Effects of Donor and Acceptor Peptides on Concomitant Hydrolysis and Transfer Reactions Catalyzed by the Exocellular DD-Carboxypeptidase–Transpeptidase from *Streptomyces* R39†

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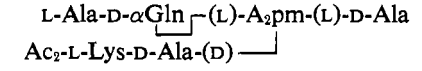
ABSTRACT: The exocellular R39 DD-carboxypeptidase–transpeptidase catalyzes bimolecular transfer reactions concomitantly with the hydrolysis of the peptide donor. Some of the transfer reactions studied have led to the synthesis of peptide dimers identical with or very similar to those found in the completed wall peptidoglycans of bacteria. Under conditions of nonsaturation of the donor site of the enzyme by the peptide donor, the increase of the rate of transfer caused by increasing concentrations of acceptor peptide is lower than can be accounted for by the decrease of the rate of hydrolysis, resulting in a progressive decrease of the total activity of the enzyme. Under conditions of saturation of the donor site of the enzyme by the peptide donor, the proportion of the enzyme activity which is diverted from the hydrolysis pathway because of the presence of low concentrations of peptide acceptor, is entirely channeled into the transfer pathway. The rate of the total reaction is equal to the maximal rate of hydrolysis of the peptide donor in the absence of peptide acceptor [*i.e.*, the $V_{Hy}(H_2O)$ max value]. High concentrations of some peptide acceptors (nonamidated peptide) cause an increase of the rate of transfer higher than the decrease of the rate of hydrolysis. The rate of total reaction then exceeds the $V_{Hy}(H_2O)$ max value. The transfer reaction itself can be inhibited by an excess of some peptide acceptors (Glu-amidated peptide). The less the enzyme is saturated by the peptide donor, the lower is the concentration of peptide acceptor at which inhibition of the transfer reaction starts to occur.

The peptide moiety of the wall peptidoglycan of *Streptomyces* strain R39 is composed of L-alanyl-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine tetrapeptides which are cross-linked through C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid linkages (Ghuysen *et al.*, 1973). These interpeptide bonds are believed to be made by transpeptidation (Wise and Park, 1965; Tipper and Strominger, 1965), a reaction during which the carboxyl group of the penultimate D-alanine residue of a pentapeptide L-alanyl-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine (*i.e.*, the donor) is transferred to the amino group located on the D carbon of the *meso*-diaminopimelic acid residue of another pentapeptide of the same composition (*i.e.*, the acceptor). Formation of the interpeptide bond is accompanied by the release of free D-alanine from the pentapeptide donor. *Streptomyces* R39 excretes a single polypeptide DD-carboxypeptidase–transpeptidase enzyme which catalyzes hydrolysis and transfer reactions according to the equations R-D-Ala-D-Ala + H_2O → D-Ala + R-D-Ala (carboxypeptidase activity) and R-D-Ala-D-Ala + NH_2-R′ → D-Ala + R-D-Ala-R′ (transpeptidase activity). The requirements of the R39 enzyme for peptide donors R-D-Ala-D-Ala (Leyh-Bouille *et al.*, 1972) in carboxypeptidase assays and for peptide acceptors NH_2-R′ (Pollock *et al.*, 1972; Perkins *et al.*, 1973; Ghuysen *et al.*, 1973) in transpeptidase assays suggested that this enzyme might be an exo form of the membrane-bound enzyme which catalyzes peptide cross-linking *in vivo*. In early studies, the model transpeptidation reactions always consisted of the transfer of the dipeptide N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanine from the synthetic tripeptide donor N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine to free amino acids or to well-defined peptide acceptors. One of the acceptors used for these assays was the natural peptide acceptor L-alanyl-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine (Ghuysen *et al.*, 1973). The products of the transfer reaction were D-alanine and the hexapeptide



These studies have now been extended by using model transfer reactions leading to the synthesis of peptide dimers which resembled much more closely the wall peptide dimers made by transpeptidation *in vivo*. Another result of these investigations was to show that the K_m and V_{max} values for peptide donors as determined in carboxypeptidase assays were important parameters directing the rates of the transfer reaction.

Materials and Methods

DD-Carboxypeptidase–Transpeptidase from Streptomyces R39. The enzyme was that used previously (Ghuysen *et al.*, 1973); it contained 0.6 unit/ml. One unit is defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmol of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (release of the C-terminal D-alanine residue) per min at 37° when 8 mM peptide is incubated with the enzyme in the absence of amino group

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TABLE I: Electrophoretic Mobilities of Radioactive Compounds at pH 6.4.[a]

Peptides	Migration (cm)	Time of Electrophoresis (hr)	Reactions in which the Compound is Involved (Table II) Reaction No.
L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala-D-*Ala	60	4	1, 4, 5
L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala	67	4	1
Ac-L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala-D-*Ala (D) Ac	60	1.5	2–6
Ac-L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala (D) Ac	60	1.5	2
Ac_2*-L-Lys-D-Ala-D-Ala	65	4	3–7
Ac_2*-L-Lys-D-Ala	75	4	3
L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala-D-*Ala (D)	80	4	4
L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala L-Ala-D-αGln┌(L)-A_2pm-(L)-D-Ala (D)	40	4	5
L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala L-Ala-(D)-αGln┌(L)-A_2pm-(L)-D-Ala (D)			
Ac-L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala (D) Ac L-Ala-D-αGln┌(L)-A_2pm-(L)-D-Ala	43	1.5	6
(D) *Ac_2-L-Lys-D-Ala	35	4	7

[a] *Ala = [^{14}C]Ala; *Ac = [^{14}C]acetyl. All compounds migrated toward the anode. D-Ala which is also a product of all the reactions was neutral and migrated about 5 cm toward the cathode. Before electrophoresis, the samples (15 μl of 0.5 M K_2HPO_4) were diluted with water (40 μl) and then spotted as bands, 4 cm width, on Whatman 3MM paper.

acceptor, in 0.03 M Tris-HCl buffer (pH 7.5) and 3×10^{-3} M $MgCl_2$ (carboxypeptidase assay). In previous studies (Leyh-Bouille *et al.*, 1972; Pollock *et al.*, 1972; Perkins *et al.*, 1973; Ghuysen *et al.*, 1973) one unit had been defined in terms of nanomoles hydrolyzed per hour.

