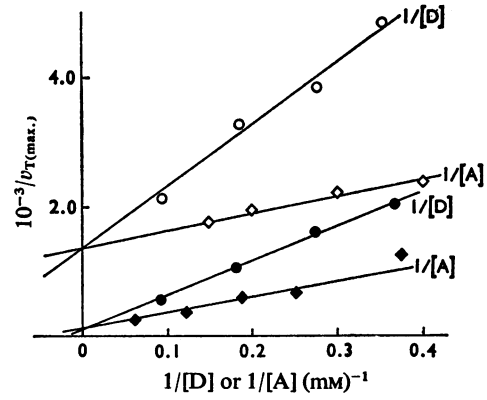


Fig. 7. *Plots of* $(1/v_T)_{max.}$, *at infinite concentration of A, versus* 1/[D], *and of* $(1/v_T)_{max.}$, *at infinite concentration of D, versus* 1/[*A*]

The $1/v_{T(max.)}$ values (○, ●) are the intercept values on the ordinate axis of Fig. 5. The $1/v_{T(max.)}$ values (◇, ◆) are the intercept values on the ordinate axis of Fig. 6. Acceptor: ○, ◇, Gly-L-Ala; ●, ◆, *meso*-diaminopimelic acid. The $1/v_{T(max.)}$ values are expressed in $M^{-1}\cdot h$ of tripeptide donor transpeptidated.

Total reaction. Fig. 8 shows the double-reciprocal plots $1/v_P$ versus 1/[D] for various concentrations of A. With *meso*-diaminopimelic acid, variations of $1/v_P$ versus 1/[D] (v_P = hydrolysis + transpeptidation)

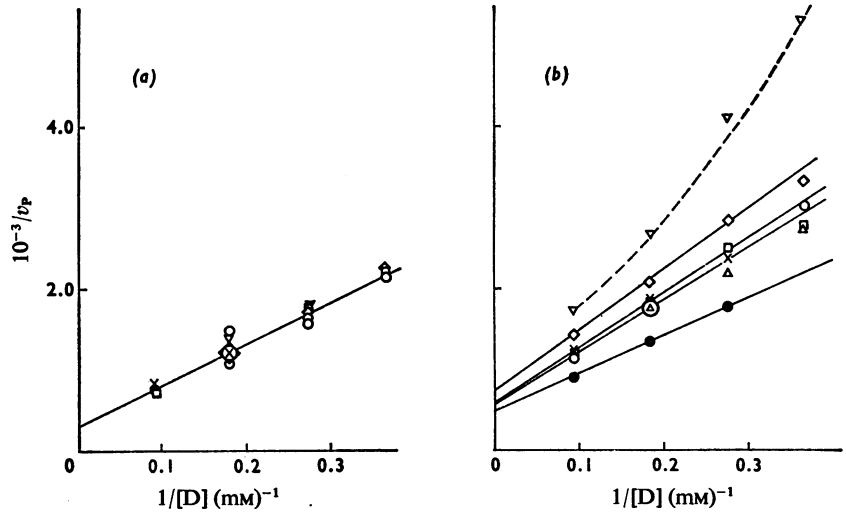


Fig. 8. *Lineweaver–Burk plots of* $1/v_P$ *versus* $1/[D]$ *for various concentrations of acceptor*

v_P was calculated as the sum v_T+v_{Hy} (Fig. 3 and Fig. 5). Acceptor: (*a*) *meso*-diaminopimelic acid; (*b*) Gly-L-Ala. The $1/v_P$ values are expressed in $M^{-1}\cdot h$ of tripeptide donor used for hydrolysis and transpeptidation. For symbols see Fig. 3 and Fig. 5.

