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The Catalytic Activity and Penicillin Sensitivity in the Liquid and Frozen States of Membrane-Bound and Detergent-Solubilised Transpeptidase of *Streptomyces* R61

Jean DUSART, Alberto MARQUET, Jean-Marie GHUYSEN, and Harold R. PERKINS

Service de Microbiologie, Faculté de Médecine, Institut de Botanique, Université de Liège and The University of Liverpool, Department of Microbiology

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The $K_{m,app.}$ values of the membrane-bound transpeptidase of *Streptomyces* R61 for the donor Ac_2-L-Lys-D-Ala-D-Ala and the acceptor Gly-Gly are not affected by temperature variations when the reaction mixtures are incubated in liquid suspensions. At $-5\,^{\circ}C$, the incubation can be carried out either in the liquid or in the frozen state. The enzyme is active in the latter state. In the frozen state, the $K_{m,app.}$ value for the acceptor remains unchanged but there is a 3-fold increase in the maximum velocity, a 10-fold decrease of the $K_{m,app.}$ value for the donor and a 10-fold increase of the benzylpenicillin concentration required to inhibit the enzyme activity by 50% (ID_{50} value). Temperatures of $-35\,^{\circ}C$ or below are required to completely inhibit the membrane-bound enzyme in the frozen state. Cetyltrimethylammonium bromide extracts the transpeptidase both from the isolated membranes and, with a much higher yield, from the intact mycelium. The extracted enzyme is not active in the frozen state, requires detergent for activity, has decreased $K_{m,app.}$ values for both donor and acceptor, exhibits the same sensitivity to benzylpenicillin and cephalosporin C as the membrane-bound transpeptidase (in liquid suspensions) and, like this latter enzyme, has no DD-carboxypeptidase activity. The detergent-extracted transpeptidase penetrates gels of Sephadex-100 and is not sedimented at $200000 \times g$.

The isolated plasma membranes of *Streptomyces* strain R61 perform a transpeptidation reaction according to the equation Ac_2-L-Lys-D-Ala-D-Ala (peptide donor) + Gly-Gly (peptide acceptor) → Ac_2-L-Lys-D-Ala-Gly-Gly (transpeptidation product) + D-Ala [1,2]. It is believed that this transpeptidase is the physiological transpeptidase [1] which catalyses the peptide crosslinking during the synthesis of the wall peptidoglycan. *Streptomyces* R61 also possesses an exocellular DD-carboxypeptidase transpeptidase that is excreted during growth [3]. This latter enzyme has been purified to protein homogeneity [4]. In addition to the above transpeptidation reaction, it catalyses the hydrolysis of the peptide donor according to the equation Ac_2-L-Lys-D-Ala-D-Ala + H_2O → Ac_2-L-Lys-D-Ala (hydrolysis product) + D-Ala (DD-carboxypeptidase activity) [5]. Both the membrane-bound transpeptidase [2] and the exocellular DD-carboxypeptidase transpeptidase [6–8] react with β-lactam antibiotics to form inactive enzyme-antibiotic complexes which, subsequently, undergo spontaneous breakdown. During this process, both enzymes are reactivated and the antibiotic molecule is released in a chemically altered form. At present, no membrane-bound bacterial transpeptidase has ever been solubilised, purified and characterized. The experiments reported here deal with the effects that the membranous environment exerts on the properties of the transpeptidase of *Streptomyces* R61 and with the solubilisation of this enzyme from the plasma membranes in a catalytically active and penicillin-sensitive form.

Abbreviations. $CeMe_3NBr$, *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide; other abbreviations follow CBN Recommendations, see *Eur. J. Biochem.* 27, 201–207 (1972).

Definition. ID_{50}, the antibiotic concentration which decreased by 50% the rate of transpeptidation.

MATERIALS AND METHODS

Membranes

Membranes were prepared as described previously [1,2]. The membranes were suspended in 0.05 M

Tris-HCl buffer pH 7.5, at a concentration of about 25 mg protein/ml as determined with the Folin-Ciocalteu reagent. The suspensions were stored at 4 °C in the presence of thymol. Some experiments were carried out in buffers other than Tris-HCl. For this purpose, the membrane suspensions were centrifuged and resuspended in the selected buffer.

The effects exerted by each of the following parameters (substrate concentrations, temperature, antibiotic concentrations, absence and presence of detergents) on the enzyme activity were always studied with the same batch of membranes. Since variations in the activity, as expressed per mg protein, occurred depending upon the membrane preparations, the reaction velocity values are not absolute. They may differ from one series of experiments to another.

Transpeptidation Assay

The standard concentrations of substrates were 1.5 mM Ac_2-L-Lys-D-Ala-D-Ala and 15 mM [^{14}C]Gly-Gly [1,2]. Enzyme (either membrane-bound or detergent-extracted) and substrates were incubated at 37 °C in final volumes of about 35 µl of the selected buffer. The transpeptidation product, Ac_2-L-Lys-D-Ala-[^{14}C]Gly-Gly, was separated from the excess of [^{14}C]Gly-Gly by paper electrophoresis at pH 5.6 and estimated as described previously [1,2]. By using membrane preparations, stored for 3–4 days, a clear-cut separation of the transpeptidation product by paper electrophoresis did not require the presence of Triton X-100. Contrary to what had been proposed previously [1,2], Triton X-100 was thus omitted in the present assays. Depending upon the particular membrane preparation and with these standard conditions, the membranes catalysed the synthesis of 20–40 pmol of transpeptidation product min^{-1} $mg\ protein^{-1}$.

