The Peptidoglycan Crosslinking Enzyme System in *Streptomyces* Strains R61, K15 and *rimosus*

Kinetic Coefficients Involved in the Interactions of the Membrane-Bound Transpeptidase with Peptide Substrates and β-Lactam Antibiotics

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(Received May 27, 1977)

The transpeptidation reaction performed by the membranes of *Streptomyces* strain R61 fits the general rate equation for an enzyme-catalysed bimolecular reaction. The same membranes (E) interact with β-lactams (I) to form inactive penicillin·enzyme·membrane complexes (EI*) of rather high stability, which subsequently break down (E + I → EI* → E + degradation products). The enzyme is regenerated and the antibiotic is released in the form of an inactive metabolite. With benzylpenicillin, the degradation product is benzylpenicilloic acid. The reaction is heat-labile. The first step of the reaction (E + I → EI*) is characterized by a second-order rate constant ($k_{\text{formation}}$ in M$^{-1}$ s$^{-1}$) and the second step (EI* → E + degradation products) by a first-order rate constant ($k_{\text{breakdown}}$ in s$^{-1}$). The effects *in vitro* of various β-lactams on the membrane-bound transpeptidase, as expressed by the relevant $k_{\text{formation}}$ and $k_{\text{breakdown}}$ values, parallel the effects *in vivo* of the same antibiotics as expressed by their ability to prevent the germination and growth of conidia. The kinetic parameters of the transpeptidase that was solubilized with N-cetyl-N,N,N-trimethylammonium bromide with respect to its interaction with both peptide substrates and β-lactam antibiotics are quantitatively different from those of the membrane-bound enzyme. Moreover, the solubilized enzyme fragments benzylpenicillin with formation of phenylacetylglycine, a reaction which is similar to that catalysed by the exocellular R61 enzyme. The membranes of *Streptomyces* strains *rimosus* and K15 possess an active 'classic' penicillinase. They were not studied but the kinetic coefficients of the corresponding solubilized transpeptidases were determined and compared with those of the solubilized enzyme from strain R61.

The isolated membranes of *Streptomyces* strain R61 catalyse both transpeptidation and hydrolysis reactions with substrates resembling natural wall peptides, the ratio of transpeptidation to hydrolysis being high when compared with the exocellular enzyme [1–4]. The enzyme activities can be solubilized by direct extraction of the mycelium with N-cetyl-N,N,N-trimethylammonium bromide and partially purified by gel filtration on Sephadex G-100. The standard transpeptidation reaction Ac$_2$-L-Lys-d-Ala-d-Ala + Gly-Gly → Ac$_2$-L-Lys-d-Ala-Gly-Gly + d-Ala catalysed by both the membrane-bound and the solubilized transpeptidases fits the general initial rate equation for an enzyme-catalysed bimolecular reaction. The kinetic coefficients were determined. In previous studies, the exocellular Dδ-carboxypeptidase-transpeptidase (E) excreted by the same *Streptomyces* R61 was shown to form with penicillin (I) a stoichiometric and inactive complex, EI*, which subsequently breaks down with regeneration of the active enzyme and release of the antibiotic in the form of degraded metabolites [5,6]. It was found that the same model applied to the interaction between the β-lactams and the R61 membrane-bound and solubilized transpeptidases. The kinetic parameters were also determined. The main goal of these investigations was to establish whether or not the membrane-bound transpeptidase was physiologically important for the growth of *Streptomyces* R61 and whether or not the detergent-solubilized transpeptidase had the same properties as the corresponding membrane-bound enzyme. For purposes of comparison, the present study was extended to the detergent-solubilized and partially purified transpeptidases from *Streptomyces* strains K15 and *rimosus* [4]. The membranes of these latter organisms were not studied in
detail because of the high ‘classic’ penicillinase activity that they possessed. Moreover, the membranes of *Streptomyces rimosus* were also able to degrade the acceptor Gly-Gly into free glycine.

**MATERIALS AND METHODS**

**Enzymes**

The isolated membranes from *Streptomyces* R61, K15 and *rimosus*, and the corresponding detergent-solubilized and partially purified transpeptidases were prepared as described previously [4]. The membranes from strains *rimosus* and K15 were prepared according to the standard procedure and those from strain R61 according to the modified procedure [4]. After partial purification by filtration on Sephadex G-100 (*K*0 0.45), the solubilized transpeptidases from strain R61 and *rimosus* were in 33 mM Tris-HCl buffer pH 7.5 and that from strain K15 was in 30 mM potassium phosphate buffer pH 7.5 [4].