Peptide Substrates. The following peptides were used: (1) the nonradioactive tetrapeptide L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine (Ghuysen *et al.*, 1973); (2) the radioactive pentapeptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-[^{14}C]alanyl-D-[^{14}C]alanine (specific activity 9 Ci/mol) [Barnett, 1973; Nguyen-Distèche *et al.*, 1974]; (3) the radioactive diacetylated tripeptide N^{α},N^{ϵ}-[^{14}C]diacetyl-L-lysyl-D-alanyl-D-alanine (specific activity 4.5 Ci/mol) [Perkins *et al.*, 1973]; (4) the radioactive diacetylated pentapeptide

Ac-L-Ala-D-Glu┌(L)-A_2pm-(L)-D-[^{14}C]Ala-D-[^{14}C]Ala
Ac-(D) —┘

(specific activity 4.5 Ci/mol). The radioactive pentapeptide was N-acetylated under the conditions described for the acetylation of the tripeptide N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (Nieto and Perkins, 1971) and was filtered on Sephadex G-25 before utilization.

Enzymic Assays. All the incubations were carried out at 37°, in 0.5 M K_2HPO_4; *i.e.*, under conditions which favored the rates of transfer reaction (Ghuysen *et al.*, 1973). The final volumes of the reaction mixtures were 15 μl. The reaction products were separated from each other by electrophoresis on Whatman 3MM paper at pH 6.5 (collidine–acetic acid–water, 9:2.65:100, v/v) by using a Gilson high-voltage (10,000 V) electrophorator (60 V/cm). The migration of the products are given in Table I. The radioactive compounds were located on the strips and estimated as described by Pollock *et al.* (1972). The results were expressed in micromoles of substrate converted to products minute^{-1} enzyme unit^{-1}.

Reaction Models. The action of the R39 enzyme in transfer and hydrolysis reactions was studied on the radioactive free pentapeptide, the radioactive diacetyl pentapeptide, and the radioactive diacetyl tripeptide in the presence and in the absence of Glu-amidated tetrapeptide. The various reactions catalyzed are shown in Table II.

Symbols. [Peptide] = peptide concentration. For hydrolysis of peptide donor in the absence of amino group acceptor (carboxypeptidase assay), the symbols $V_{Hy}(H_2O)$ (rate of hydrolysis), $V_{Hy}(H_2O)$max, and $K_m(H_2O)$ (as derived from the double reciprocal plots $1/V_{Hy}(H_2O)$ *vs.* 1/[peptide]) were

TABLE II: Reaction Models.[a]

Reaction 1: Hydrolysis of the Radioactive Pentapeptide

L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala-D-*Ala + H_2O → D-*Ala + L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala

Reaction 2: Hydrolysis of the Radioactive Diacetyl Pentapeptide

Ac-L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala-D-*Ala + H_2O → D-*Ala + Ac-L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala

(D) Ac (D) Ac

Reaction 3: Hydrolysis of the Radioactive Diacetyl Tripeptide

Ac_2*-L-Lys-D-Ala-D-Ala + H_2O → D-Ala + Ac_2*-L-Lys-D-Ala

Reaction 4: Homogeneous Transfer between Two Radioactive Pentapeptides

L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala-D-*Ala + L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala-D-*Ala

L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala-D-*Ala

→ D-*Ala + (D)

L-Ala-D-Glu (L)-A_2pm-(L)-D-*Ala

Reaction 5: Hybrid Transfer between Radioactive Pentapeptide and Glu-Amidated Tetrapeptide

L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala-D-*Ala + L-Ala-D-αGln┌(L)-A_2pm-(L)-D-Ala

L-Ala-D-αGln┌(L)-A_2pm-(L)-D-Ala

→ D-*Ala + (D)

L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala

Reaction 6: Hybrid Transfer between Radioactive Diacetyl Pentapeptide and Glu-Amidated Tetrapeptide

Ac-L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala-D-*Ala + L-Ala-D-αGln┌(L)-A_2pm-(L)-D-Ala

(D) Ac

L-Ala-D-αGln┌(L)-A_2pm-(L)-D-Ala

(D)

→ D-*Ala + Ac-L-Ala-D-Glu┌(L)-A_2pm-(L)-D-*Ala

(D) Ac

Reaction 7: Hybrid Transfer between Radioactive Diacetyl Tripeptide and Glu-Amidated Tetrapeptide

*Ac_2-L-Lys-D-Ala-D-Ala + L-Ala-D-αGln┌(L)-A_2pm-(L)-D-Ala

L-Ala-(D)-αGln┌(L)-A_2pm-(L)-D-Ala

→ D-Ala + (D)

*Ac_2-L-Lys-D-Ala

[a] *Ala = [^{14}C]Ala; *Ac = [^{14}C]acetyl. All the reactions were carried out in 0.5 M K_2HPO_4. It is not known whether the homogeneous dimer which originated through dimerization of the radioactive pentapeptide (reaction 4) had two or three radioactive D-alanine residues since the C-terminal D-[^{14}C]alanine in the peptide might be removed in a hydrolysis reaction. The yield of conversion was calculated by multiplying the percentage of radioactivity found in the isolated peptide dimer by a factor of 1.5.

used. For hydrolysis of peptide donor occurring concomitantly with a transfer reaction involving the same peptide donor and an amino group acceptor, the symbols V_{Hy} (rate of hydrolysis), V_T (rate of transfer), $V_p = V_{Hy} + V_T$ (rate of total reaction or of release of D-alanine), $V_T$max and K_mT (as derived from the double reciprocal plots $1/V_T$ *vs.* 1/[peptide]) were used.

Experimental Section

Carboxypeptidase Activity of the R39 Enzyme. Hydrolysis of Peptide Donors in the Absence of Amino Acceptor. The $K_m(H_2O)$ and $V_{Hy}(H_2O)$max values (see Symbols in Materials and Methods) for reactions 1, 2, and 3 (Table II) were determined in 0.5 M K_2HPO_4 from initial velocity measurements and are given in Table III. The free pentapeptide was the most sensitive substrate. When compared to it, the diacetyl pentapeptide had an unaltered $K_m(H_2O)$ value but an eightfold decreased $V_{Hy}(H_2O)$max value. Conversely, the diacetyl tripeptide had a $V_{Hy}(H_2O)$max value of the same order of magnitude but a 250-fold increased $K_m(H_2O)$ value.

