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Solubilization and Isolation of the Membrane-Bound DD-Carboxypeptidase of *Streptococcus faecalis* ATCC 9790

Properties of the Purified Enzyme

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Streptococcus faecalis ATCC 9790 possesses six membrane-bound, penicillin-binding proteins. That numbered 6 (M_r 43000) is the most abundant one and is the DD-carboxypeptidase studied previously. The enzyme has been solubilized and purified to the stage where one single protein band can be detected by gel electrophoresis. The purification procedure does not alter the properties that the enzyme exhibits when it is membrane-bound. The DD-carboxypeptidase itself may be a killing target for penicillin in *S. faecalis*.

Streptococcus faecalis ATCC 9790 possesses a membrane-bound DD-carboxypeptidase (standard reaction catalysed: Ac_2-LLys-DAla-DAla + H_2O → D-alanine + Ac_2-LLys-DAla) [1–3] which at alkaline pH is able to perform simple exchange reactions (standard reaction catalysed: Ac_2-LLys-DAla-DAla + D-[^{14}C]alanine → D-alanine + Ac_2-LLys-DAla-D[^{14}C]alanine) [2]. The isolated membranes bind about 100 pmol of [^{14}C]benzylpenicillin/mg of protein and about 30% of these binding sites are on the DD-carboxypeptidase [3]. The enzyme (E) forms with β-lactam antibiotics (I) inactive complexes EI* which, depending upon the antibiotics, have half-lives either equivalent or, more often, much longer than the generation time of the bacterium [3]. During breakdown of complex EI*, the enzyme is reactivated and the β-lactam is released in a degraded form. Phenylacetylglycine is formed from benzylpenicillin, a reaction which requires fragmentation of the antibiotic molecule [3]. With various β-lactams it was observed that the higher the ability of the antibiotic to form an inactive complex EI*, the higher was the ability of the same antibiotic to inhibit cell growth, suggesting that in *S. faecalis* the DD-carboxypeptidase was an important target for penicillin [3]. Attempts were therefore undertaken to solubilize and purify the enzyme without altering the properties that it exhibits in its natural membranous environment.

Enzymes. DD-Carboxypeptidase (EC 3.4.12.6); penicillinase (EC 3.5.2.6); phospholipase C (EC 3.1.4.3).

MATERIALS AND METHODS

Membranes

Membranes, in 1 mM phosphate pH 7, 1 mM $MgCl_2$ and at 20 mg protein/ml, were prepared as described previously [2].

Measurement of Enzyme Activities

Enzyme preparation (either the isolated membranes or the solubilized fractions) and 3 mM Ac_2-LLys-DAla-DAla were incubated in 20 µl (final volume) of 50 mM carbonate buffer pH 10 for 30 min at 37 °C in the absence of D-[^{14}C]alanine (DD-carboxypeptidase activity) or in the presence of 10 mM D-[^{14}C]alanine (0.49 Ci/mol; exchange activity). The amounts of D-alanine liberated (enzymic procedure) or of Ac_2-LLys-DAla-D[^{14}C]Ala formed (after separation by paper electrophoresis) were estimated as described previously [2, 3]. Specific enzyme activity is expressed in µmol of Ac_2-LLys-DAla-DAla utilized × min^{-1} × (mg protein)$^{-1}$. Unless otherwise stated, the experiments were carried out with the same batch of Ac_2-LLys-DAla-DAla as that used previously [2, 3]. This tripeptide was a gift from UCB, Brussels, Belgium. Another tripeptide preparation, a gift from Reanal, Budapest, Hungary, was used in one experiment (see text). Variations in the K_m and V values were observed depending upon the tripeptide preparation used.

Protein Estimation

The technique of Lowry et al. [4] was used with bovine serum albumin as standard. However, all the reagents and solutions were supplemented with 1% (final concentration) of sodium dodecyl sulfate.

Organic Phosphate Estimation

The technique of Lowry et al. [5] was used with glycerol phosphate as standard.