**General Initial Rate Equation for an Enzyme-Catalysed Bimolecular Reaction:**

\[ S_1 + S_2 \rightleftharpoons S_1^+ + S_2^- \]

The equation proposed by Dalziel [7] for such a reaction is \([E_0]/v = \Phi_0 + \Phi_2/[S_2] + (\Phi_1 + \Phi_1/[S_1])/[S_1]\). From initial rate measurements made at constant initial concentrations of S2 and different concentrations of S1, the plot of \([E_0]/v\) vs \(1/[S_1]\) gives a straight line with both slope and intercept linear functions of \(1/[S_2]\). If several such series of measurements are made each with a different constant value of \(S_2\), the slopes and intercepts of these primary plots may be plotted against \(1/[S_2]\) to give the four coefficients directly as slopes and intercepts: Secondary plots of intercepts: slope = \(\Phi_2\); intercept = \(\Phi_0\). Secondary plots of slopes: slope = \(\Phi_1\); intercept = \(\Phi_1\). [S2] can equally well be chosen as the variable in the primary plots and [S1] as the variable in the secondary plots. Two sets of values for the four coefficients can thus be obtained from the secondary plots. \(\Phi_0\) has the dimension of a reciprocal first-order rate constant and is equal to the reciprocal of the maximum rate with unit enzyme concentration. \(\Phi_1\) and \(\Phi_2\) are equivalent to reciprocal second-order rate constants and are related to the Michaelis constants by \(\Phi_1/\Phi_0 = K_{m1}\), and \(\Phi_2/\Phi_0 = K_{m2}\). \(\Phi_1\) has the dimension of the reciprocal of a third-order rate constant.

**Conditions for the Determination of the Kinetic Coefficients Involved in the Bimolecular Transpeptidation Reaction**

The yields of the transpeptidation reaction Ac2-L-Lys-D-Ala-D-Ala + [¹⁴C]Gly-Gly → D-Ala + Ac2-Lys-D-Ala-[¹⁴C]Gly-Gly were measured as described previously [4]. In the present analyses, A (for acceptor, i.e. the dipetide Gly-Gly) and D (for donor, i.e. the tripeptide Ac2-L-Lys-D-Ala-D-Ala) replaced the general terms \(S_1\) and \(S_2\) in the equation of Dalziel; the \(\Phi_d/\Phi_0\) values thus calculated were equivalent to the \(K_m\) values for Gly-Gly (at saturating concentrations of Ac2-L-Lys-D-Ala-D-Ala), the \(\Phi_d/\Phi_0\) values were equivalent to the \(K_m\) values for Ac2-L-Lys-D-Ala-D-Ala (at saturating concentrations of Gly-Gly) and the \(\Phi_d/\Phi_0\) values were equivalent to the reciprocals of the \(V\) values (under conditions of saturating concentrations of donor and acceptor). The experiments were carried out at 37 °C in the following buffers: 14 mM phosphate buffer, pH 7.5, for the membranes of strain R61; 33 mM Tris-HCl buffer pH 7.5 for the solubilized enzymes from strains *rimosus* and R61; and 30 mM phosphate, pH 7.5, for the solubilized enzyme from strain K15. All reaction mixtures with solubilized enzymes contained 0.02% cetyltrimethylammonium bromide.

**¹⁴C Benzy1penicillin Binding**

Binding of [¹⁴C]benzylpenicillin to the membrane-bound transpeptidase of strain R61 has been described previously [3]. For the solubilized and partially purified transpeptidases of strain R61, the following technique was used. Enzyme samples, each containing 15 µg protein, and various concentrations (10–100 µM) of either non-radioactive or radioactive benzylpenicillin (with the ¹⁴C label on the carbonyl group of the benzyl side-chain; 54 Ci/mol; Amersham) were incubated together in 20 µl (final volumes) 10 mM phosphate buffer for various times (10–60 min) at 37 °C. The samples containing non-radioactive benzylpenicillin were used for the estimation of the residual enzyme activity. The samples containing [¹⁴C]benzylpenicillin were supplemented with an excess of un-labeled benzylpenicillin and the [¹⁴C]benzylpenicillin bound was estimated as the radioactivity remaining at the origin of thin-layer plates after chromatography in solvent II (see below). In the control samples, benzylpenicillin was added to the reaction mixtures before treatment with [¹⁴C]benzylpenicillin.

**Determination of the Kinetic Coefficients Involved in the Interactions with β-Lactam Antibiotics**

The interactions between β-lactams (I) and both membrane-bound and detergent-solubilized transpeptidases (E) fit the general equation \(E + I \overset{k_{breakdown}}{\rightarrow} E^* \overset{k_{binding}}{\rightarrow} E + \text{degradation products}\). \(E^*\) is an inactive complex of rather high stability.

**Determination of the k_{breakdown} Values: Isolated Membranes of Strain R61.** The values used in the present study were those determined previously [3].
Determination of the $k_{\text{breakdown}}$ Values: Solubilized and Partially Purified Enzymes. Each enzyme was incubated for 15 min at 37 °C in 10 mM phosphate buffer pH 7.5 with a large excess of $\beta$-lactam (1–10 mM) in order to obtain complete inhibition. The excess of free antibiotic was destroyed by further incubation for 5 min at 30 °C with 0.1 I.U. penicillinase (Penicillinase Riker, Neutrapen). Enzyme reactivation was then followed by using the two following techniques. (a) Ac2-L-Lys-d-Ala-d-Ala (5 mM) and Gly-Gly (14 mM) were added to the reaction mixtures immediately after the penicillinase treatment and the amount of d-alanine progressively liberated as a function of time at 37 °C was estimated (d-amino acid oxidase technique). All samples (with the exception of the complexes formed between the solubilized R61 transpeptidases and either cloxacillin or methicillin) progressively recovered their activities with the result that after a certain time, the transpeptidation rates were identical to those of the untreated enzymes. From the time values obtained by extrapolating the straight-line portions of the graphs, the first-order rate constant $k_{\text{breakdown}}$ values (in s$^{-1}$) were calculated as explained by Marquet et al. [3]. (b) The penicillinase-treated samples were further incubated at 37 °C in the absence of peptide substrates and after increasing times, the extent of enzyme recovery was estimated. From the data thus obtained, and after correction for the fact that breakdown of the enzyme·antibiotic complexes continued to proceed during incubation with the substrates (for details, see [8]), the $k_{\text{breakdown}}$ values were estimated. Both techniques yielded the same constant values.