Concomitant Hydrolysis and Transfer Reactions with Radioactive Pentapeptide L-*Alanyl*-γ-D-*glutamyl*-(L)-*meso-diaminopimelyl*-(L)-D-[^{14}*C*]*alanyl*-D-[^{14}*C*]*alanine.* When increasing concentrations of pentapeptide were exposed to the R39 enzyme, the nature of the reaction products in fact depended upon the concentration of the pentapeptide in the mixture. At concentrations lower than 0.2 mM, *i.e.*, lower than 20 times the $K_m(H_2O)$ value, only hydrolysis to tetrapeptide and D-alanine occurred (reaction 1, Table II). At concentrations ranging from 0.2 to 2 mM (Table IV, A), *i.e.*, between 20 and 200 times the

TABLE III: $K_m(H_2O)$ and V_{Hy} (H_2O)max Values: Hydrolysis of Peptide Donor in the Absence of Amino Group Acceptor.

Reaction No. (Table II)	Plots	$K_m(H_2O)$	V_{Hy}-(H_2O)max[a]
1	$\frac{1}{V_{Hy}}$ *vs.* $\frac{1}{[\text{pentapeptide}]}$	10 μM	1.2
2	$\frac{1}{V_{Hy}}$ *vs.* $\frac{1}{[\text{Ac}_2\text{ pentapeptide}]}$	10 μM	0.15
3	$\frac{1}{V_{Hy}}$ *vs.* $\frac{1}{[\text{Ac}_2\text{ tripeptide}]}$	2.5 mM	2.0

[a] $V_{Hy}(H_2O)$max values were expressed in μmoles of peptide hydrolyzed per min per enzyme unit.

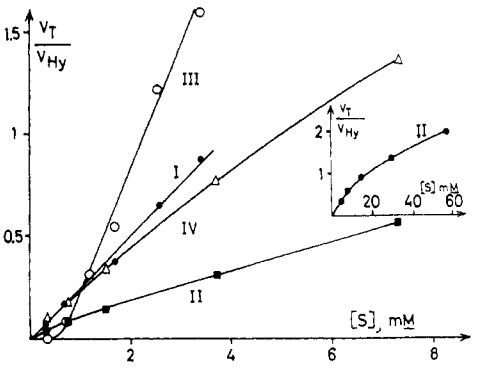

FIGURE 1: Plots of V_T/V_{Hy} *vs.* [peptide]: (I) (reactions 1 and 4, Table II) V_T(homo)/V_{Hy} *vs.* [pentapeptide] (Table IV, A); (II) (reactions 1 and 5, Table II) V_T(hybr)/V_{Hy} *vs.* [Glu-amidated tetrapeptide] under conditions of saturation of the donor site of the enzyme by 0.3 mM pentapeptide (Table V, A); (III) (reactions 1 and 4, Table II) V_T(homo)/V_{Hy} *vs.* [pentapeptide] in the presence of 3.7 mM Glu-amidated tetrapeptide (Table IV, B); (IV) (reactions 2 and 6, Table II) V_T(hybr)/V_{Hy} *vs.* [Glu-amidated tetrapeptide] under conditions of saturation of the donor site of the enzyme by 0.3 mM diacetyl pentapeptide (Table V, B).

$K_m(H_2O)$ value, hydrolysis and dimerization through the formation of a C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid linkage (reaction 4, Table II) occurred concomitantly. Under these conditions of concentration, the pentapeptide was utilized by the R39 enzyme both as a donor and an acceptor (Table IV, A). The increase of the rate of transfer reaction [V_T(homo)] and the decrease of the rate of hydrolysis caused by increased concentrations of pentapeptide were commensurate so that the rate of the total reaction V_p (*i.e.*, the release of D-alanine which is the common product of reactions 1 and 4) remained virtually constant and equal to the $V_{Hy}(H_2O)$max value. At concentrations higher than 2 mM, *i.e.*, higher than 200 times the $K_m(H_2O)$ value, the increase in the rate of dimerization exceeded the decrease in the rate of hydrolysis, resulting in an increased total enzyme activity [$V_p > V_{Hy}$-(H_2O)max] (Table IV, A).

The question of the "activation" of the R39 enzyme by very high concentrations of pentapeptide is reexamined in a later section of this paper. One should note, however, that from the double reciprocal plot $1/V_T$(homo) *vs.* 1/[pentapeptide] an apparent K_mT value of about 10 mM and a $V_T$max value of about 3 μmol of pentapeptide converted to dimer min^{-1} enzyme $unit^{-1}$ could be calculated. Thus, at infinite concentration of pentapeptide, the rate of transfer reaction would be higher than the $V_{Hy}(H_2O)$max value. As shown in Figure 1 (line I), the ratio V_T(homo)V_{Hy} was directly proportional to the pentapeptide concentration.

Concomitant Hydrolysis and Hybrid Transfer Reactions with Radioactive Pentapeptide L-*Alanyl*-γ-D-*glutamyl*-(L)-*meso-diaminopimelyl*-(L)-D-[^{14}C]*alanyl*-D-[^{14}C]*alanine and Glu-Amidated Tetrapeptide* L-*Alanyl*-D-*isoglutaminyl*-(L)-*meso-diaminopimelyl*-(L)-D-*alanine*. The Glu-amidated tetrapeptide lacks the C-terminal D-alanyl-D-alanine donor group but has the proper amino group (located on the D carbon of *meso*-diaminopimelic acid in α position to a free carboxyl) needed for transpeptidation. In a first series of experiments, the Glu-amidated

TABLE IV: Effect of Pentapeptide Concentration on: (A) Concomitant Hydrolysis and Dimerization (reactions 1 and 4, Table II) and on Total Reaction in the Absence of Glu-Amidated Tetrapeptide, and (B) Concomitant Hydrolysis, Dimerization, and Hybrid Transpeptidation (reactions 1, 4, and 5; Table II) and on Total Reaction in the Presence of 3.7 mM Glu-Amidated Tetrapeptide.[a]

Radioactive pentapeptide mM	(A) Glu-amidated tetrapeptide = 0			(B) Glu-amidated tetrapeptide = 3.7 mM				
	V_{Hy}	V_T(homo)	V_p	V_{Hy}	V_T(homo)	V_T(hybr)	V_T(total)	V_p
0.17	1.25	0.01	1.26	0.78	0	0.28	0.28	1.06
0.34	1.12	0.08	1.20	1.04	0	0.37	0.37	1.41
0.68	1.02	0.16	1.18	1.05	0.09	0.47	0.56	1.61
1.20	0.96	0.28	1.24	0.91	0.28	0.50	0.78	1.69
1.70	0.92	0.34	1.26	0.80	0.43	0.53	0.96	1.76
2.56	0.86	0.56	1.42	0.60	0.73	0.56	1.29	1.89
3.40	0.81	0.71	1.52	0.50	0.80	0.58	1.38	1.88