Symbols

[D] = concentration of tripeptide donor; [A] = concentration of dipeptide acceptor; v = initial velocity of transpeptidation; $V_{[D]=\infty}$ = maximal velocity of transpeptidation at infinite concentration of donor and a finite concentration of acceptor; $V_{[A]=\infty}$ = maximal velocity at infinite concentration of acceptor and a finite concentration of donor; $V_{[A,D]=\infty}$ = maximal velocity at infinite concentrations of both donor and acceptor; $K_{m,app.}$ values: the apparent K_m value for the acceptor is the acceptor concentration at which the reaction rate is half the $V_{[A]=\infty}$ value (at a given concentration of the donor). Similarly, the apparent K_m value for the donor is the donor concentration at which the reaction rate is half the $V_{[D]=\infty}$ value (at a given concentration of the acceptor).

ID_{50} Values for Benzylpenicillin

The effect of benzylpenicillin on transpeptidase activity was expressed as the concentration (ID_{50} value) which decreased by 50 % the amount of product formed under standard conditions of transpeptidation [1].

Detergents

The following detergents were used: Triton X-100 (Serva, no. 37240), Brij 35 (Pierce Chemical Co, no. 20800), sodium deoxycholate (Fluka), Nonidet P40 (BDH Chemicals, no. 56009), sodium dodecylsulphate (BDH Chemicals, no. 30176) and cetyltrimethylammonium bromide ($CeMe_3NBr$) (Merck, no. 2342, *pro analysi*).

RESULTS

MEMBRANE-BOUND TRANSPEPTIDASE

Effects of pH and Ionic Strength on the Activity of the Membrane-Bound Transpeptidase

At an ionic strength of 0.046, the membrane-bound enzyme exhibited a broad pH optimum between 7 and 9 (Fig. 1A). At identical pH values, the activity was higher in cacodylate-HCl buffer (pH 6.0) or in Tris-HCl buffer (pH 8.0) than in sodium phosphate. In Tris-HCl buffer, pH 7.5, the activity was maximal at ionic strengths lower than 0.04 and was completely inhibited at ionic strengths higher than 0.2.

Kinetic Parameters of the Membrane-Bound Transpeptidase at 37 °C, in Liquid Suspensions

Reciprocal plots $1/v$ *versus* 1/[D] for various concentrations of A (Fig. 2A) and $1/v$ *versus* 1/[A] for various concentrations of D (Fig. 2B) gave rise, at 37 °C, to a series of non-parallel straight lines, hence excluding a ping-pong bi-bi mechanism for the transpeptidation reaction. The $K_{m,app.}$ values for one substrate increased as the concentration of the other substrate increased. Similarly, the velocity of the reaction at infinite concentration of one substrate

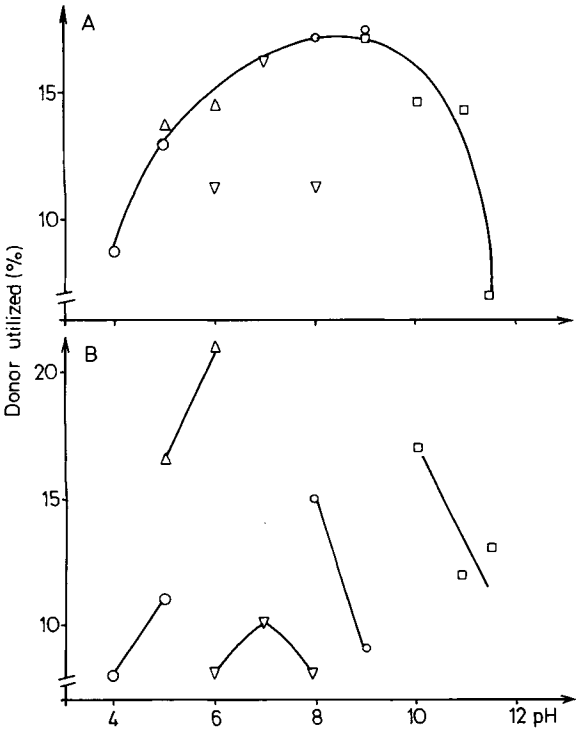

Fig. 1, *Effect of pH on the activity of (A) the membrane-bound transpeptidase and (B) the $CeMe_3NBr$-solubilised transpeptidase.* (A) Membranes were centrifuged and resuspended at a concentration of 25 mg protein/ml in the following buffers (ionic strength = 0.08): (○) acetate; (△) cacodylate; (▽) phosphate; (○) Tris-HCl; (□) L-Ala–NaOH. Samples (20 µl, containing 500 µg protein) of the membrane suspensions were incubated with 1.5 mM Ac_2-L-Lys-D-Ala-D-Ala and 15 mM [^{14}C]Gly-Gly in final volumes of 37 µl, for 5 h at 37 °C. Final ionic strength was 0.046. Under the best conditions, 17% of the donor was utilized in the reaction. (B) Samples of the $CeMe_3NBr$-solubilised and partially purified enzyme (by filtration on Sephadex, see text) were incubated with 1.5 mM Ac_2-L-Lys-D-Ala-D-Ala and 15 mM [^{14}C]Gly-Gly, for 4 h at 37 °C, in final volumes of 37 µl containing 20 µl of the same buffers as those given in (A). Under the best conditions, 21% of the donor was utilized in the reaction

(*i.e.* $V_{[A]=\infty}$ or $V_{[D]=\infty}$) increased as the concentration of the other substrate increased. On the basis of these maximal velocity values, the double-reciprocal plot $1/V_{[A]=\infty}$ *versus* 1/[D] and the double-reciprocal plot $1/V_{[D]=\infty}$ *versus* 1/[A] (Fig. 2C) gave rise to two straight lines intersecting on the ordinate at a $V_{[A,D]=\infty}$ value of 1.7 nmol of transpeptidation product formed per min per mg protein. Extrapolation of the two lines on the abscissa gave $K_{m,app.}$ values of about 4 mM for the acceptor (at [D] = ∞) and about 40 mM for the donor (at [A] = ∞).