Determination of the $k_{\text{formation}}$ Values: Isolated Membranes of Strain R61. Two techniques were used. (a) Direct technique. In those cases where the EI* complexes had long half-lives, 500 µg membrane protein and various amounts of antibiotic (final concentrations: 1–600 µM) were incubated together at 37 °C in 30 µl 14 mM phosphate pH 7.5. The incubation times were varied depending upon the nature and the concentrations of the antibiotic used. Formation of complex EI* was stopped by destroying the excess of antibiotic with penicillinase and the residual activity was estimated. In practice, the 30 µl of the antibiotic/membrane suspensions were supplemented with 10 µl of a solution containing the penicillinase and the substrates. The final substrate concentrations were 9 mM. The mixtures were incubated for 30 min at 37 °C, after which time the reaction was stopped by immersing the tubes in a boiling water bath for 1 min and the amount of transpeptidation product formed was estimated. For each antibiotic concentration used, the apparent rate constant value for complex formation ($k_{a}$) was obtained from plots ln ($A_{i}/A_{o}$) versus time ($A_{o}$ = activity in the absence of the antibiotic; $A_{i}$ = residual activity after time $t$ in the presence of the antibiotic) after correction for the fact that breakdown of the EI* complexes occurred during incubation with the substrates. In all cases, these primary plots gave rise to straight lines and the secondary plots $k_{a}$ versus [antibiotic] also gave rise to straight lines passing through the origin of the coordinates. From these secondary plots, the second-order rate constant, $k_{\text{formation}}$, values (in M$^{-1}$ s$^{-1}$) were calculated. (b) Steady-state technique. In those cases where the EI* complexes had short half-lives (3.5 min, for example) the above technique could not be used. The $k_{\text{formation}}$ values were estimated from measurements, made at the steady state, of the rates of transpeptidation in the presence of various antibiotic concentrations. If one assumes that the inhibition of the transpeptidation by $\beta$-lactam is competitive with respect to the peptide donor; that the mechanism of the transpeptidation is such that the peptide acceptor binds first to the enzyme; and that the formation of complex EI* is a two-step process E + I $\rightleftharpoons$ EI $\rightleftharpoons$ EI*, where $K$ (in M) is the dissociation constant of the intermediate complex EI and $k_{3}$ (in s$^{-1}$) is the first-order rate constant for the isomerization of complex EI into complex EI*, the general equation is:

$$\frac{1}{V_{\text{steady state}}} = \frac{\Phi_{0}}{[A]} + \frac{\Phi_{AD}}{[AD]} + \frac{\Phi_{D}}{[D]} \left(1 + [I] \frac{k_{3} + k_{\text{breakdown}}}{k_{\text{breakdown}} K} \right)$$

where the ratio $(k_{3} + k_{\text{breakdown}})/k_{\text{breakdown}} K$ is equivalent to the Henri-Michaelis constant for the overall reaction E + I $\rightleftharpoons$ E + degradation products (J. M. Frère; personal communication). From this equation, the plot $1/V_{\text{steady state}}$ versus [I] gives rise to a straight line with the slope equal to $[(k_{3} + k_{\text{breakdown}})/k_{\text{breakdown}} K \times \Phi_{D}/[D]]$. If $k_{3} \gg k_{\text{breakdown}}$, then the $k_{3}/K$ ratio, i.e. the $k_{\text{formation}}$ constant, is given by the product $k_{\text{breakdown}} \times [D]/\Phi_{D}$ × slope. For control purposes, this technique was applied to the interaction with benzylpenicillin (which is characterized by a low $k_{\text{breakdown}}$ value and for which the $k_{\text{formation}}$ value could be determined by the direct technique). Both direct and steady-state techniques yielded similar values.

Determination of the $k_{\text{formation}}$ Values: Solubilized and Partially Purified Enzymes. The same direct technique as above was used, i.e. the $k_{\text{formation}}$ values were obtained from secondary plots $k_{a}$ versus [antibiotic].

Identification of the Degradation Products Arising from Interaction with [14C]Benzy1penicillin

Various techniques were used. (a) By electrophoresis on strips of 3MM Whatman paper for 1 h at 60 V/cm and at pH 6.5, benzylpenicillin, benzyl-
penicilloic acid and phenylacetylglycine migrated 19, 31 and 27 cm towards the anode respectively. (b) By silica gel thin-layer chromatography, the RF values of benzylpenicillin, phenylacetylglycine and benzylpenicilloic acid were 0.92, 0.64 and 0 respectively in solvent I (see below) and 0.65, 0.76 and 0.55 respectively in solvent II. (c) By chromatography on a cation-exchange column and elution with a lithium citrate buffer gradient under conditions applied for amino acid analysis [9]. A technicon amino acid analyser was used with a 1.4/0.6-cm column of chromobeads type B in the Li⁺ form. All samples were buffered with 0.2 M lithium citrate buffer, pH 2.2, before loading. [¹⁴C]-Benzy!penicilloic acid and [¹⁴C]phenylacetylglycine were used as reference compounds. They were prepared as previously described [10].