[a] V_{Hy} = rate of hydrolysis of pentapeptide; V_T(homo) = rate of dimerization between two radioactive pentapeptides (reaction 4, Table II); V_T(hybr) = rate of hybrid transfer between radioactive pentapeptide and Glu-amidated tetrapeptide (reaction 5, Table II); V_T(total) = V_T(homo) + V_T(hybr); V_p = V_{Hy} + V_T(homo) + V_T(hybr). The rates of reaction were expressed in μmoles of pentapeptide converted into product min^{-1} enzyme $unit^{-1}$. The pentapeptide at the indicated concentrations was incubated for 30 min at 37° with 5×10^{-5} enzyme unit in 15 μl of 0.5 M K_2HPO_4, in the absence (experiment A) or in the presence of 3.7 mM Glu-amidated tetrapeptide (experiment B). In the above experiments, 5–40% of the pentapeptide was utilized in the total reaction (except for the data obtained at the lowest, 0.17 mM, concentration of pentapeptide where 65–75% of the substrate was utilized).

tetrapeptide was used to study the rate of hybrid transfer reaction 5 [V_T(hybr), Table II] under conditions of pentapeptide concentrations such that the homogeneous transfer reaction 4 (Table II) did not occur at all or occurred at an extremely low rate.

Fixed concentrations of pentapeptide (either 2, 20, or 0.3 mM) were exposed to the R39 enzyme in the presence of increasing concentrations of Glu-amidated tetrapeptide (Table V, A). At the 2 μM concentration of pentapeptide, *i.e.*, 0.2 times the $K_m(H_2O)$ value, the hybrid transfer reaction was below the level of detection (or did not occur at all) even in the presence of very high concentrations of Glu-amidated tetrapeptide, but hydrolysis of the pentapeptide (reaction 1; Table II) was progressively inhibited and eventually completely blocked.

At the 20 μM concentration of pentapeptide, *i.e.*, 2 times the $K_m(H_2O)$ value, hybrid transpeptidation occurred. The increase of the rate of transfer reaction caused by increased concentrations of Glu-amidated tetrapeptide was lower than could be accounted for by the decrease of the rate of hydrolysis, resulting in a progressive decrease of the total activity V_p of the enzyme.

At the 0.3 mM concentration of pentapeptide, *i.e.*, 30 times the $K_m(H_2O)$ value, and with concentrations of Glu-amidated tetrapeptide not higher than 30 mM, the increase of the rate of hybrid transpeptidation and the decrease of the rate of hydrolysis were commensurate. The rate of total reaction V_p remained constant and equal to the $V_{Hy}(H_2O)$max value. Hence, providing that its donor site was fully saturated by the pentapeptide donor, the R39 enzyme was able to channel into the hybrid transfer pathway that part of the activity which was diverted from the hydrolysis pathway because of the presence of the Glu-amidated tetrapeptide acceptor.

Attempts to enhance the total enzyme activity V_p beyond the $V_{Hy}(H_2O)$max value by increasing the concentration of the Glu-amidated tetrapeptide acceptor (as it occurred when the R39 enzyme was exposed to very high concentrations of pentapeptide alone; see previous section and Table IV, A) failed because the hybrid transfer reaction itself was subject to inhibition by excess of Glu-amidated tetrapeptide (Table V, A). Optimum concentrations of Glu-amidated tetrapeptide acceptor conferring maximum hybrid transpeptidation were 1.5 mM when 20 μM pentapeptide donor was present in the reaction mixture and 30 mM for a 0.3 mM pentapeptide concentration (Table V, A).

Under conditions of saturation of the donor site of the enzyme by 0.3 mM pentapeptide, the plots $V_T(hybr)/V_{Hy}$ *vs.* [Glu-amidated tetrapeptide] gave rise to a curve exhibiting an upward convexity (Figure 1, curve II). At a given concentration of Glu-amidated tetrapeptide, increasing concentrations of pentapeptide from 20 μM to 0.3 mM caused a slight increase of the ratios $V_T(hybr)/V_{Hy}$ (Table V, A).

Concomitant Hydrolysis, Hybrid Transfer, and Homogeneous Transfer Reactions with Radioactive Pentapeptide L-*Alanyl*-γ-D-*glutamyl*-(L)-*meso-diaminopimelyl*-(L)-D-[^{14}C]*alanyl*-D-[^{14}C]-*alanine and Glu-Amidated Tetrapeptide* L-*Alanyl*-D-*isoglutaminyl*-(L)-*meso-diaminopimelyl*-(L)-D-*alanine*. In a second series of experiments, a fixed concentration of Glu-amidated tetrapeptide (3.7 mM) was exposed to the R39 enzyme in the presence of increasing concentrations of radioactive pentapeptide (from 0.2 to 3.4 mM) (Table IV, B). The 3.7 mM concentration of Glu-amidated tetrapeptide was chosen because it did not inhibit the hybrid transfer reaction even at the lowest (0.17 mM) concentration of pentapeptide used. Under these conditions, reactions 1 (hydrolysis), 4 (homogeneous transfer), and 5 (hybrid transfer) (see Table II) occurred simultaneously. By comparing the results of Table IV, B with those of Table IV, A obtained when the R39 enzyme was exposed to the same concentration of pentapeptide in the absence of Glu-amidated tetrapeptide, the following observations were made.

At concentrations of pentapeptide lower than 1 mM, *i.e.*, lower than 100 times the $K_m(H_2O)$ value, the Glu-amidated tetrapeptide caused a decrease of the rate of homogeneous transfer reaction. Obviously, this observation reflected a competition for the same acceptor site on the enzyme between the Glu-amidated tetrapeptide and the amount of pentapeptide in excess of that needed to saturate the donor site.