At 37 °C and under standard conditions of donor concentration (1.5 mM), the $K_{m,app.}$ value for the acceptor was 0.8 mM. Similarly, under standard conditions of acceptor concentration (15 mM), the $K_{m,app.}$ value for the donor was 26 mM. It thus follows that the standard tests for transpeptidation were carried out under conditions where the acceptor and donor concentrations were equivalent to about 20 times and 0.06 times the relevant $K_{m,app.}$ values.

Effects of Temperature and Freezing on the Activity of the Membrane-Bound Enzyme

Membranes and substrates were incubated under standard conditions (500 µg protein, 1.5 mM donor and 15 mM acceptor) at temperatures ranging from −70 °C to 66 °C. For temperatures below −5 °C, the samples were first frozen in liquid nitrogen and then placed in the cold bath at the requisite temperature for incubation. It was observed that at −5 °C, the sample frozen in liquid nitrogen remained solidly frozen whereas a sample that was incubated directly at −5 °C remained in the liquid state. Thus, an opportunity was presented for comparing the activity of the enzyme at the same temperature in both the liquid and solid states.

After incubation, the enzyme was inactivated by brief immersion (30 s) in boiling water. From the extent of donor utilised, Arrhenius plots were prepared (Fig. 3A–D). The following observations were made: (a) the enzyme was denatured at temperatures greater than 45 °C, (b) there was a transition at approximately 15 °C, (c) the yield of transpeptidation product at −20 °C in the frozen state was identical to that at 30 °C in the liquid state, (d) incubation at −5 °C in the frozen state caused a 6-fold increase in product formation compared to that obtained at the same temperature in the liquid state, and (e) the slopes of the lines for liquid (from −5 °C to 15 °C) and frozen suspensions were parallel, indicating identical energies of activation.

In a separate series of experiments, reaction mixtures were frozen in liquid nitrogen, thawed and incubated as liquid suspensions at temperatures equal to and greater than −5 °C. The transpeptidation yields were identical to those obtained from samples which were incubated without prior freezing. Hence, the favourable conditions for transpeptidation imparted by the frozen state are reversible.

Kinetic Parameters of the Membrane-Bound Transpeptidase at −5 °C in the Frozen State

Reaction mixtures, once prepared in an ice-bath, were immediately frozen in liquid nitrogen and then incubated at −5 °C, under which conditions they remained frozen. After incubation, the frozen samples were immersed in boiling water for 30 s and the amounts of transpeptidation product formed were

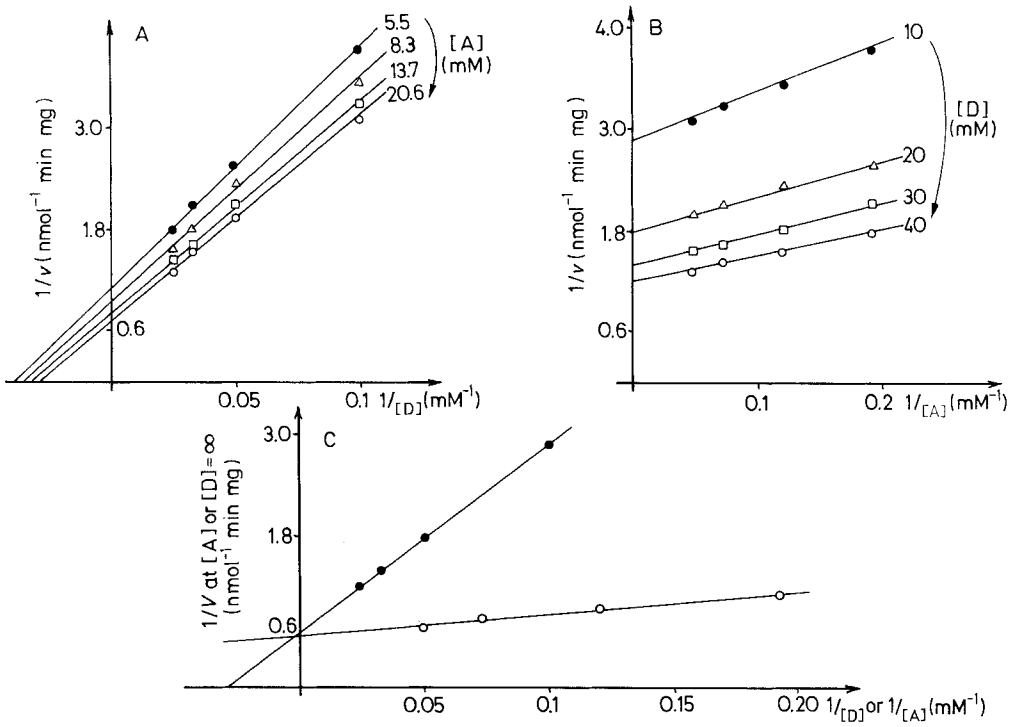

Fig. 2. *Kinetics of the transpeptidation reaction catalysed by the membrane-bound transpeptidase at 37 °C in liquid suspension.* Double-reciprocal plots of: (A) $1/v$ *versus* 1/[D] for various concentrations of acceptor [(●——●) 5.5 mM; (△——△) 8.3 mM; (□——□) 13.7 mM; (○——○) 20.6 mM]; (B) $1/v$ *versus* 1/[A] for various concentrations of donor [(●——●) 10 mM; (△——△) 20 mM; (□——□) 30 mM; (○——○) 40 mM]; (C) $1/V_{[D]=\infty}$ *versus* 1/[A] (○——○) and $1/V_{[A]=\infty}$ *versus* 1/[D] (●——●). The $1/V_{[D]=\infty}$ and $1/V_{[A]=\infty}$ values are those of (A) and (B) respectively. Experimental conditions: membranes (500 μg), Ac_2-L-Lys-D-Ala-D-Ala (the donor, from 10 to 40 mM, final concentrations) and [^{14}C]Gly-Gly (the acceptor, from 5.5 to 20.6 mM) were incubated for 7 h at 37 °C in 37 μl, final volumes, of 0.025 M Tris-HCl buffer pH 7.5. Results (v) are expressed in nmol of transpeptidation product formed per min per mg protein