**Results**

**Kinetic Parameters Involved in Transpeptidation Reactions (Table 1)**

The transfer reactions that were catalysed could be analysed as simple cases of bimolecular reactions owing to the fact that the rates of hydrolysis of the tripeptide donor Acz-L-Lys-D-Ala-D-Ala were negligible when compared with the rates of transpeptidation [4]. With the membrane-bound transpeptidase of strain R61, the $\Phi_A/\Phi_0$, $\Phi_D/\Phi_0$ and $1/\Phi_0$ values were calculated from the data published previously [2]. With the R61 detergent-solubilized transpeptidase, the reaction was inhibited by excess of acceptor (Fig. 1) but not by donor concentrations up to 7.14 mM even in the presence of a low acceptor concentration (8 μM). Consequently, the kinetic parameters were determined at acceptor concentrations ranging from 8 to 50 μM and at donor concentrations ranging from 0.48 to 2.38 mM.

**Table 1. Kinetic coefficients $\Phi_0$, $\Phi_A$, $\Phi_D$ and $\Phi_{AD}$ for the bimolecular transfer reactions catalysed by Streptomyces transpeptidases (at 37 °C)**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme</th>
<th>Primary plots</th>
<th>$\Phi_0$</th>
<th>$\Phi_A$</th>
<th>$\Phi_D$</th>
<th>$\Phi_{AD}$</th>
<th>$1/\Phi_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>R61</td>
<td>isolated membranes</td>
<td>$1/v$ vs $1/[D]$</td>
<td>23 000</td>
<td>2500</td>
<td>27 000</td>
<td>690</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1/v$ vs $1/[A]$</td>
<td>23 000</td>
<td>4500</td>
<td>13 500</td>
<td>580</td>
<td>37</td>
</tr>
<tr>
<td>R61</td>
<td>transpeptidase (solubilized)</td>
<td>$1/v$ vs $1/[D]$</td>
<td>20</td>
<td>0.17</td>
<td>0.28</td>
<td>2.5</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1/v$ vs $1/[A]$</td>
<td>20.4</td>
<td>0.15</td>
<td>0.31</td>
<td>1.4</td>
<td>10.3</td>
</tr>
<tr>
<td>K15</td>
<td>transpeptidase (solubilized)</td>
<td>$1/v$ vs $1/[D]$</td>
<td>39.6</td>
<td>2.1</td>
<td>0.36</td>
<td>9.3</td>
<td>4.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1/v$ vs $1/[A]$</td>
<td>40.7</td>
<td>2.1</td>
<td>0.31</td>
<td>8.8</td>
<td>4.44</td>
</tr>
<tr>
<td><strong>rimosus</strong></td>
<td>transpeptidase (solubilized)</td>
<td>$1/v$ vs $1/[D]$</td>
<td>11.6</td>
<td>0.5</td>
<td>0.14</td>
<td>3.0</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1/v$ vs $1/[A]$</td>
<td>11.2</td>
<td>0.56</td>
<td>0.14</td>
<td>3.0</td>
<td>3.8</td>
</tr>
</tbody>
</table>
With the detergent-solubilized transpeptidase of strain K15, concentrations of the donor Ac-\(\text{L-Lys-D-Ala-D-Ala}\) higher than 2.3 mM decreased the rate of transpeptidation. The lower the acceptor concentration, the stronger was the inhibition (Fig. 2). Therefore, the primary reciprocal plots \(1/v \ vs \ 1/[D]\) and \(1/v \ vs \ 1/[A]\) were obtained at concentrations of Ac-\(\text{L-Lys-D-Ala-D-Ala}\) ranging from 0.476 to 2.3 mM and at concentrations of \[^{14}\text{C}]\text{Gly-Gly}\) ranging from 0.034 to 0.26 mM.

With the detergent-solubilized transpeptidase from strain \textit{rimosus}, the kinetic parameters were determined at acceptor concentrations ranging from 0.034 to 0.143 mM and at donor concentrations ranging from 0.47 to 2.3 mM. Fig. 3 A and B shows the secondary plots of intercepts and slopes obtained with this enzyme preparation.

**Occurrence of Membrane-Bound Penicillinase**

Membranes (500 \(\mu\text{g}\) protein) and \[^{14}\text{C}]\text{benzylpenicillin}\) (1.2 mM; final concentration) were incubated in 26 \(\mu\text{l}\), final volume, of 14 mM phosphate buffer, pH 7.5, for 2, 5, 10, 30 and 60 min at 37 °C. With the membranes of strain R61, formation of \[^{14}\text{C}]\text{benzylpenicilloic acid}\) could not be detected by paper electrophoresis nor by thin-layer chromatography. The membranes of strains \textit{rimosus} and K15 yielded \[^{14}\text{C}]\text{benzylpenicilloic acid}\). The rates of hydrolysis were 5 nmol and 1 nmol min\(^{-1}\) mg protein\(^{-1}\) respectively. This penicillin-degrading activity must be attributed to the occurrence of a membrane-bound penicillinase and not to the action of the membrane-bound transpeptidase. The transpeptidases of the membranes of strains \textit{rimosus} and K15 were inactivated by a large excess of benzylpenicillin. After elimination of the unbound antibiotic by centrifugation, and on the basis of the rates of enzyme reactivation at 37 °C, the half-lives of the membrane-bound transpeptidase - benzylpenicillin complexes were found to be 170 min for the enzyme from strain \textit{rimosus} and 200 min for the enzyme from strain K15. Within the limits of experimental error, the solubilized...
and partially purified transpeptidase preparations were devoid of penicillinase activity.