At concentrations of pentapeptide higher than 1 mM, *i.e.*, higher than 100 times the $K_m(H_2O)$ value, (1) the rate of homogeneous transpeptidation was at least as great as in the absence of Glu-amidated tetrapeptide; (2) there was a small but progressive increase in the hybrid transfer reaction. The plots $1/V_T(hybr)$ *vs.* 1/[pentapeptide] (in the presence of 3.7 mM Glu-amidated tetrapeptide) gave rise to a straight line with a shallow slope, a 20-fold increase in the pentapeptide concentration (from 0.17 to 3.4 mM) resulting in a twofold increase in the rate of hybrid transpeptidation (0.28–0.58 μmol min^{-1} enzyme $unit^{-1}$); (3) the increase of the rate of the overall (homogeneous + hybrid) transpeptidation was much greater than could be accounted for by the decrease of the rate of hydrolysis. As a consequence, the rate of the total reaction V_p [V_{Hy} + V_T(homo) + V_T(hybr)] eventually reached a value of 1.9 μmol of pentapeptide converted into products min^{-1} enzyme $unit^{-1}$. This value was at least 50% higher than the $V_{Hy}(H_2O)$max value for the pentapeptide. Hence, as already suggested above, concentrations of free pentapeptide higher than 1 mM [*i.e.*, 100 times the $K_m(H_2O)$ value] enhanced the total activity of the R39 enzyme, in terms of D-alanine liberated, beyond the maximal hydrolytic activity performed in the absence of peptide acceptor; (4) the plots $V_T(homo)/V_{Hy}$ *vs.* [pentapeptide] obtained in the presence of 3.7 mM Glu-amidated tetrapeptide (Figure 1, line III), when compared to the same plots obtained in the absence of Glu-amidated tetrapeptide (Figure 1, line I), showed that at concentrations of pentapeptide higher than 1 mM the presence of the Glu-amidated tetrapeptide acceptor in the reaction mixture considerably favored the dimerization of the pentapeptide (reaction 4, Table II) *vs.* its hydrolysis. This last observation well illustrates the complexity of the enzyme system.

Concomitant Hydrolysis and Transfer Reactions with Radioactive Diacetylated Pentapeptide Diacetyl-L-*alanyl*-γ-D-*glutamyl*-(L)-*meso-diaminopimelyl*-(L)-D-[^{14}C]*alanyl*-D-[^{14}C]*alanine and Glu-Amidated Tetrapeptide* L-*Alanyl*-D-*isoglutaminyl*-(L)-*meso-diaminopimelyl*-(L)-D-*alanine*. The experiments described in this paragraph and in the ensuing one were devised in order to establish whether the $V_{Hy}(H_2O)$max and $K_m(H_2O)$ values for peptide donor (carboxypeptidase activity) were important parameters for determining the rates of transfer and total reactions. Since the diacetyl pentapeptide had the same $K_m(H_2O)$ value as the free pentapeptide (10 μM) but a much lower $V_{Hy}(H_2O)$max value (0.15 μmol min^{-1} enzyme $unit^{-1}$ instead of 1.2) (Table III), the influence of this latter parameter on transfer reactions was studied by replacing the free pentapeptide by the diacetyl pentapeptide as donor in the reaction mixture.

A fixed 0.3 mM concentration of radioactive diacetyl pentapeptide was exposed to the R39 enzyme in the presence of increasing concentrations of Glu-amidated tetrapeptide. Hydrolysis of the diacetyl pentapeptide (reaction 2; Table II) and hybrid transfer reaction 6 (Table II) occurred concomi-

TABLE V: Effect of Glu-Amidated Tetrapeptide Concentration on: (A) Concomitant Hydrolysis, Dimerization, and Hybrid Transpeptidation (reactions 1, 4, and 5; Table II) and on Total Reaction in the Presence of a Fixed Concentration of Radioactive Pentapeptide (2 μM, 20 μM, and 0.3 mM, respectively); (B) Concomitant Hydrolysis and Hybrid Transpeptidation (reactions 2 and 6; Table II) and on Total Reaction in the Presence of a Fixed Concentration of Radioactive Diacetyl Pentapeptide (0.3 mM); (C) Concomitant Hydrolysis and Hybrid Transpeptidation (reactions 3 and 7; Table II) and on Total Reaction in the Presence of a Fixed Concentration of Radioactive Diacetyl Tripeptide (0.27 and 1.4 mM, respectively).[a]

Non-radioactive Glu-Amidated Tetrapeptide (mM)	(A) Radioactive Pentapeptide										(B) Radioactive Diacetyl pentapeptide			(C) Radioactive Diacetyl Tripeptide					
	2 μM	20 μM				0.3 mM					0.3 mM			0.27 mM			1.4 mM		
	V_{Hy}/V_p	V_{Hy}	V_T(hybr)	V_p	V_T(hybr)/V_{Hy}	V_{Hy}	V_T-(homo)	V_T-(hybr)	V_p	V_T-(hybr)/V_{Hy}	V_{Hy}	V_T(hybr)	V_p	V_{Hy}	V_T(hybr)	V_p	V_{Hy}	V_T(hybr)	V_p
0	0.24	0.30	0	0.30		1.20	0.08	0	1.28		0.143	0	0.143	0.18	0	0.18	0.67	0	0.67
0.35						1.15	trace	0.05	1.20		0.137	0.014	0.151	0.11	0.033	0.14	0.45	0.10	0.55
0.70	0.14					1.12	trace	0.09	1.22		0.118	0.021	0.139	0.073	0.037	0.11			
0.80														0.060	0.030	0.09	0.26	0.15	0.41
1.50	0.08	0.22	0.02	0.24	0.10	1.12	trace	0.16	1.28	0.14	0.108	0.037	0.145						
1.60														0.02	0.030	0.05	0.18	0.20	0.38
2.40														0.011	0.028	0.039	0.09	0.20	0.29
3.20														0	0.009	0.009			
3.70	0.025	0.15	0.04	0.20	0.27	1.02	0	0.33	1.35	0.32	0.075	0.058	0.133	0	0	0	0.028	0.10	0.128
7.30	0	0.12	0.057	0.18	0.48	0.81	0	0.46	1.28	0.57	0.053	0.072	0.125						
14.60	0	0.08	0.060	0.14	0.75	0.68	0	0.63	1.32	0.93									
29.20						0.50	0	0.73	1.23	1.46									
58.40		0	0	0		0.34	0	0.67	1.01	3									