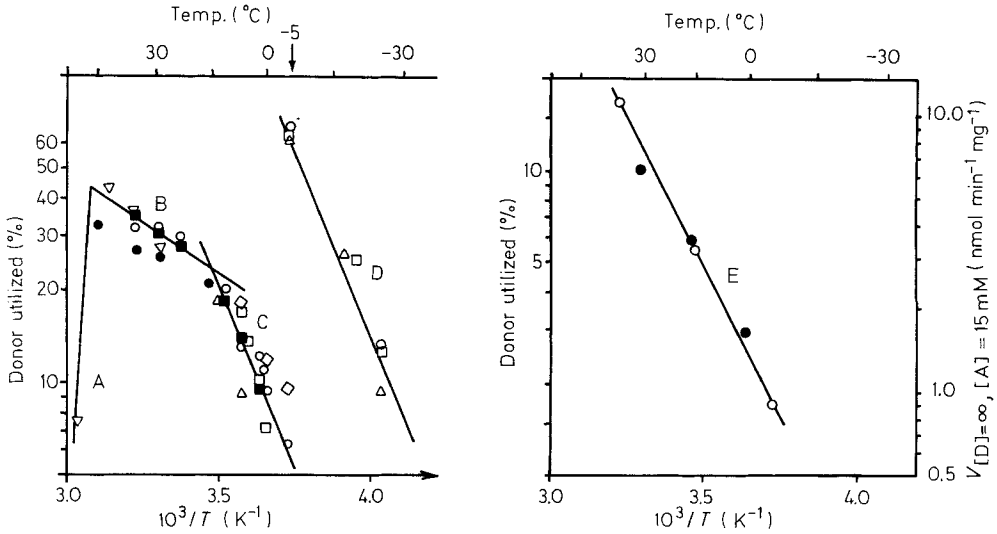

Fig. 3. *Arrhenius plots for the activity of the membrane-bound enzyme in the liquid and frozen states and of the detergent-extracted enzyme.* (A, B, C) Membranes (500 μg protein), 1.5 mM Ac_2-L-Lys-D-Ala-D-Ala and 15 mM [^{14}C]Gly-Gly in 35 μl of 0.025 M Tris-HCl buffer, pH 7.5, were incubated for 15 h, at temperatures ranging from 66 °C to −5 °C, in the liquid state. The graph shows the results obtained from seven independent experiments made with various membrane preparations (□, ▽, ◇, ○, △, ■, ●). The lines (B) and (C), with a transition at 15 °C, were drawn from the data (■——■) of one of these experiments. (D) The experimental conditions were identical to those described above, except that the membrane-containing reaction mixtures were first frozen in liquid nitrogen and then incubated in the frozen state, from −5 °C to −30 °C. Results from three separate experiments are shown (○, △, □). (E) (○) $V_{[D]=\infty,[A]=15\,mM}$: the data are those of Table 1, with membrane-bound enzyme; (●) donor utilized: $CeMe_3NBr$ extracted enzyme, 1.5 mM Ac_2-L-Lys-D-Ala-D-Ala and 15 mM [^{14}C]Gly-Gly in 35 μl of 0.025 M Tris-HCl buffer, pH 7.5, were incubated for 1 h at the indicated temperatures. An energy of activation E^* of about 10 kcal/mol (41.8 kJ/mol) was calculated from the slopes of the lines (C, D, E) according to the equation: ln activity = const − (E^*/RT) where T is the absolute temperature and R is equal to 1.987 cal (8.313 J) mol^{-1} K^{-1}