\[^{14}C\text{]}\text{Benzylpenicillin Binding}\]

As measured previously [3], 30 \text{\textmu M} benzylpenicillin was required to inhibit completely the transpeptidase activity of the isolated membranes of strain R61. Under these conditions, 25 pmol \[^{14}C\text{]}\text{benzylpenicillin} was fixed/mg protein.

With the membranes of strain rimosus, 20 \text{\textmu M} benzylpenicillin was required to inhibit the transpeptidase activity by 80\% (in 14 mM phosphate buffer pH 7.5; 20 min at 37 °C). Under these conditions, 70 pmol \[^{14}C\text{]}\text{benzylpenicillin} was fixed/mg protein. The membranes of strain K15 were not examined in this respect.

With the solubilized and partially purified transpeptidases, the benzylpenicillin concentrations required to inhibit the activity by 80--100\% were 50 \text{\textmu M} for the rimosus enzyme, 100 \text{\textmu M} for the R61 enzyme and 200 \text{\textmu M} for the K15 enzyme. The amounts of benzylpenicillin fixed/mg protein were 8 nmol for both R61 and K15 enzymes and 20 nmol for the rimosus enzyme.

**Kinetic Coefficients Involved in the Interactions between \beta-Lactams and the Membrane-Bound Transpeptidase of Streptomyces R61 (Table 2)**

The \(k_{\text{breakdown}}\) values were determined previously [3]. The complexes formed with benzylpenicillin, phenoxymethylpenicillin, carbenicillin and oxacillin have low \(k_{\text{breakdown}}\) values (0.73--2.8 \times 10^{-4} \text{ s}^{-1}) and therefore the \(k_{\text{formation}}\) values could be determined by the direct procedure (Materials and Methods). Fig. 4 illustrates the primary plots \(\ln (A_i/A_0)\) versus time and Fig. 5 the secondary plots \(k_a\) versus [I] obtained with benzylpenicillin. With all the other antibiotics, the primary plots were also linear, showing that the ratios of antibiotic to enzyme remained high throughout the duration of the experiments.

**Table 2. Interaction between \beta-Lactams and the membrane-bound transpeptidase of Streptomyces R61**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>(k_{\text{formation}}) at 37 °C</th>
<th>(k_{\text{breakdown}}) at 37 °C</th>
<th>Half-life of complexes E1* at 37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>400</td>
<td>33 \times 10^{-4}</td>
<td>3.5</td>
</tr>
<tr>
<td>Phenoxymethylpenicillin</td>
<td>140</td>
<td>2.8 \times 10^{-4}</td>
<td>41</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>53^a</td>
<td>1.1 \times 10^{-4}</td>
<td>104</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>15</td>
<td>0.73 \times 10^{-4}</td>
<td>160</td>
</tr>
<tr>
<td>Cloxacillin</td>
<td>13</td>
<td>1.23 \times 10^{-4}</td>
<td>94</td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>5</td>
<td>33 \times 10^{-4}</td>
<td>3.5</td>
</tr>
</tbody>
</table>

\(^a\) A value of 40 M\(^{-1}\) s\(^{-1}\) was obtained by the steady-state procedure. Values of 55 and 65 M\(^{-1}\) s\(^{-1}\) were obtained in two distinct experiments by using the direct procedure.

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**Fig. 4. Determination of the apparent rate constant values (\(k_a\)) for the formation of complex E1* between the membranes of Streptomyces R61 and various concentrations (5, 10 and 20 \text{\textmu M}, respectively) of benzylpenicillin. Primary plots as determined on the basis of measurements of residual enzyme activity. Membranes (500 \text{\mu g} protein) and benzylpenicillin were incubated in 30 \mu l final volumes, of 14 mM phosphate buffer, pH 7.5, for increasing times at 37 °C. After the times indicated on the graph, the reaction mixtures were supplemented with penicillinase, Ac2-L-Lys-D-Ala-D-Ala and \[^{14}C\text{]}\text{Gly-Gly} (both substrates at 9 mM) and incubated for 30 min at 37 °C. The reaction mixtures were treated at 100 °C for 1 min and the amount of transpeptidation product formed was estimated after separation by paper electrophoresis at pH 5.6. \(A_i\) = residual enzyme activity after incubation in the presence of benzylpenicillin; \(A_0\) = enzyme activity in the absence of benzylpenicillin; \(k_a\) values = slopes of the straight lines; [I] = benzylpenicillin concentrations.**