[a] V_{Hy} = rate of hydrolysis of pentapeptide (reaction 1, Table II), diacetyl pentapeptide (reaction 2, Table II) or diacetyl tripeptide (reaction 3, Table II). V_T(homo) = rate of dimerization between two radioactive pentapeptides (reaction 4, Table II). Dimerization did not occur at 2 and 20 μM pentapeptide. V_T(hybr) = rate of hybrid transpeptidation between Glu-amidated tetrapeptide and either radioactive pentapeptide (reaction 5, Table II), radioactive diacetyl pentapeptide (reaction 6, Table II), or radioactive diacetyl tripeptide (reaction 7, Table II). $V_p = V_{Hy} + V_T$. The rates of reaction were expressed in μmoles of radioactive peptide donor (pentapeptide, diacetyl pentapeptide, or diacetyl tripeptide, respectively) converted into products min^{-1} enzyme $unit^{-1}$. All incubations were carried out at 37° in 15 μl of 0.5 M K_2HPO_4. In experiments A, 5×10^{-5} enzyme unit was used and the incubation times were 2 min (for 2 μM pentapeptide), 20 min (for 20 μM pentapeptide), and 60 min (for 0.3 mM pentapeptide). In experiments B and C, 6×10^{-4} enzyme unit was used and the incubation times were 30 min.

tantly (Table V, B). The 0.3 mM concentration of diacetyl pentapeptide, *i.e.*, 30 times the $K_m(H_2O)$ value, was chosen in order to saturate the enzyme at its donor site. Under these conditions, and as expected from the experiments described above, the rate of the total reaction V_p (*i.e.*, reaction 2 + reaction 6) remained virtually constant and equal to the $V_{Hy}(H_2O)$max value for the diacetyl pentapeptide, *i.e.*, was much lower than the $V_{Hy}(H_2O)$max value for the free pentapeptide (Table III). The plots V_T(hybrid)/V_{Hy} *vs.* [Glu-amidated tetrapeptide] at saturation of the donor site of the enzyme by 0.3 mM diacetyl pentapeptide, gave rise to a curve exhibiting an upward convexity (Figure 1, curve IV). By comparing these plots with those obtained at saturation of the donor site by the free pentapeptide (Figure 1, line II), it appeared that the acetylation of this peptide donor, although causing a drastic decrease of all the enzyme activities, resulted nevertheless in increased V_T/V_{Hy} values.

Concomitant Hydrolysis and Transfer Reactions with Radioactive Acetylated Tripeptide [^{14}C]*Diacetyl-*L*-lysyl-*D*-alanyl-*D*-alanine and Glu-Amidated Tetrapeptide* L*-Alanyl-*D*-isoglutaminyl-*(L)*-meso-diaminopimelyl-*(L)*-*D*-alanine.* The diacetyl tripeptide had a $V_{Hy}(H_2O)$max value which was of the same order of magnitude as that of the free pentapeptide (1.7 instead of 1.2) but a much higher $K_m(H_2O)$ value (2.5 mM instead of 10 μM; Table III). Hence the influence of this latter parameter on transfer reactions was in turn studied by replacing the free pentapeptide by the diacetyl tripeptide as donor in the reaction mixture.

In a first series of experiments, fixed concentrations of radioactive diacetyl tripeptide (0.27 and 1.4 mM, respectively; *i.e.*, 0.11 and 0.56 times the $K_m(H_2O)$ value) were incubated with the R39 enzyme in the presence of increasing concentrations of Glu-amidated tetrapeptide (Table V, C). Hydrolysis of the diacetyl tripeptide (reaction 3, Table II) and hybrid transfer reaction 7 (Table II) occurred concomitantly.

Despite the fact that the $V_{max}(H_2O)$ value for the diacetyl tripeptide was slightly higher than that of the free pentapeptide, the rates of hydrolysis, transpeptidation, and total reaction observed with both 0.27 and 1.4 mM diacetyl tripeptide were considerably smaller than those obtained when 0.3 mM free pentapeptide was exposed under the same conditions to the R39 enzyme in the presence of Glu-amidated tetrapeptide (Table V, C, left-hand column and Table V, A, third column on the right). Obviously, these differences of rate were related to the differences between the $K_m(H_2O)$ values of the two peptides (Table III) which ensured that the donor site on the enzyme was largely saturated by 0.3 mM free pentapeptide whereas it was far from being saturated by 0.3 and 1.4 mM diacetyl tripeptide. The expected consequence of the nonsaturation of the R39 enzyme by the diacetyl tripeptide was that the increase of transpeptidation caused by increasing concentions of Glu-amidated tetrapeptide was lower than the decrease of the rate of hydrolysis, resulting in a progressive decrease of the total enzyme activity.

Like the hybrid transfer reaction 5 (Table II), the hybrid transfer reaction 7 (Table II) was inhibited by excess Glu-amidated tetrapeptide. Whereas the hybrid transfer reaction 5 carried out at saturation of the donor site of the enzyme by 0.3 mM free pentapeptide was inhibited by concentrations of Glu-amidated tetrapeptide higher than 30 mM (Table V, A), the hybrid transfer reaction 7 carried out under conditions of nonsaturation of the enzyme by 0.27 mM diacetyl tripeptide started to be inhibited by a concentration of Glu-amidated tetrapeptide as low as 1 mM (Table V, C). When the concentration of diacetyl tripeptide was increased to 1.4 mM, inhibition of the transfer reaction still started to occur in a 2.4 mM Glu-amidated tetrapeptide (Table V, C). Hence, the further the donor site on the enzyme was from being saturated by the peptide donor, the more strongly was the transfer reaction inhibited by low concentrations of Glu-amidated tetrapeptide acceptor.

TABLE VI: Effect of Diacetyl Tripeptide Concentration on Concomitant Hydrolysis and Transpeptidation (Reactions 3 and 7, Table II) and on Total Reaction in the Presence of 0.8 mM Glu-Amidated Tetrapeptide.[a]

Radioactive Ac_2-L-Lys-D-Ala-D-Ala (mM)	Nonradioactive Glu-Amidated Tetrapeptide, 0.8 mM			
	V_{Hy}	V_T	V_p	V_T/V_{Hy}
0.27	0.06	0.03	0.09	0.50
0.55	0.113	0.055	0.168	0.48
0.80	0.157	0.08	0.237	0.51
1.07	0.213	0.093	0.306	0.43
1.60	0.27	0.11	0.38	0.41

[a] V_{Hy} = rate of hydrolysis of diacetyl tripeptide. V_T = rate of transpeptidation (reaction 7, Table II). $V_p = V_{Hy} + V_T$. The rates of reaction were expressed in μmoles of diacetyl tripeptide converted into products min^{-1} enzyme $unit^{-1}$. The diacetyl tripeptide at the indicated concentrations was incubated for 30 min at 37° with 6×10^{-4} enzyme unit, in 15 μl of 0.5 M K_2HPO_4 and in the presence of 0.8 mM Glu-amidated tetrapeptide. In this experiment, 29–40% of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala was utilized in the total reaction.

