The Exchange Reaction of Peptides R-D-Alanyl-D-alanine with D-[\(^{14}\)C]Alanine to R-D-Alanyl-D-[\(^{14}\)C]alanine and D-Alanine, Catalysed by the Membranes of \textit{Streptococcus faecalis} ATCC 9790

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Under alkaline conditions, the membrane-bound dd-carboxypeptidase of \textit{Streptococcus faecalis} ATCC 9790 catalyses exchange reactions in which the X-L-R\textsubscript{3}-D-Ala moiety of peptides of the type X-L-R\textsubscript{3}-D-Ala-D-Ala is transferred to simple amino compounds such as D-alanine, glycine and glycyl-glycine. The enzyme system is unable, however, to catalyse complex reactions that would simulate the natural transpeptidation reaction.

Isolated membranes of \textit{Streptococcus faecalis} ATCC 9790 hydrolyse the C-terminal peptide bonds of peptides ending in a L-R\textsubscript{3}-D-Ala-D-Ala sequence where R\textsubscript{3} is an amino acid residue bearing a long side-chain [1]. The standard reaction catalysed by this dd-carboxypeptidase activity is Ac\textsubscript{2}-L-Lys-D-Ala-D-Ala + H\textsubscript{2}O \rightarrow D-Ala + Ac\textsubscript{2}-L-Lys-D-Ala. Maximal activity occurs at pH 6-7. In 50 mM sodium cacodylate buffer pH 6 and at 37°C, the \(K_m\) value is about 4 mM and the \(V\) value is about 20 nmol of tripeptide hydrolysed min\(^{-1}\) (mg membrane protein\(^{-1}\)). During these studies, a corresponding exchange reaction that would yield Ac\textsubscript{2}-L-Lys-D-Ala-D-[\(^{14}\)C]Ala from the system Ac\textsubscript{2}-L-Lys-D-Ala-D-Ala + D-[\(^{14}\)C]alanine could not be detected. Reexamination of the enzymic properties of the isolated membranes has revealed that such a reaction occurs at high pH values. Since an exchange reaction of this type might be the expression of the membrane-bound dd-transpeptidase activity involved in peptide crosslinking during wall peptidoglycan synthesis [2,3], experiments were undertaken in order to characterize the properties of the enzyme activity responsible for this exchange reaction.

\textit{Enzyme, dd-Carboxypeptidase (EC 3.4.12.6).}

\textbf{MATERIALS AND METHODS}

\textbf{Plasma Membranes}

The procedure used previously [1] was modified as follows. Cells (from 15\% of culture), grown in the same medium as used previously, up to near the end of the exponential phase, were chilled by addition of water/ice to the medium, collected by continuous centrifugation with a Westphalia LWA 205 centrifuge (12000 rev./min), washed twice with cold water by centrifugation with a Sorvall RC2B centrifuge at 10000 \(\times\) g for 10 min and resuspended in 300 ml of 5 mM sodium phosphate buffer pH 7.0 containing 1 mM MgCl\(_2\), 30 mg of hen egg-white lysozyme, 1.5 mg of DNase (pancreatic) and 0.75 mg of RNase (pancreatic). The cell suspension was maintained at 37°C for about 1 h after which time lysis was complete. The lysate was centrifuged at 4°C, at 40000 \(\times\) g for 30 min and the pellet was washed twice by centrifugation with the 1 mM MgCl\(_2\), 5 mM sodium phosphate buffer pH 7.0. Intact cells and wall debris were eliminated by centrifugation at 500 \(\times\) g for 10 min and the supernatant fraction was centrifuged at 40000 \(\times\) g for 30 min. The plasma membranes thus obtained were suspended in 15 ml of water and the suspension divided into 1.5-ml samples. These samples
were stored at \(-25^\circ C\). In most cases, no detectable loss of activity occurred during storage; with some batches, however, a slow inactivation was observed (about 50\% after 3 months).

*Amino Acids and Peptides*

All amino acids and peptides were those used previously [1].