**Fig. 5. Determination of the \(k_{\text{formation}}\) value for the interaction between benzylpenicillin and the membrane-bound transpeptidase of Streptomyces R61. Secondary plots using the data of Fig. 4. [I] = benzylpenicillin concentrations.**
In order to check the validity of the assumptions involved in the steady-state procedure (Materials and Methods), the $k_{\text{formation}}$ for the interaction with benzylpenicillin was also determined by this latter technique. From the transpeptidation rate values ($v$) obtained at the steady state, the secondary plots $1/v$ versus $[I]$ gave rise to a straight line (Fig. 6) and from the slope of this line the $k_{\text{formation}}$ value was calculated on the basis of the equation $k_{\text{formation}} = k_{\text{breakdown}} \times [D] / \Phi_D \times \text{slope}$. A value of $40 \text{ M}^{-1} \text{s}^{-1}$ (at $37 ^\circ \text{C}$) was obtained, which was in good agreement with that obtained by the direct procedure ($55$ and $65 \text{ M}^{-1} \text{s}^{-1}$ in two different experiments). The $k_{\text{formation}}$ values for ampicillin and cephalosporin C (where the half-lives of the EI* complexes are short, $3.5$ min) were then estimated by using the same steady-state technique (Table 2).

One of the assumptions required for the determination of the $k_{\text{formation}}$ value by the steady-state procedure was that the inhibition of the enzyme activity by the $\beta$-lactam was competitive (Materials and Methods). Kinetically, from Lineweaver-Burk plots $1/v$ versus $1/[\text{Ac}_{2}-\text{Lys}-\text{D-Ala}-\text{D-Ala}]$ at various concentrations of ampicillin (Fig. 7), the inhibition of the transpeptidation was indeed competitive with respect to the peptide donor.

### Relationship between the Kinetic Coefficients Involved in the Interactions between $\beta$-Lactams and the R61 Membrane-Bound Transpeptidase and the Effects of the $\beta$-Lactams on the Germination of the Conidia of Streptomyces R61 (Table 3)

Previous studies [3] had shown that the effectiveness of a particular penicillin or cephalosporin was similar with respect to its ability (a) to inhibit in vitro the membrane-bound transpeptidase ($ID_{50}$ values; i.e. antibiotic concentrations required to inhibit the membrane activity by $50\%$ under standard conditions); (b) to bind to isolated membranes (exclusion $D_{50}$ values; as measured by competition experiments); and (c) to inhibit the formation of single-cell colonies from conidia on agar nutrient plates ($LD_{50}$ values). Table 3 shows that the relative effectiveness both

### Table 3. Effects of $\beta$-lactams on the formation of colonies from conidia of Streptomyces R61 on agar nutrient plates ($LD_{50}$ values), on the membrane-bound transpeptidase ($ID_{50}$, exclusion $D_{50}$, $K_i$ values), on the detergent-solubilized transpeptidase ($K_i$ values) and on the exoenzyme ($K_i$ values)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Spores: $LD_{50}$</th>
<th>Membrane-bound enzyme</th>
<th>Detergent-solubilized enzyme: $K_i$</th>
<th>Exoenzyme: $K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$ M</td>
<td>$ID_{50}$</td>
<td>exclusion $D_{50}$</td>
<td>$K_i$</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>5</td>
<td>8</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Phenoxymethylpenicillin</td>
<td>5</td>
<td>18</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>2.2</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>33</td>
<td>35</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>Cloxacillin</td>
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<td>50</td>
<td>45</td>
<td>10</td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>112</td>
<td>540</td>
<td>220</td>
<td>660</td>
</tr>
</tbody>
</table>

* From [3].

$K_i = k_{\text{breakdown}} / k_{\text{formation}}$ (see Table 2).

(From Table 4).

(From [6]).
Peptidoglycan-crosslinking Enzyme System in Streptomyces sp.  

Table 4. Interaction between β-lactams and the solubilized and partially purified transpeptidases from Streptomyces strains R61, K15 and rimosus

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Strain R61</th>
<th>Strain K15</th>
<th>Strain rimosus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k_{\text{formation}} )</td>
<td>( k_{\text{breakdown}} )</td>
<td>half-life</td>
</tr>
<tr>
<td></td>
<td>( M^{-1}s^{-1} )</td>
<td>( s^{-1} )</td>
<td>min</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>15</td>
<td>1.8 x 10^{-4}</td>
<td>62</td>
</tr>
<tr>
<td>Phenoxymethylpenicillin</td>
<td>15</td>
<td>0.9 x 10^{-4}</td>
<td>125</td>
</tr>
<tr>
<td>Benzylicillin</td>
<td>10</td>
<td>0.13 x 10^{-4}</td>
<td>900</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>8</td>
<td>0.9 x 10^{-4}</td>
<td>125</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>--</td>
<td>--</td>
<td>&gt;900</td>
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<tr>
<td>Cloxacillin</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Methicillin</td>
<td>--</td>
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</table>

Fig. 7. Lineweaver-Burk plots of reciprocal of initial velocity of transpeptidation versus reciprocal of peptide donor concentration \([S]\) for various concentrations of ampicillin \([1]\). Membranes of strain R61 (500 μg), \( ^{14}\text{C}\)Gly-Gly (1.25 mM, final concentration), \( \text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}\) (from 3.9 to 9.75 mM) were incubated together in 40 μl of 7 mM phosphate buffer for 30 min at 37°C in the absence and in the presence of various ampicillin concentrations (from 0.04 mM to 0.2 mM). The reaction mixtures were heated at 100°C for 1 min and the amount of transpeptidation product was estimated after paper electrophoresis at pH 5.6

in vitro and in vivo, of those antibiotics for which the \( k_{\text{formation}} \) and \( k_{\text{breakdown}} \) values were determined, corresponded closely with their \( K_i \) values as expressed by the \( k_{\text{breakdown}}/k_{\text{formation}} \) ratio values. For purposes of comparison, Table 3 also gives the corresponding \( K_i \) values for the R61 exocellular enzyme.