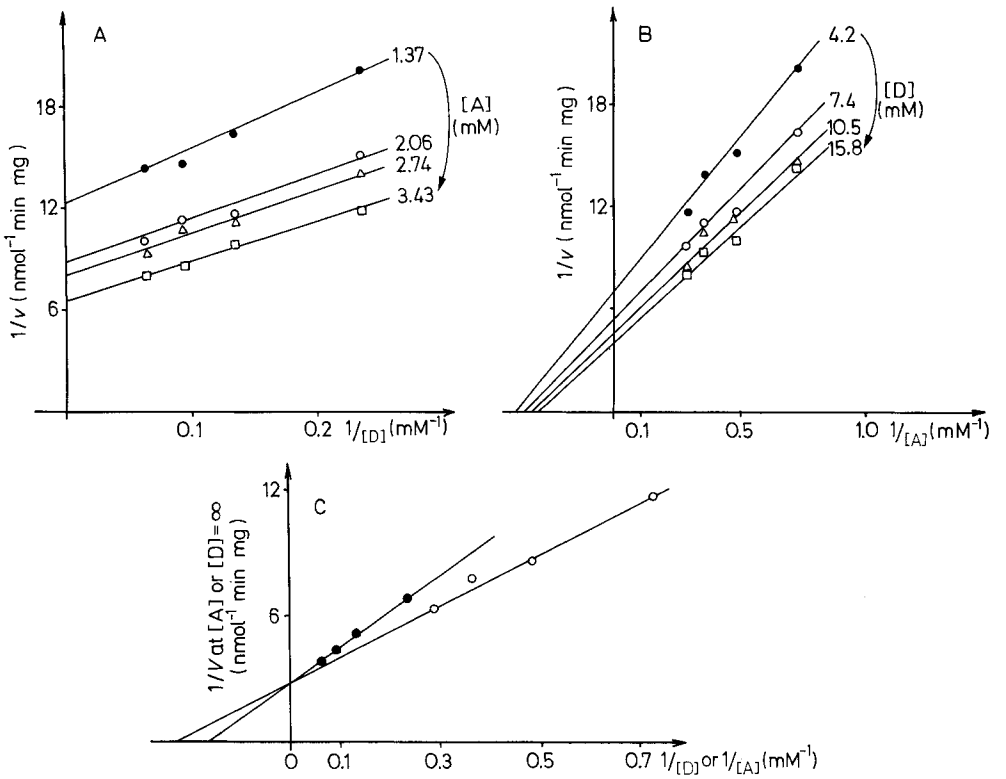

Fig. 4. *Kinetics of the transpeptidation reaction catalysed by the membrane-bound transpeptidase at −5 °C in the frozen state.* Double-reciprocal plots of: (A) $1/v$ *versus* 1/[D] for various concentrations of acceptor: [(●——●) 1.37 mM; (○——○) 2.06 mM; (△——△) 2.74 mM; (□——□) 3.43 mM]; (B) $1/v$ *versus* 1/[A] for various concentrations of donor: [(●——●) 4.2 mM; (○——○) 7.4 mM; (△——△) 10.5 mM; (□——□) 15.8 mM]; (C) $1/V_{[D]=\infty}$ *versus* 1/[A] (○——○) and $1/V_{[A]=\infty}$ *versus* 1/[D] (●——●). The $1/V_{[D]=\infty}$ and $1/V_{[A]=\infty}$ values are those of Fig. 3A and 3B, respectively. Experimental conditions: membranes (500 µg), A_2-L-Lys-D-Ala-D-Ala (the donor, from 4.2 to 15.8 mM, final concentrations) and [^{14}C]Gly-Gly (the acceptor, from 1.37 to 3.43 mM, final concentrations) in 37 µl, final volumes, of 0.025 M Tris-HCl buffer, pH 7.5, were mixed at 0 °C, frozen in liquid nitrogen, and incubated for 5 h at −5 °C. Results (v) are expressed in nmol of transpeptidation product formed per min per mg protein

measured. As observed previously at 37 °C (Fig. 2), the reciprocal plots $1/v$ *versus* 1/[D] for various concentrations of A (Fig. 4A) and $1/v$ *versus* 1/[A] for various concentrations of D (Fig. 4B) also gave rise to a series of non-parallel lines, hence excluding a ping-pong mechanism for the reaction. From the plots $1/V_{[A]=\infty}$ *versus* 1/[D] and $1/V_{[D]=\infty}$ *versus* 1/[A] (Fig. 4C), a $K_{m,app.}$ value for the acceptor (at [D] = ∞) of 6 mM, a $K_{m,app.}$ value for the donor (at [A] = ∞) of 4 mM and a $V_{[A,D]=\infty}$ value of 0.35 nmol of transpeptidation product formed per min per mg protein, were calculated. Hence, a shift of the incubation temperature from 37 °C to −5 °C with a change from a membrane liquid suspension to a frozen membrane suspension had no significant effect on the $K_{m,app.}$ value for the acceptor (at [D] = ∞) but caused a 10-fold decrease in the $K_{m,app.}$ value for the donor (at [A] = ∞).

In order to ascertain that the effect observed on the $K_{m,app.}$ value for the donor was attributable to the frozen state under which the incubation was performed, reaction mixtures containing 15 mM acceptor and various concentrations of donor were incubated at 37 °C, 15 °C and −5 °C (liquid state), respectively. Simultaneously, identical reaction mixtures were first frozen in liquid nitrogen and then incubated at −5 °C (frozen state). The data (Table 1) show the following: (a) with liquid membrane suspensions, a decrease of the temperature from 37 °C to −5 °C has no effect on the $K_{m,app.}$ value for the donor (27−29 mM) but causes a decrease of the maximal velocity of the reaction from 1.17 to 0.10 nmol min^{-1} mg protein^{-1}; (b) at −5 °C, the frozen state enhances the activity of the enzyme both by decreasing the $K_{m,app.}$ value for the donor from 27 to 5 mM and by increasing the maximal velocity value from 0.10 to 0.43 nmol min^{-1} mg protein^{-1}; (c) the $V_{[D]=\infty,[A]=15\,mM}$ value at −5 °C in the frozen state is about one-third that at 37 °C. The efficiency values, however, *i.e.* the ratios $V_{[D]=\infty}/K_{m,app.}$ which express the catalytic activity of the enzyme at low concentrations of donor, are 2-fold higher at −5 °C under freezing conditions than at

Table 1. *Effects of temperature and the frozen state on the* $K_{m,app.}$ *value for donor, the* $V_{[D]=\infty}$ *value and the efficiency of the membrane-bound transpeptidase*
For conditions, see text. The acceptor concentration (15 mM) was equivalent to about 4 times the $K_{m,app.}$ value (for [D] = ∞). The $V_{[D]=\infty}$ values are expressed in nmol of transpeptidation product formed per min per mg protein. Enzyme efficiency = $V_{[D]=\infty}/K_{m,app.}$. In Expt A, the samples were first frozen in liquid nitrogen and then incubated at −5 °C; in Expt B, the samples were directly incubated at the indicated temperatures

Expt	Temperature	State of the reaction mixture during incubation	$K_{m,app.}$ for donor	$V_{[D]=\infty}$ at [A] = 15 mM	Efficiency
	°C		mM	nmol min^{-1} mg^{-1}	
A	−5	frozen	5	0.43	0.090
B	−5	liquid	27	0.10	0.0037
	14	liquid	29	0.33	0.0121
	37	liquid	28	1.17	0.042

37 °C. This conclusion fits well the data of Fig. 3 (B,C,D) which show that in the presence of a low (1.5 mM) concentration of donor the enzyme has a higher activity at −5 °C (in the frozen state) than at 37 °C.