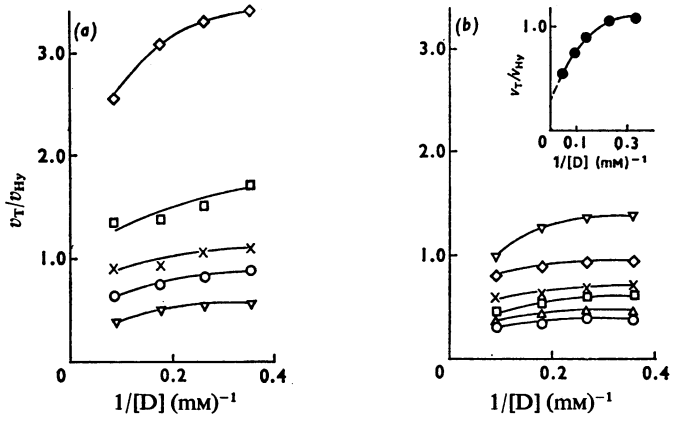
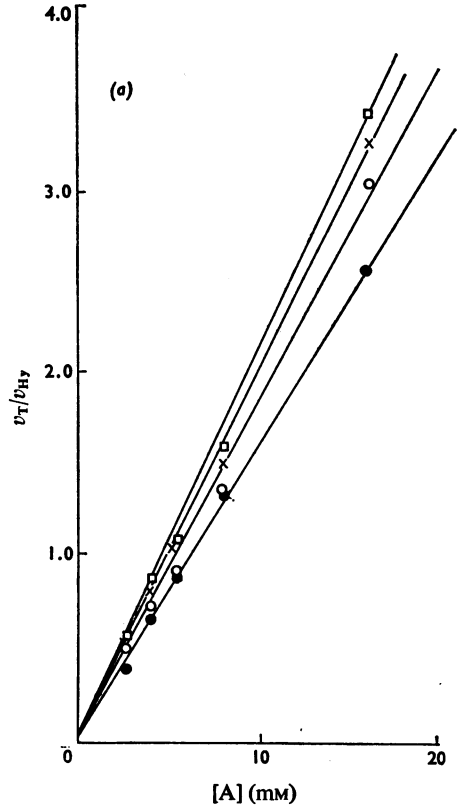
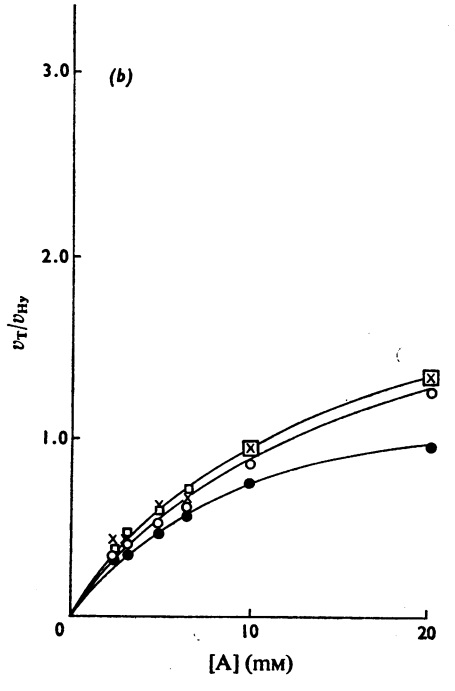


Fig. 9. *Plots of* v_T/v_{Hy} *versus* $1/[D]$ *for various concentrations of acceptor*

The ratios v_T/v_{Hy} were calculated from data of Fig. 3 and Fig. 5. Symbols are identical with those of Fig 3 and Fig. 5. Acceptor: (*a*) *meso*-diaminopimelic acid; (*b*) Gly-L-Ala. For the curve inset in (*b*), the highest concentration of tripeptide donor utilized was increased up to 19.3mM. Gly-L-Ala (20mM) and tripeptide donor (3–19.3mM) were incubated for 80min at 37°C with 1.3×10^{-3} units of enzyme in 4mM-phosphate buffer, pH7 (final vol. 35μl).

were linear, but v_P was independent of the concentration of acceptor. Hence the total activity of the enzyme, in terms of D-alanine liberated, is only a function of the amount of peptide donor present in the reaction mixture. There was a marked inhibitory effect of Gly-L-Ala on the rate of the total reaction. As suggested by the upward concavity of the curve, Gly-L-Ala at high concentration (20mM) caused a