Kinetic Coefficients for the Interaction between β-Lactams and the Solubilized and Partially Purified Transpeptidases (Table 4)

The \( k_{\text{breakdown}} \) values were measured as described in Materials and Methods. Fig. 8 illustrates the progressive recovery of the transpeptidase activity in the ampicillin-inactivated R61 preparation. The peptide substrates were introduced in the reaction mixture just after the penicillinase treatment. Fig. 9 illustrates the progressive recovery of the transpeptidase activity in the R61 preparation previously inactivated by benzylpenicillin and ampicillin, respectively. In these experiments, enzyme recovery
proceeded in the absence of added substrates. Irrespective of the method used, the same $k_{\text{breakdown}}$ value was obtained.

The $k_{\text{formation}}$ values were also measured as described in Materials and Methods. Fig. 10 illustrates the primary plots $\ln \left( \frac{A_t}{A_0} \right)$ versus time and Fig. 11 the secondary plots $k_a$ versus [I] obtained for the interaction between carbenicillin and the solubilized transpeptidase of strain K15. In all the cases studied, the primary plots were linear.

**Nature of the Degradation Products Arising from the Interactions between [14C]Benzylicillin and the Isolated Membranes of Strains R61 and rimosus**

Previous studies [3] had shown that the membranes of strain R61, when first treated with [14C]benzylicillin and then reincubated at 37°C in phosphate buffer in the absence of free β-lactam, released a radioactive degradation product which both by paper electrophoresis and by thin-layer chromatography in solvent II behaved as [14C]benzylicillinol acid.
plot of recovery of enzyme activity. If one assumes that the membrane-bound transpeptidase and benzylpenicillin form a stoichiometric complex, then on the basis of the benzylpenicilloic acid released, the isolated membranes would contain about 20 pmol enzyme/mg protein.

Chromatography on the cation-exchange column (Materials and Methods) of the supernatant fraction obtained after full enzyme recovery of the [14C]-benzylpenicillin-treated membranes revealed that [14C]benzylpenicilloic acid was the main degradation product released (70% of the total radioactivity), thus confirming the results obtained previously. However, [14C]phenylacetylglycine (10%) and an unidentified [14C]-labeled 'B' product (20%) were also present. The characterization of this latter product is under current investigation (Adriaens, Meesschaert and Leyh-Bouille, unpublished results).

The same techniques as above were applied to the membranes of strain *rimosus*. [14C]Benzylenpicillicolic acid and the unidentified [14C]-labeled 'B' product were the two released compounds occurring in the molar ratio of 2 to 1. [14C]Phenylacetylglycine was not detected.

Finally, boiling of the complexes formed between [14C]benzylpenicillin and both R61 and *rimosus* membranes and treatment with sodium dodecylsulphate (2%), inhibited the release of the radioactivity by 80–90%.

**Nature of the Degradation Products Arising from the Interactions between [14C]Benzylenpicillicillin and the Solubilized and Partially Purified Transpeptidases**

The *rimosus* enzyme was especially suitable for this type of study because of the high $k_{\text{formation}}$ value for benzylpenicillin (340 M$^{-1}$ s$^{-1}$). Under these conditions, complex formation could be achieved at a low antibiotic concentration. The enzyme preparation (100 µl containing 340 µg protein in 33 mM Tris-Cl buffer pH 7.5) was supplemented with 7 nmol of [14C]benzylpenicillin (final concentration: 50 µM) with the result that the enzyme activity was inhibited by 75%.

(In the present experiment the excess of penicillin was not destroyed by penicillinase action for the reason that in the presence of the Tris-Cl buffer used, the unbound penicillin was rapidly transformed into penicilloyl-Tris.) After varying times at 37°C, samples were removed from the reaction mixture and used for the estimation of enzyme activity and for analysis of the breakdown products by paper electrophoresis. Release of a [14C]phenylacetylglycine-like compound and enzyme reactivation appeared to be concomitant events (Fig. 12). Radioactive compounds having the same mobilities as [14C]benzylenpicilloic acid (about 5%) and benzylpenicilloyl-Tris (about 25%) respectively, were also detected on the electrophoreograms but the amounts of these latter compounds either did not vary or varied only slightly throughout the time course experiment. Finally, the amount of radioactivity which remained at the origin of the electrophoreograms; for conditions, see text. (●) Radioactivity remaining at the origin of the electrophoreograms; (○) [14C]phenylacetylglycine; (■) [14C]benzylpenicilloy-Tris-like compound; (Δ) [14C]benzylpenicilloic-acid-like compound; (□) enzyme reactivation as estimated on the basis of the amount of α-alanine released.