Energy of Activation of the Membrane-Bound Transpeptidase

An Arrhenius plot of log $V_{[D]=\infty,[A]=15\,mM}$ (Table 1) *versus* $1/T$ (K^{-1}) indicated an energy of activation for the reaction in liquid suspension (from −5 °C to 37 °C) of about 10 kcal/mol or 41.8 kJ/mol (Fig. 3E). The same energy of activation was obtained by using the values obtained at [D] = 1.5 mM and [A] = 15 mM either in the frozen state (from −25 °C to −5 °C, Fig. 3D) or in the liquid state (at least from −5 °C to 15 °C, Fig. 3C). At this low [D] value of 1.5 mM, however, a change of slope occurred at temperatures higher than 15 °C (Fig. 3B).

Sensitivity of the Membrane-Bound Transpeptidase in the Liquid and Frozen States to Benzylpenicillin

The ID_{50} values for benzylpenicillin, as determined under standard substrate concentrations (1.5 mM donor; 15 mM acceptor), were found to be 1−2 μM at 37 °C, 0 °C and −5 °C in the liquid state (Fig. 5A). In the frozen state, both at −5 °C and −25 °C, the enzyme had a 10-fold decreased sensitivity to benzylpenicillin (ID_{50} value = 10−20 μM) (Fig. 5B).

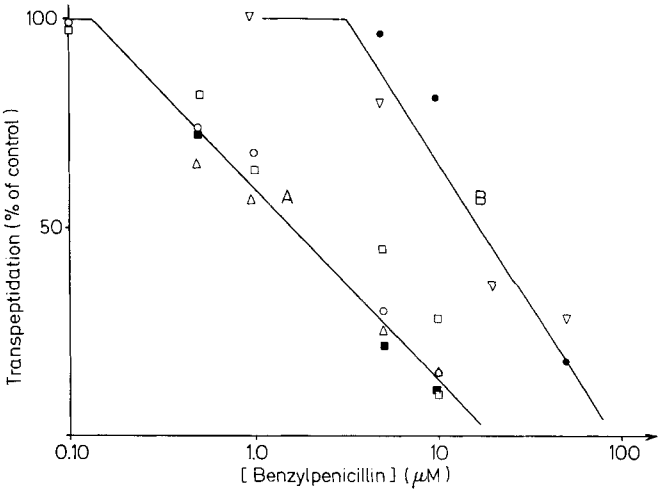

Fig. 5. *Effect of benzylpenicillin on transpeptidase activity.* (A) Detergent-solubilised enzyme at 37 °C (■——■) and membrane-bound enzyme in the liquid state at 37 °C (□——□), 0 °C (○——○) and −5 °C (△——△), respectively. (B) Membrane-bound enzyme in the frozen state at −5 °C (▽——▽) and −25 °C (●——●), respectively. Results are expressed as a percentage of the enzyme activity measured in the absence of benzylpenicillin. Experimental conditions for membrane-bound enzyme: membranes (500 μg protein), 1.5 mM donor and 15 mM acceptor in 37 μl of 0.025 M Tris-HCl buffer, pH 7.5, were incubated, in the absence and in the presence of various concentrations of benzylpenicillin, for 2 h at 37 °C, and −5 °C (frozen state), and for 22 h at 0 °C, −5 °C (liquid state) and −25 °C (frozen state). In all cases, less than 20% of donor was utilized in the reaction. At −25 °C and −5 °C in the frozen state, the reaction mixtures were frozen in liquid nitrogen before incubation. Conditions for detergent-solubilised enzyme: enzyme, 1.5 mM donor, 15 mM acceptor in 37 μl of 0.025 M Tris-HCl buffer, pH 7.5, were incubated in the absence and in the presence of various concentrations of benzylpenicillin, for 2 h at 37 °C. A maximum of 25% of the donor was utilized in the reaction

DETERGENT-SOLUBILISED TRANSPEPTIDASE

Effects of Detergents on the Activity of Membrane-Bound Transpeptidase

Membrane suspensions (25 mg protein/ml) were incubated with detergents (final concentrations: 0.5–2%) in 0.025 M Tris-HCl buffer, pH 7.5. Unless otherwise stated, they were maintained at 37 °C for 30−60 min, after which time they were centrifuged (40000 × *g*; 30 min) and the transpeptidase activity

was estimated both in the supernatants and in the pellets under standard conditions. Triton X-100 (2%), Brij 35 (1%), sodium deoxycholate (1%) and Nonidet P40 (0.9%) had no effect on the membrane-bound transpeptidase which remained associated with the sedimented pellets. At a concentration of 15%, Nonidet P40 inactivated the enzyme. Treatment with sodium dodecylsulphate (0.5%; 90 min at 0 °C) caused a complete dissolution of the membranes and a complete inactivation of the enzyme. Treatment with 0.9% $CeMe_3NBr$ caused a 2–3-fold increase of the transpeptidase activity which, however, remained membrane-bound. After treatment with 1.5% $CeMe_3NBr$, the increased transpeptidase activity was found entirely in the supernatant fraction.

Solubilisation of Transpeptidase from Intact Mycelium with Cetyltrimethylammonium Bromide