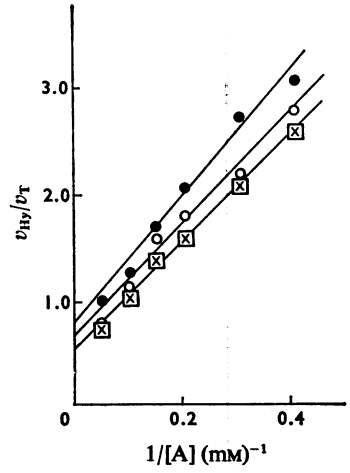


Fig. 11. *Plots of* v_{Hy}/v_T *versus* $1/[A]$ *for various concentrations of donor*

The ratios v_{Hy}/v_T were calculated from the data of Fig. 4 and Fig. 6. Acceptor: Gly-L-Ala. Symbols are identical with those of Fig. 4 and Fig. 6.

disproportionate decrease of the total catalytic activity as the concentration of the tripeptide donor decreased.

Ratio of transpeptidation to hydrolysis. (*a*) At any concentration of acceptor, the ratio v_T/v_{Hy} decreased with increasing concentrations of tripeptide donor (Fig. 9). The inhibitory effect upon transpeptidation of the tripeptide donor was more pronounced at higher concentrations of donor. (*b*) At any concentration of tripeptide donor, the ratio v_T/v_{Hy} was directly proportional to the concentration of *meso*-diaminopimelic acid, whereas it was a function of the concentration of Gly-L-Ala according to an equation of the general form $v_T/v_{Hy} = (a[A])/(b[A]+c)$ (Fig. 10). Plotting the inverse ratio therefore yields straight lines, since $v_{Hy}/v_T = b/a+(c/a[A])$ (Fig. 11), where *a*, *b* and *c* are constants with values depending upon [D].

Discussion

The proportion of the catalytic activity of the exocellular R61 enzyme that is channelled into the transpeptidation and the hydrolysis pathways greatly

Fig. 10. *Plots of* v_T/v_{Hy} *versus* $[A]$ *for various concentrations of donor*

The ratios v_T/v_{Hy}, were calculated from the data of Fig. 4 and Fig. 6. Symbols are identical with those of Fig. 4 and Fig. 6.

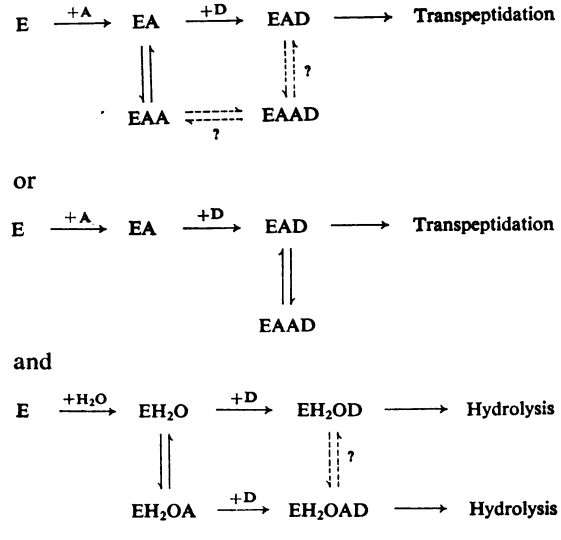
depends upon the environmental conditions, especially the pH and the polarity of the reaction mixtures. The transpeptidase activity sharply increases from pH6 to pH8 and then levels off, whereas the carboxypeptidase activity continuously decreases at pH values higher than 6 (Fig. 1). A similar effect of the pH on the carboxypeptidase and transpeptidase activities of the R39 enzyme was previously observed (Ghuysen *et al.*, 1973*b*). To be able to perform a nucleophilic attack on the carbon atom bearing the carbonyl group in the peptide donor, the amino group of the peptide acceptor must be unprotonated. The curves of transpeptidation versus pH present an inflexion point at pH7.0, i.e. a value considerably lower than the pK values of the amino groups of both acceptor molecules (8.15 for Gly-L-Ala and above 9 for *meso*-diaminopimelic acid, as determined by electrotitration). Hence, at pH7, both acceptors must be largely protonated ($R\text{-}NH_3^+$) and the pK value of 7 experimentally observed might therefore be assigned to a specific enzyme–substrate complex.