*Estimation of Free Amino Acids*

Non-radioactive amino acids were measured with the fluorodinitrobenzene technique [4]. Free d-alanine was also estimated enzymatically by using a modification of the technique described previously [5]. In order to avoid precipitations, however, the potassium pyrophosphate buffer was replaced by 0.1 M Tris-HCl buffer pH 8. Moreover, the last methanol/water (v/v) solution used was supplemented with 2\% sodium dodecylsulfate. The reactions were carried out without prior elimination of the membranes from the reaction mixtures. Under the conditions used for both d-carboxypeptidase and exchange activities, the membranes did not perform determinable racemase activity through which d-alanine would be transformed into L-alanine.

*Estimation of Radioactive Compounds*

Radioactive compounds were separated by electrophoresis on strips of Whatman 3MM paper at pH 6.5 (collidine/acetic acid/water, 9.1/2.65/1000, v/v/v) or at pH 2 (0.5 M formic acid), at 60 V/cm for 1–4 h. The radioactive compounds were located on the strips and the radioactivity was estimated as described previously [1].

*Buffers*

Cacodylate, borate and carbonate buffers were prepared and the ionic strength (I) estimated according to Bates and Bower (see [6]). At pH 10, both standard borate buffer (0.0125 M Na2B4O7·10 H2O + 0.018 M NaOH) and standard carbonate buffer (0.025 M NaHCO3 + 0.01 M NaOH) had approximately the same I value of 0.045 M. Effects of the salt concentration at this pH were studied by varying the concentrations of the above buffers.

*Detergents*

Triton X-100 was purchased from Serva, Nonidet P-40 from BDH, N-cetyl-N,N,N-trimethylammonium bromide from Merck and Genapol was a gift from Hoechst, Belgium.

![Fig. 1. Effects of pH on d-carboxypeptidase and exchange activities. Cacodylate (pH 6) and borate buffers (pH 8–10.5) (I = 0.025 or 0.045 M). For other conditions, see text. Results were expressed as percentage of Ac2-L-Lys-d-Ala-d-Ala converted into Ac2-L-Lys-d-Ala-d-[14C]Ala (exchange activity, curve 1) or hydrolyzed (d-carboxypeptidase activity, curve 2)](image)

**RESULTS**

*Occurrence of a Membrane-Bound Enzyme Activity Catalysing the Exchange Reaction Ac2-L-Lys-d-Ala-d-Ala + d-[14C]Alanine → Ac2-L-Lys-d-Ala-d-[14C]Ala + d-Alanine*

Membranes (40 \(\mu\)g protein) and either 6 mM Ac2-L-Lys-d-Ala-d-Ala alone or a mixture of 6 mM Ac2-L-Lys-d-Ala-d-Ala and 10 mM d-[14C]alanine (0.49 Ci/mol) were incubated for 30 min at 37°C in 20 \(\mu\)l (final volumes) of buffers of pH values from 6 to 10.5 (at I = 0.025 M and 0.045 M for the carboxypeptidase activity and the exchange activity, respectively) (Fig. 1). Both the hydrolysis of the tripeptide (in the absence of added d-[14C]alanine) and the exchange reaction occurred with maximal rates at pH 10. A shift of the pH of the reaction mixture from 6 to 10 caused a 2-fold increase of the rate of hydrolysis and a 30-fold increase of the rate of exchange (Fig. 1). At pH 10, the tripeptide was transformed into radioactive tripeptide more rapidly than it was hydrolysed into dipeptide.

In borate buffer pH 10, both reactions had maximal rates at \(I = 0.02 – 0.03\) M and were inhibited at higher ionic strengths (Fig. 2). In carbonate buffer pH 10, variations of the ionic strength above the 0.045 M value of the standard buffer (Materials and Methods) did not affect the enzyme activities (Fig. 2). Irrespective of the ionic strength, both reactions were faster in carbonate buffer than in borate buffer (Fig. 2).
The favorable effect of the carbonate buffer was observed late during the course of the present studies and a series of experiments that are reported in the ensuing paragraphs had been carried out in borate buffer.