It was subsequently found that a transpeptidase activity, identical in all respects to the one extracted from the isolated membranes, was directly solubilised from the washed mycelium by treatment with 2% $CeMe_3NBr$ under the same conditions as above. Remarkably, however, the yield in solubilised transpeptidase activity, was about 10-fold higher than that obtained *via* prior isolation of the plasma membranes. The following procedure was used. *Streptomyces* R61 was grown (volume of inoculation: 2%) for 48 h at 28 °C with vigorous shaking in 1-l flasks containing 500 ml of peptone-oxoid medium [9]. The mycelium was washed 4 times with distilled water by decantation, resuspended in water, homogenised either with a Potter Elvehjem tissue homogeniser or a disintegrator ILA (type X-1020) and centrifuged at $10000 \times g$ for 20 min. The pellet (from 15 l of culture) was washed once by centrifugation with 0.033 M Tris-HCl buffer pH 7.5 and resuspended in 500 ml of the same buffer. The suspension to which an equal volume of buffer containing 4% $CeMe_3NBr$ was added, was maintained at 37 °C for 45 min with occasional stirring after which time the cells were removed by centrifugation at room temperature. The supernatant fraction was further clarified by filtration Schleicher and Schüll paper filter no. 589. A major part of the detergent was precipitated without loss of transpeptidase activity by maintaining the extract at 0 °C overnight. The supernatant fraction obtained by centrifugation at 0 °C was concentrated 10-fold (final volume: 100 ml) by ultrafiltration at 5 °C with an Amicon DC2 apparatus equipped with a H1DP10 cartridge. Further elimination of $CeMe_3NBr$ from the concentrated fraction was achieved by maintaining it at 0 °C as above. Under standard conditions of substrate concentrations, the final preparation exhibited an activity of about 2.8 pmol of transpeptidation product formed per min per µl or about 1 nmol of transpeptidation product formed per min per mg protein (a value to be compared to that of about 60–90 pmol min^{-1} mg $protein^{-1}$ obtained with the isolated membranes in the presence of 2% $CeMe_3NBr$).

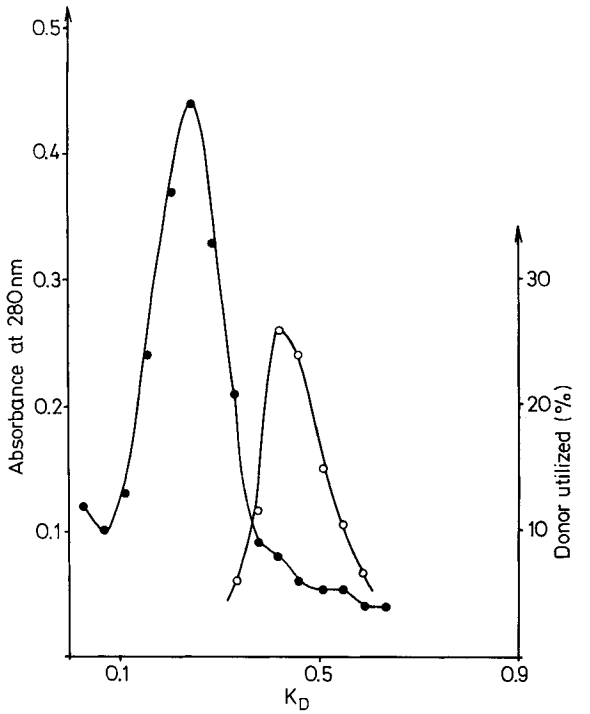

Fig. 6. *Partial purification of the $CeMe_3NBr$-extracted transpeptidase by filtration on Sephadex G-100.* Elution profiles of the transpeptidase activity (○——○) and of materials absorbing at 280 mM (●——●). Samples (20 µl) of the eluted fractions were incubated with 1.5 mM donor and 15 mM acceptor in 37 µl (final volumes) of 0.025 M Tris-HCl buffer, pH 7.5, (final concentration) for 4 h at 37 °C. Results are expressed as a percentage of donor utilized

Ultrafiltration and High-Speed Centrifugation of the Detergent-Solubilised Transpeptidase

The solubilised enzyme freely passed through Amicon UM50 ultrafiltration membranes (designed to retain substances of molecular weight higher than 50000). It did not sediment by centrifugation at $200000 \times g$ for 3 h.

Gel Filtration of the Detergent-Solubilised Transpeptidase

A 20-ml sample of the concentrated extract was chromatographed (at 5 °C) on a column of Sephadex G-100 (3.6×80 cm) previously equilibrated with 0.033 M Tris-HCl buffer, pH 7.5, and the transpeptidase activity was eluted at a 0.43 K_D value (Fig. 6).

The yield was 60–70%. Further purification of the transpeptidase could be achieved by additional filtrations on the same column of Sephadex G-100 with 0.033 M Tris-HCl buffer, pH 7.5, containing 0.05% $CeMe_3NBr$. The presence of detergent was necessary to avoid denaturation of the enzyme after the first filtration. The activity was always eluted at the same 0.43 K_D value. Concentration of the active fractions to be applied on the Sephadex G-100 column was carried out without loss of activity by ultrafiltration (at 5 °C) on Amicon UM10 membranes. Three successive filtrations under the above conditions yielded a partially purified enzyme with a 50-fold increased specific activity as compared to that of the concentrated extract. The final yield of activity was 50–60%.

Effects of pH and Ionic Strength on the Activity of the Detergent-Solubilised Transpeptidase

Like the membrane-bound enzyme, the detergent-solubilised enzyme had a broad pH optimum between 6 and 8 (Fig. 1 B) and in Tris-HCl buffer, pH 7.5, it had a maximal activity at ionic strengths lower than 0.03. The solubilised enzyme, however, was more dependent on the nature of the buffers used than the membrane-bound enzyme (Fig. 1 B).

Effects of Temperature and Freezing on the Activity of the Detergent-Solubilised Transpeptidase

The solubilised enzyme was rapidly denatured at temperatures above 45 °C. At temperatures below 0 °C, the activity of the frozen enzyme was completely inhibited. The Arrhenius plot between 0 °C and 40 °C gave rise to a single line (Fig. 3 E). The energy of activation calculated from this plot was about 10 kcal/mol (41.8 kJ/mol), the same as for the membrane-bound enzyme either in the liquid or frozen state (Fig. 3 C, D).