![Fig. 12. Degradation products arising from the interaction between [14C]benzylpenicillin and the solubilized and partially purified transpeptidase of *Streptomyces* strain *rimosus*. Time course experiment. For conditions, see text.](image-url)
increasing amounts of [14C]phenylacetylglycine were released during enzyme reactivation.

DISCUSSION

The three detergent-solubilized transpeptidases have similar $\Phi/D$/$\Phi_0$ values (3.8 - 10.3 mM) and similar $\Phi_A/\Phi_0$ values (0.08 - 0.23 mM), indicating that they behave similarly with respect to the peptide substrates. The enzyme preparations also exhibit similar $1/\Phi_0$ values ($0.1 - 0.5 \mu$mol min$^{-1}$ mg protein$^{-1}$) indicating that they are probably roughly at the same degree of purity. On the basis of the $\Phi/D/\Phi_0$ and $\Phi_A/\Phi_0$ values obtained with the isolated membranes of Streptomyces R61 (Table 1), it appears that the solubilization of the transpeptidase facilitates donor binding and, to a greater extent, acceptor binding. Assuming that the enzyme in the membrane consists of a lipoprotein complex (as suggested by the fact that the membrane remains catalytically active at very low temperature [2]), disruption of the complex by cetlytrimethylammonium bromide results in the enzyme having a better fit for the two artificial substrates used in the transpeptidation test in vitro. From the $1/\Phi_0$ values, the specific activity of the detergent-solubilized and partially purified transpeptidase of Streptomyces R61 is about 300-fold higher than that of the isolated membranes (as they were prepared).

Determination of the $k_{\text{formation}}$ and $k_{\text{breakdown}}$ values for the interaction between $\beta$-lactams and the membrane-bound transpeptidase of Streptomyces R61 (Table 2) were possible because the membranes of this organism did not possess any ‘classic’ penicillinase. Penicillinase activity would degrade penicillin into penicilloic acid at a much faster rate than transpeptidase activity is regenerated through breakdown of the complex formed with penicillin. On the basis of the data of Table 3, it appears that one (or the) enzyme which has to be inactivated by $\beta$-lactams in order to prevent the early development of the mycelia from the conidia is the membrane-bound transpeptidase. This membrane-bound transpeptidase is less sensitive to $\beta$-lactams than the exocellular enzyme. Thus the $K_s$ values for benzylpenicillin are 10 nM with the exocellular enzyme and 2.2 $\mu$M with the membrane-bound enzyme. The corresponding values for cephalosporin C are 1 nM and 660 $\mu$M. From this extremely high sensitivity of the exocellular enzyme, it is concluded that binding of $\beta$-lactams to this enzyme has no effect on the early mycelial development. The same conclusion applies to the lysozyme-releasable enzyme [4] which, on the basis of its ID$_{50}$ value for benzylpenicillin, is as sensitive as the exocellular enzyme (unpublished data).

Quantitatively, the solubilization of the membrane-bound transpeptidase of strain R61 by the cationic detergent causes alterations of both the $k_{\text{formation}}$ and $k_{\text{breakdown}}$ values involved in the interaction with $\beta$-lactams (Tables 2 and 4). In particular, the solubilized enzyme has the same low $k_{\text{formation}}$ value for ampicillin, phenoxymethylpenicillin, benzylpenicillin and carbenicillin, whereas the corresponding values for the membrane-bound enzyme exhibit large differences. However, both membrane-bound and solubilized transpeptidases have similar $K_s$ values for ampicillin, phenoxymethylpenicillin, benzylpenicillin and carbenicillin (Table 3). Qualitatively, benzylpenicillic acid appears to be the (main) degradation product arising from the interaction between benzylpenicillin and the membrane-bound enzyme. In marked contrast, the solubilized enzyme from strain R61, as well as those from strain K15 and rimosus, cause fragmentation of the $\beta$-lactam with formation of phenylacetylglycine, a reaction which is identical to that catalysed by the exocellular R61 enzyme. On the basis of the $k_{\text{formation}}$ values, the solubilized K15 transpeptidase has the same low sensitivity to $\beta$-lactams as the solubilized R61 transpeptidase, whereas the solubilized transpeptidase from strain rimosus has a much higher sensitivity.

The isolated membranes from the three strains examined and the corresponding solubilized enzyme preparations contain penicillin-binding components other than the transpeptidase enzyme. These ‘additional’ binding components necessarily immobilize a certain amount of $\beta$-lactams. Hence, the $\beta$-lactam concentrations which were actually involved in the interaction with the transpeptidase enzyme target were lower than the total concentrations used and therefore, the $k_{\text{formation}}$ values, as they were determined, were somewhat underestimated. Finally, the present experiments neither prove nor disprove that the transpeptidase activity is catalysed either by one single enzyme or by a set of iso-enzymes exhibiting identical kinetic parameters.

The work was supported in part by the National Institutes of Health, Washington, D.C. (contract no. R01 AI 13364-01 MBC), and by UCB, Brussels, Belgium. J.D. is Chargé de Recherches of the Fond National de la Recherche Scientifique, Brussels, Belgium. We thank Drs P. Adriaens and B. Meesschaert (Rega Instituut, Katholieke Universiteit, Leuven, Belgium), who performed the analyses of the benzylpenicillin degradation products by chromatography on cation-exchange columns.

REFERENCES