**Kinetic Parameters of Hydrolysis and Exchange Reactions at pH 10**

On the basis of initial velocity measurements of the hydrolysis of tripeptide $Ac_2$-L-Lys-D-Ala-D-Ala into dipeptide $Ac_2$-L-Lys-D-Ala + free D-alanine in carbonate buffer ($I = 0.13$ M) pH 10, Lineweaver-Burk plots indicated a $K_m$ value for the tripeptide of 11 mM and a $V$ value of 33 nmol of tripeptide hydrolysed min$^{-1}$ (mg membrane protein)$^{-1}$.

Similarly, from initial rate measurements made in the same carbonate buffer pH 10 ($I = 0.13$ M) at constant initial concentrations of $Ac_2$-L-Lys-D-Ala-D-Ala (6.25, 12.5, 18.75 and 23.25 mM, respectively) and, for each of them, different concentrations of D-$[^{14}$C]$\text{alanine}$ (2.5, 5, 10 and 15 mM, respectively), the double-reciprocal plots gave straight lines indicating $K_{m(opp)}$ value for $Ac_2$-L-Lys-D-Ala-D-Ala (at infinite concentration of D-$[^{14}$C]$\text{alanine}$) of 7 mM, a $K_{m(opp)}$ value for D-$[^{14}$C]$\text{alanine}$ (at infinite concentration of $Ac_2$-L-Lys-D-Ala-D-Ala) of 6 mM and a $V$ value (at infinite concentrations of both substrates) of 50 nmol of tripeptide utilized in the exchange reaction min$^{-1}$ (mg membrane protein)$^{-1}$. Hence, at pH 10 and at saturating substrate concentrations, the exchange reaction was almost twice as fast as hydrolysis.

**Time Course of Hydrolysis and Exchange Reaction at pH 10**

The D-alanine that is endogenously released from $Ac_2$-L-Lys-D-Ala-D-Ala during the course of a simple hydrolysis reaction, can be utilized for an exchange reaction in which the same tripeptide $Ac_2$-L-Lys-D-Ala-D-Ala plays the role of peptide donor. Conversely, in an exchange reaction between $Ac_2$-L-Lys-D-Ala-D-Ala and D-$[^{14}$C]$\text{alanine}$, the reaction product $Ac_2$-L-Lys-D-Ala-D-Ala can be hydrolysed into D-$[^{14}$C]$\text{alanine}$ and dipeptide $Ac_2$-L-Lys-D-Ala by the membrane-bound Dd-carboxypeptidase. Time course kinetics were thus necessarily complex. As shown in Fig. 3, the $Ac_2$-L-Lys-D-Ala-D-$[^{14}$C]$\text{alanine}$ formed by exchange reaction at the beginning of the incubation in borate buffer pH 10 ($I = 0.045$ M) was seen to disappear from the reaction mixture as a result of a prolonged incubation (curve 3). Also, by following the liberation of $[^{14}$C]$\text{alanine}$ from D-$[^{14}$C]$\text{alanine}$, the hydrolysis of the tripeptide was seen to be inhibited by its reaction product D-alanine (curves 1 and 2).
From the data of Table 1, it is obvious that the requirements for carbonyl donors in the exchange reaction at pH 10 are extremely similar to the substrate requirements for hydrolysis at pH 6. Both enzymatic profiles show a considerable specificity for peptides with a C-terminal L-R_3-D-Ala-D-Ala sequence and for a long side-chain at the L-R_3 position. The effects caused by the introduction of a charged ionic group at the end of the L-R_3 side chain were quantitatively different. In particular the natural compounds 11-13, which at pH 6 had been found considerably more sensitive to the dd-carboxypeptidase activity than Ac_2-L-Lys-D-Ala-D-Ala, were at pH 10 utilized less efficiently in the exchange reaction than the synthetic tripeptide. This apparent discrepancy, however, was only a pH effect. At pH 10, the rate of hydrolysis of compound 11 was 45% of the rate of hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala at the same pH 10.