The high $K_m(H_2O)$ value for the diacetyl tripeptide made it possible to study the effect of donor concentration under conditions of nonsaturation of the enzyme, on concomitant hydrolysis and transpeptidation. Increasing concentrations of diacetyl tripeptide (from 0.27 to 1.6 mM, *i.e.*, from 0.1 to 0.6 times the $K_m(H_2O)$ value) and 0.8 mM Glu-amidated tetrapeptide were incubated together with the R39 enzyme (Table VI). The 0.8 mM concentration of Glu-amidated tetrapeptide was chosen because it did not inhibit the transfer reaction 7 (Table II) even at the lowest, 0.27 mM, concentration of diacetyl tripeptide used, under which conditions the V_T(hybr) was still very close to its maximal value (Table V, C). The double reciprocal plots $1/V_{Hy}$, $1/V_T$, and $1/V_p$ *vs.* 1/[diacetyl tripeptide], in the presence of 0.8 mM Glu-amidated tetrapeptide, gave rise to straight lines (Figure 2) from which apparent K_m values ranging between 3 and 10 mM were calculated. The double reciprocal plots $1/V_p$ *vs.* 1/[diacetyl tripeptide] which expressed the total activity of the enzyme in the presence of Glu-amidated tetrapeptide, and the double reciprocal plots $1/V_{Hy}(H_2O)$ *vs.* 1/[diacetyl tripeptide] (dotted line in Figure 2) which expressed its hydrolytic activity in the absence of Glu-amidated tetrapeptide, intersected at a point on or very close to the ordinate. Hence, as previously observed with the other donor–acceptor systems, the total activity of the enzyme in concomitant hydrolysis and transfer reactions (V_p) was equivalent to its hydrolytic activity in carboxypeptidase assay [$V_{Hy}(H_2O)$], only under conditions of saturation of the donor site by the diacetyl tripeptide ($V_p = V_{Hy}(H_2O)$max]. (For a concentration of diacetyl tripeptide 20 times the $K_m(H_2O)$ value, the ratio 1/[diacetyl tripeptide) would be equal

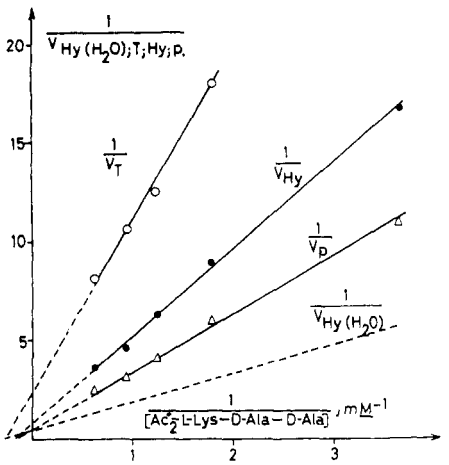

FIGURE 2: Double reciprocal plots $1/V_T$, $1/V_{Hy}$, *and* $1/V_p$ *vs.* 1/[diacetyl tripeptide] in concomitant hydrolysis and transfer reactions catalyzed by the R39 enzyme in the presence of 0.8 mM Glu-amidated tetrapeptide. Rates of reaction are expressed in μmoles min^{-1} enzyme $unit.^{-1}$ For model reactions, see reactions 3 and 7 in Table II. For data, see Table VI. Bottom line represents the double reciprocal plot $1/V_{Hy}(H_2O)$ *vs.* 1/[diacetyl tripeptide] in the absence of Glu-amidated tetrapeptide (carboxypeptidase assay).

to 0.015, *i.e.*, practically on the ordinate axis of Figure 2). Under these conditions of nonsaturation of the donor site of the enzyme, increasing concentration of diacetyl tripeptide donor caused a progressive increase of the total activity V_p and a small decrease of the V_T/V_{Hy} values (Table VI). The increased V_p values, however, remained lower than the corresponding increased $V_{Hy}(H_2O)$ values (Figure 2).

Discussion

For the first time, the exocellular DD-carboxypeptidase–transpeptidase R39 has been shown to be able to catalyze from peptide monomers, the synthesis of peptide dimers that are identical with or very similar to those which occur in the completed wall peptidoglycans of bacteria. The nonamidated peptide dimer formed according to reaction 4 (Table II) and the monoamidated peptide dimer formed according to reaction 5 (Table II) are exactly those formed by transpeptidation *in vivo* in many Gram negative bacteria and in many *Bacilli*, respectively (Ghuysen, 1968; Schleifer and Kandler, 1972). Moreover this latter monoamidated peptide dimer is the one occurring in the *Streptomyces* strain R39 itself, except, however, that the two glutamic acid residues are amide substituted in the natural peptidoglycan (Ghuysen *et al.*, 1973). This close analogy reinforces the concept that the exocellular enzyme may well be an exo form of the physiological membrane-bound enzyme.

The effects of concentrations of peptide donors and peptide acceptors on the functioning of the R39 enzyme have been studied with four different systems (free pentapeptide + free pentapeptide), (free pentapeptide + Glu-amidated tetrapeptide), (diacetyl pentapeptide + Glu-amidated tetrapeptide) and (diacetyl tripeptide + Glu-amidated tetrapeptide). All these studies support the following conclusions. (1) At concentrations of peptide donor equivalent to at least 20 times the $K_m(H_2O)$ value, *i.e.*, under conditions where the donor site of the enzyme is fully saturated by the peptide donor, the rate of the total reaction ($V_p = V_T + V_{Hy}$) for concomitant transfer and hydrolysis is equal to the maximal rate of hydrolysis of the peptide donor in the absence of peptide acceptor [$V_{Hy}(H_2O)$max]. The enzyme has the same turnover number (expressed as D-alanine released) whether it works as a simple hydrolase or is engaged in bimolecular transfer reactions occurring concomitantly with the hydrolysis of the peptide donor. Increasing concentrations of peptide acceptor (within a certain limit, *vide infra*) cause a progressive decrease of the rate of hydrolysis and an equivalent increase of the rate of transfer, resulting in an increase of the V_T/V_{Hy} values. The proportion of the enzyme activity which is diverted from the hydrolysis pathway because of the presence of the peptide acceptor, is entirely channeled into the transfer pathway. The peptide acceptor thus behaves as an inhibitor of the hydrolysis but not of the total activity which remains constant.