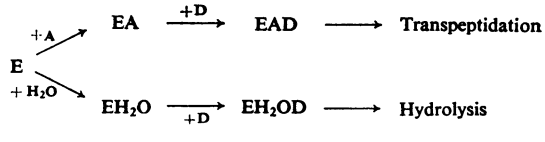
Replacement of part of the water of the reaction mixtures by solvents of low polarity preferentially decreases the carboxypeptidase activity of the enzyme so that transpeptidation then largely supersedes hydrolysis (Table 1). In this respect, one should mention that the R61 enzyme, when integrated into the membrane, functions solely as a transpeptidase (Dusart *et al.*, 1973), whereas it performs both transpeptidase and carboxypeptidase activities after extraction from the membrane by various means (J. Dusart, unpublished work). Finally, the exocellular R61 enzyme has maximum carboxypeptidase and transpeptidase activities at low ionic strength (Fig. 2). The carboxypeptidase activity of the R39 enzyme was little affected by the ionic strength of the reaction mixture whereas its transpeptidase activity was specifically enhanced in solvents of high ionic strength (0.5M-K_2HPO_4) (Ghuysen *et al.*, 1973*b*).

Both *meso*-diaminopimelic acid and Gly-L-Ala behave as non-competitive inhibitors of the hydrolysis reaction. The plots $1/v_T$, $1/v_{Hy}$ and $1/v_P$ versus 1/[D], at any given concentration of A, do not intercept the abscissa at the same 1/[D] value. The v_T/v_{Hy} ratios are not independent of the concentration of tripeptide donor but significantly decrease as the concentrations of donor increase. These two latter kinetic features are not compatible with a Ping-Pong mechanism, a random mechanism or an ordered mechanism in which the tripeptide donor would bind first to the enzyme, but rather suggest for the transfer reaction an ordered pathway mechanism (Scheme 1) in which the acceptor molecule binds first to the enzyme (Frère, 1973).

With such a mechanism, however, the double-reciprocal plots $1/v$ versus 1/[D] are theoretically non-linear. Experimentally, straight lines were observed in some cases, depending upon the concentration of acceptor used (Figs. 3, 5, 8). As discussed elsewhere (Frère, 1973), curves characterized by general non-linear equations may seemingly present themselves as straight lines under the experimental conditions used and within the limits of experimental errors. For this reason, the influence exerted by [D] upon the v_T/v_{Hy} ratio is the most dependable and useful parameter in making a choice from among various mechanisms. The experimental data, however, are not incompatible with non-symmetrical pathways, i.e. mechanisms in which the acceptor binds first to the enzyme in the transpeptidation reaction and the donor binds to the enzyme in the hydrolysis reaction.

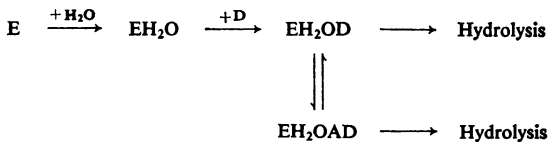
To explain both the inhibition of the transpeptidation by high concentrations of Gly-L-Ala and the presence of the term b[A] in the denominator of the equation $v_T/v_{Hy} = (a[A])/(b[A]+c)$ (which makes the ratio v_T/v_{Hy} not directly proportional to the concentration of Gly-L-Ala), the above simple mechanism must be modified by assuming the occurrence on the enzyme of an additional inhibitory binding site for the dipeptide acceptor. The reactions would then proceed according to the following possible mechanisms:





Scheme 1. *Suggested ordered pathway mechanism for the hydrolysis and transfer reactions in the presence of diaminopimelic acid*

or



The significance of the inhibition of the transpeptidation reaction by excess of acceptor is not known at present. This inhibition was also observed with peptides other than Gly-L-Ala, such as Gly-L-Glu, α-Ac-ε-Gly-L-Lys, α-Ac-α′-Gly-LL-A_2pm (Perkins *et al.*, 1973) and with the natural peptide which undergoes transpeptidation in *Streptomyces* R61 [L-Ala-D-αGln-(L_1); Gly-(L_2)-LL-A_2pm; unpublished work]. The exocellular DD-carboxypeptidase–transpeptidase from *Streptomyces* R39 also exhibited similar properties (Ghuysen *et al.*, 1973).

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