**Specificity Profile for Amino Acceptor in the Exchange Reaction at pH 10**

The following compounds were tested as possible amino acceptors in an exchange reaction with Ac_2-L-Lys-D-Ala-D-Ala as carbonyl donor: D-alanine, L-alanine, D-aspartate, L-aspartate, D-lysine, D-Ala-L-Ala, L-Ala-D-Ala, Gly-L-Ala, Gly-Gly, Ac-L-Lys-Gly, L-Ala-D-Glu-(L)-msA_2pm-(L)-D-Ala, Lac-L-Ala-D-Gln-L-Lys(D-Asp)-D-Ala and GlcNAc(β(1-4)-MurNAc-L-Ala-D-Gly-L-Lys(D-Asp)-D-Ala-D-Ala. The tests were carried out under the same conditions as those used for the determination of the specificity profile for carbonyl donors. In most cases, [^14C]Ac_2-L-Lys-D-Ala-D-Ala (60000 counts/min) and non-radioactive amino compound were used except for the systems Ac_2-L-Lys-D-Ala-D-Ala + D-[^14C]alanine, L-[^14C]alanine, [^14C]glycine or [^14C]Gly-Gly, respectively (0.4 Ci/mol each).

In addition to D-alanine, glycine (with 70% of the efficiency of D-alanine) and Gly-Gly (with 25% of the efficiency of D-alanine) were the only two compounds that were utilized as amino acceptors in the exchange reaction. All the other compounds were inactive. A positive result was obtained with L-[^14C]alanine (with 25% of the efficiency of D-alanine). The incorporation of the radioactivity was suppressed by the addition of 10 mM d-cycloserine to the reaction mixture. This observation was puzzling since the membrane did not contain any racemase able to transform L-alanine into D-alanine under the experimental conditions used. The exchange reaction with [^14C]Gly-Gly as acceptor led to the formation of the radioactive tetrapeptide Ac_2-L-Lys-D-Ala-[^14C]Gly-Gly. In contrast to Ac_2-L-Lys-D-Ala-[^14C]Ala formed from the standard substrates system, Ac_2-L-Lys-D-Ala[^14C]-

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**Table 1. Substrate requirements of the S. faecalis dd-carboxypeptidase at pH 6 and specificity profile for carbonyl donors in the exchange reaction at pH 10**

Results are expressed as a percentage of the efficiency of peptide 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Peptide</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>hydrolysis at pH 6</td>
</tr>
<tr>
<td>1</td>
<td>Ac_2-L-Lys-D-Ala-D-Ala</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Ac_2-L-Lys-D-Ala-Gly</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Ac_2-L-Lys-D-Ala-L-Leu</td>
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<td>4</td>
<td>Ac_2-L-Lys-D-Ala-L-Ala</td>
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<tr>
<td>5</td>
<td>Ac_2-L-Lys-Gly-D-Ala</td>
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</tr>
<tr>
<td>6</td>
<td>Ac_2-L-Lys-D-Leu-D-Ala</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Ac_2-L-Lys-L-Ala-D-Ala</td>
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</tr>
<tr>
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<tr>
<td>9</td>
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</tr>
<tr>
<td>10</td>
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</tr>
<tr>
<td>11</td>
<td>L-Ala-D-Gly-(Lys(Gly)-D-Ala-D-Ala</td>
<td>185</td>
</tr>
</tbody>
</table>

[^2]: This value is decreased to 45% when the rates of hydrolysis of both compounds 1 and 11 are measured at pH 10.

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**Specificity Profile for Carbonyl Donors in the Exchange Reaction at pH 10**

In previous studies [1], the substrate requirements of the membrane-bound dd-carboxypeptidase had been defined by measuring the rate of hydrolysis of various peptides in 50 mM cacodylate buffer pH 6. The specific activity for the hydrolysis of the standard tripeptide Ac_2-L-Lys-D-Ala-D-Ala (at a 3 mM initial concentration) was 7.6 nmol min^-1 (mg membrane protein)^-1. In Table 1, the efficiency values of these peptides as substrates of the dd-carboxypeptidase activity at pH 6 were recalculated and expressed as a percentage of the efficiency of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala.