(2) Under conditions of nonsaturation of the donor site of the enzyme by the peptide donor, the proportion of the enzyme activity which is diverted from the hydrolysis pathway by the peptide acceptor is not entirely channeled into the transfer pathway. Some of it is lost and the rate of the total reaction ($V_p = V_T + V_{Hy}$) is lower than the corresponding $V_{Hy}(H_2O)$ value. The peptide acceptor thus behaves as an inhibitor of both hydrolysis and total reaction. At a given concentration of peptide donor, the increase of the rate of transfer reaction caused by increasing concentrations of peptide acceptor (within a certain limit, *vide infra*) is lower than can be accounted for by the decrease of the rate of hydrolysis, resulting in both a progressive increase of the V_T/V_{Hy} values and a progressive decrease of the total activity V_p. At a given concentration of peptide acceptor, increasing concentrations of peptide donor (up to saturation of the enzyme donor site, see case 1 above) cause an increase of both the rate of hydrolysis and the rate of transfer so that the rate of the total reaction V_p progressively approaches the corresponding $V_{Hy}(H_2O)$ value (until $V_p = V_{Hy}(H_2O)$max). The effects of peptide donor concentration on the V_T/V_{Hy} ratios appear to be small.

(3) The Glu-amidated tetrapeptide acceptor inhibits the hydrolysis pathway. At high concentrations, it also inhibits the transfer pathway itself. This property is attributable to the occurrence of an amide group on the α-carboxyl group of D-glutamic acid (Ghuysen *et al.*, 1973). As a result, the plots V_T/V_{Hy} *vs.* [acceptor] do not give rise to straight lines but to curves exhibiting an upward convexity (for a theoretical analysis, see Frère, 1973). The present studies have also shown that the less the enzyme is saturated by the peptide donor, the lower is the concentration of Glu-amidated tetrapeptide at which inhibition of transpeptidation starts to occur. It thus follows that a low concentration of peptide donor combined with a high concentration of peptide acceptor can "freeze" the enzyme to the point where both substrates remain completely unattacked.

(4) The nonamidated pentapeptide can act both as donor and acceptor. Because of the low $K_m(H_2O)$ value (10 μM), saturation of the donor site on the enzyme is achieved by a concentration as low as 0.2 mM and the amount of pentapeptide exceeding this latter concentration is utilized by the enzyme as an acceptor (reaction 4, Table II). As an acceptor, the pentapeptide which has no amide group on the D-glutamic acid residue, does not inhibit the transfer reaction by excess of concentration. Accordingly, the V_T/V_{Hy} values are directly proportional to the pentapeptide concentration. Moreover, the use of this pentapeptide has revealed that high concentrations of an acceptor that does not inhibit the transfer reaction can cause, under conditions of saturation of the donor site of the enzyme by the donor peptide, an increase of the transfer reaction which is higher than the corresponding decrease of the rate of hydrolysis. Consequently, the rate of total reaction V_p

is higher than the $V_{Hy}(H_2O)$max value; the acceptor still behaves as an inhibitor of the hydrolysis, but as an activator of the total reaction (when compared to the maximal rate of hydrolysis of the peptide donor in the absence of acceptor).

(5) The *in vitro* functioning of the soluble R39 DD-carboxypeptidase–transpeptidase, with regard to the absolute and relative rates of the hydrolysis and transfer reactions catalyzed (the V_T, V_{Hy}, and V_T/V_{Hy} values), very much depends upon the concentrations of the peptide donors and acceptors involved in the reaction. The effect of the peptide donor concentration is itself a function of the $K_m(H_2O)$ and $V_{Hy}(H_2O)$-max values for this peptide in carboxypeptidase assay. These values can be drastically modified by seemingly small chemical alterations of the peptide. N-Acetylation of the pentapeptide donor results in a eightfold decrease of the $V_{Hy}(H_3O)$max value. Parallel to this, the total activity V_p is decreased but the ratios V_T/V_{Hy} are increased. N-Acetylation of the wall peptidoglycan nucleotide precursor in *Corynebacterium insidiosium* and *C. sepedonicum* has been described (Perkins, 1968). The effects of the peptide acceptor concentration also depend upon minute chemical alterations such as the substitution of the α-carboxyl group of D-glutamic acid by an amide group. Such a structural feature is encountered in the peptidoglycans of many bacteria including that of the *Streptomyces* R39. It thus follows that mechanisms able to change the acceptor and donor concentrations or to alter some well-defined structural features of these peptides, are important control mechanisms for the regulation of the activity of the exocellular DD-carboxypeptidase–transpeptidase. Similar mechanisms might be involved in the control of the peptide cross-linking system *in vivo*.

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A Study of the Binding of Thiamine Diphosphate and Thiochrome Diphosphate to the Pyruvate Dehydrogenase Multienzyme Complex†

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ABSTRACT: The binding of thiamine diphosphate and thiochrome diphosphate to the pyruvate dehydrogenase enzyme complex from *Escherichia coli* has been studied using equilibrium dialysis and steady-state kinetics. At 4° in 0.01 M potassium phosphate buffer (pH 6.2)–0.5 mM $MgCl_2$, 22.4 mol of thiamine diphosphate are bound per mole of multienzyme complex with a dissociation constant of 12.1 μM. This stoichiometry is in good agreement with the proposed subunit structure of the enzyme (Eley, M. H., Namihira, G., Hamilton, C., Munk, P., and Reed, L. J. (1972), *Arch. Biochem. Biophys. 152*, 655). Both kinetic and equilibrium experiments indicate that thiochrome diphosphate is a competitive inhibitor of thiamine diphosphate, with a dissociation constant of 15 μM. In addition, thiochrome diphosphate binds less strongly to a large number of unidentified other sites. The binding of thiochrome diphosphate to the enzyme complex is unaltered when >70% of the FAD is removed from the complex. The binding and fluorescence properties of thiochrome diphosphate are suitable for its use as a donor molecule in energy-transfer experiments with FAD as the energy acceptor.

The pyruvate dehydrogenase multienzyme complex from *Escherichia coli* catalyzes the oxidative decarboxylation of pyruvate (Koike *et al.*, 1960). This multienzyme complex has

$$\text{pyruvate} + \text{coenzyme A} + NAD^+ \longrightarrow \text{acetyl-coenzyme A} + CO_2 + NADH + H^+ \quad (1)$$

been separated into three component enzymes: pyruvate dehydrogenase, utilizing thiamine diphosphate as a cofactor; dihydrolipoyl transacetylase, containing covalently attached lipoic acid; and dihydrolipoyl dehydrogenase, containing tightly bound FAD (Koike *et al.*, 1963).

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