Kinetic Parameters of the Detergent-Solubilised Transpeptidase

The double-reciprocal plots $1/v$ *versus* 1/[D] (at [A] = 30 mM and with [D] ranging from 4 to 40 mM) and $1/v$ *versus* 1/[A] (at [D] = 13.7 mM and with [A] ranging from 1.4 to 7 mM) obtained with the detergent-solubilised enzyme gave rise to straight lines (at 37 °C and in 0.025 M Tris-HCl buffer, pH 7.5) whose extrapolated values on the abscissa gave $K_{m,\,app.}$ values of about 5 mM for the donor and 0.2 mM for the acceptor. With the membrane-bound enzyme in the liquid state, the $K_{m,\,app.}$ value for the donor (at [A] = 30 mM) was 35 mM and the $K_{m,\,app.}$ value for the acceptor (at [D] = 13.7 mM) was 2.1 mM. Hence, $CeMe_3NBr$ caused approximately a 10-fold decrease of the $K_{m,\,app.}$ values of the transpeptidase for its synthetic substrates. This property explains well how, at the low substrate concentrations used for the estimation of the activity under standard conditions (1.5 mM donor; 15 mM acceptor), $CeMe_3NBr$ treatment of the membrane caused a 2–3-fold increase of the enzyme activity.

Like the membrane-bound transpeptidase and unlike the exocellular enzyme (see the introductory remarks), the detergent-solubilised enzyme did not catalyse, in the absence of Gly-Gly acceptor, the release of the C-terminal D-alanine residue from the tripeptide Ac_2-L-Lys-D-Ala-D-Ala, *i.e.* it did not possess DD-carboxypeptidase activity.

Sensitivity of the Detergent-Solubilised Transpeptidase to Benzylpenicillin

The detergent-solubilised transpeptidase exhibited at 37 °C the same ID_{50} value for benzylpenicillin as the membrane-bound transpeptidase, when the incubation with this latter enzyme was carried out in the liquid state (37 °C, 0 °C, −5 °C). The ID_{50} value was 1–2 μM (Fig. 5 A). Both detergent-solubilised and membrane-bound transpeptidase also had at 37 °C the same ID_{50} values (600 μM) for cephalosporin C. One should note that according to previous studies [1], the membrane-bound enzyme was about 1000 times more resistant to cephalosporin C than the R61 exocellular DD-carboxypeptidase transpeptidase.

DISCUSSION

The membrane-bound transpeptidase of *Streptomyces* R61 closely depends upon environmental conditions for its functioning. In liquid suspensions, temperature variations affect the maximum velocity of the reaction but neither alter the $K_{m,\,app.}$ values of the enzyme for its synthetic substrates (40 mM for the tripeptide donor at [A] = ∞ and 4 mM for the dipeptide acceptor at [D] = ∞) nor its penicillin sensitivity (as determined by the ID_{50} value: about 1–2 μM for benzylpenicillin). Surprisingly, the membrane-bound enzyme is active in the frozen state. The frozen state has no effect on the $K_{m,\,app.}$ value for the acceptor but causes a 10-fold decrease of the $K_{m,\,app.}$ value for the donor and, parallel to this, causes a 10-fold increase of the ID_{50} value for benzylpenicillin. Temperatures of −35 °C are required in order to completely inhibit the membrane-bound enzyme, and at −5 °C, the velocity of the reaction is considerably higher in frozen suspensions than in liquid suspensions. Such behaviour suggests that the transpeptidase func-

tions in a lipid environment which remains remarkably fluid at low temperatures and that the frozen state imparts to the enzyme a conformation which favours its interaction with the donor substrate but does not disfavour its interaction with benzylpenicillin. Assuming that the enzyme in the membrane consists of a lipoprotein complex, it is not surprising that after disruption of this complex with $CeMe_3NBr$, the extracted protein has lost the property of functioning in the frozen state and that it exhibits modified properties. The $CeMe_3NBr$-extracted enzyme has much decreased $K_{m,\,app.}$ values for both donor and acceptor, but its sensitivity to benzylpenicillin and cephalosporin C is identical to that of the membrane-bound enzyme (in liquid suspensions). Hence, both the frozen state and $CeMe_3NBr$ treatment differently affect the $K_{m,\,app.}$ value of the enzyme for the peptide donor and its ID_{50} value for benzylpenicillin. This lack of correlation makes it unlikely that penicillin acts as a structural analogue of the substrate donor.

On the basis of ultracentrifugation, ultrafiltration and gel chromatography experiments, the $CeMe_3NBr$-extracted transpeptidase appears to be a soluble enzyme. It occurs, however, in a form which is different from that of the transpeptidase that is spontaneously excreted during growth by *Streptomyces* R61 (see introductory remarks). In particular, the $CeMe_3NBr$-extracted enzyme requires the presence of the detergent for activity, has no DD-carboxypeptidase activity and has a much higher resistance to cephalosporin C. Agents other than $CeMe_3NBr$ were tested as possible means of solubilising the membrane-bound transpeptidase in a stable, catalytically active, penicillin-sensitive and gel-filtratable form. Neutral detergents had no solubilising activity. Extraction with *n*-butanol (by dropwise addition of the solvent to a membrane suspension slightly acidified with acetic acid and maintained at 0 °C), yielded an aqueous phase exhibiting transpeptidase activity. As previously described [3], treatment of isolated membranes with 2 M urea in the presence of 10 mM ethylenediaminetetracetate gave a fraction which exhibited some DD-carboxypeptidase activity in addition to the transpeptidase activity. Both urea and *n*-butanol procedures, however, lacked reproducibility and yielded unstable enzymes.

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J. Dusart and J.-M. Ghuysen, Service de Microbiologie, Département de Botanique, Université de Liège au Sart-Tilman, B-4000 par Liège 1, Belgium

A. Marquet, Instituto de Biología Cellular, Centro de Investigaciones Biológicas, C.S.I.C., Velázquez 144, Madrid 6, Spain

H. R. Perkins, Department of Microbiology, University of Liverpool, P.O. Box 147, Liverpool, Great Britain L69 3BX