The same peptides as above were tested as possible carbonyl donors in the exchange reaction at pH 10. Peptides (6 mM), D-[^14C]alanine (10 mM, 0.49 Ci/mol) and membranes (115 μg protein) were incubated together in 20 μl (final volumes) of borate buffer pH 10 (I = 0.045 M) for 30 min at 37°C and the amount of radioactive peptide formed was estimated. With the standard system Ac_2-L-Lys-D-Ala-D-Ala + D-[^14C]alanine the rate of exchange was 6.6 nmol min^-1 mg^-1. The efficiency of the various peptides as carbonyl donors was also expressed as a percentage of that of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala (Table 1).

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[^14C]: This value is decreased to 45% when the rates of hydrolysis of both compounds 1 and 11 are measured at pH 10.
Gly-Gly was not hydrolysed by the membrane-bound DD-carboxypeptidase activity (Fig. 3, curve 4), an observation which was in agreement with the known substrate requirement of this latter enzyme. Finally, in a control experiment, [14C]Gly-Gly alone was incubated with the membrane preparation under the same conditions as those used for the exchange reaction. Free [14C]glycine was not formed in any detectable amount.

**Thermal Denaturation**

At 55°C and in water, both membrane-bound DD-carboxypeptidase and exchange activities had identical half-lives of 240 min.

**Effects of Detergents**

The enzyme activities were measured in carbonate buffer pH 10 (I = 0.045 M) in the presence of various detergents. Cetyltrimethylammonium bromide (1%) inhibited both activities. Nonidet P-40, Triton X-100 and Genapol X-100 (up to 10%) had no effects. Detergents were also used as possible solubilizing agents. At concentrations higher than 0.5%, and in buffers of pH values higher than 8, Genapol X-100 and Nonidet P-40 caused solubilization of the membranes without altering the enzyme activities.

**DISCUSSION**

All the experiments reported hereby support the view that the membrane-bound DD-carboxypeptidase of *S. faecalis* that catalyses the hydrolysis of the D-Ala-D-Ala peptide bonds of Ac2-L-Lys-D-Ala-D-Ala and other peptides of the type X-L-R3-D-Ala-D-Ala, also catalyses exchange reactions in which the X-L-R3-D-Ala moiety of the above peptides is transferred to simple amino compounds such as D-alanine, glycine and glycyglycine. Hydrolysis occurs both at pH 6 and at pH 10. Exchange reactions occur in a narrow range of alkaline pH values (with maximal rates at pH 10) suggesting that in order to be utilized the acceptor must have its amino group largely unprotonated (at pH 6, the exchange reaction is 30 times slower than at pH 10). By incubating the tripeptide Ac2-L-Lys-D-Ala-D-Ala at pH 10 with the isolated membranes in the presence of 10 mM D-[14C]alanine, the exchange reaction occurs faster than hydrolysis showing that 10 mM D-alanine competes successfully with 55 M water. In spite of the high efficiency of the system, neither D-amino acids other than D-alanine, nor peptides that are closely related to the natural peptides, function as acceptors in place of D-alanine, glycine or glycyglycine at least with Ac2-L-Lys-D-Ala-D-Ala as carbonyl donor. Similar observations have been made with the DD-carboxypeptidases of various bacilli ([2, 7, 8]). These latter enzymes also catalyse simple transfer reactions but not complex reactions that would simulate the natural transpeptidation reaction. One may hypothesize that the *S. faecalis* membrane-bound DD-carboxypeptidase, as revealed by the hydrolysis and exchange reaction described above, is the physiological transpeptidase but that its proper functioning as a polymerizing enzyme requires a strict positioning of the natural substrates involved. In fact, it is impossible to state whether in *S. faecalis*, DD-carboxypeptidase and physiological transpeptidase are distinct enzymes or not, even if, as shown in the following paper [9], this DD-carboxypeptidase appears to be physiologically important.

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