Penicillinase and Phospholipase C

Penicillinase Riker (Neutrapen) was purchased from Serva and was used with a specific activity of 0.68 IU/μl; 1 IU catalyzes the hydrolysis of 1 μmol of benzylpenicillin/min. Phospholipase C, type I, was purchased from Sigma and was used with a specific activity of 10 U/mg protein; 1 U catalyzes the liberation of 1 μmol of water-soluble organic phosphate from lecithin per min (at 37 °C and pH 7.3).

Detergents

Genapol X-100 (isotridecanolpolyglycol ether, $\bar{n} = 10$) was a gift from Farbwerke Hoechst A.G., Belgium. Nonidet P-40 (polyoxyethylene *p-t*-octyl phenol) and *N*-cetyl-*N*,*N*,*N*-trimethyl ammonium bromide were purchased from BDH Chemicals Ltd.

Radioactive Benzylpenicillin

[^{14}C]Benzylpenicillin (with the radioactive label on the carbonyl group of the phenylacetyl side chain; either 52 or 54 Ci/mol) was purchased from the Radiochemical Center, Amersham.

Affinity Chromatography on Ampicillin-Bound CH-Sepharose 4B

CH-Sepharose 4B (4 g) was equilibrated against a 0.5 M NaCl solution for 15 min at 22 °C, washed successively with 800 ml of 0.5 M NaCl and 200 ml of water (pH 4.5) and collected by filtration on a sintered glass filter. Sepharose was resuspended in 30 ml of water containing 160 mg ampicillin (pH 4.8) and after dropwise addition of 1 ml of a water solution containing 575 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl, the suspension was slowly stirred for 90 min. During this treatment, the pH was maintained between 4.8 and 6 by adding 0.1 M HCl. The ampicillin-bound Sepharose was washed with 500 ml of 10 mM phosphate pH 8 + 0.2 M NaCl and finally, with 500 ml of 10 mM phosphate pH 8 + 0.1% Genapol X-100.

Preparative Polyacrylamide Gel Electrophoresis in Genapol X-100

Proteins were separated by electrophoresis on cylindrical polyacrylamide gels (9 × 0.7 cm) for 4 h at 22 °C (4 mA per gel) according to [6] except that both the gels and the cathode buffer contained 0.1% Genapol X-100. The buffers used were 0.42 M Tris pH 9.18 at the anode and 40 mM Tris/40 mM boric acid (pH 8.64) at the cathode. The stacking gel was 3% polyacrylamide and the separation gel 5% polyacrylamide.

Analytical Polyacrylamide Slab Gel Electrophoresis in Sodium Dodecyl Sulfate

The method used was essentially that described by Laemmli and Favre [7]. The slab gels were 20-cm long, 15-cm wide and 1-mm thick. The stacking and separation gels were 6% and 10% polyacrylamide, respectively. The electrophoreses were carried out for 6 h at 4 °C and 20 mA constant current (the voltage varied from 30 to 300 V) in 50 mM Tris/383 mM glycine buffer pH 8.3 + 0.1% sodium dodecyl sulfate. Bromophenol blue migrated 15 cm toward the anode. Proteins were stained with Coomassie brilliant blue (0.07% in methanol/acetic acid/water, 5/1/4, v/v/v) for 2–3 h. Destaining was carried out overnight in methanol/acetic acid/water (5/7.5/87.5, v/v/v). The molecular weight of the proteins was estimated on the basis of the migration of the following standard proteins: *Escherichia coli* RNA polymerase, β and β' subunits (mean M_r 160000), bovine serum albumin (dimer M_r 136000, monomer M_r 68000), catalase (M_r 60000), leucine aminopeptidase (M_r 53000), ovalbumin (M_r 43000) and *E. coli* RNA polymerase, α subunit (M_r 40000).

Detection of Penicillin-Binding Proteins

Samples smaller than 20 μl were treated with [^{14}C]benzylpenicillin at a 20 μM, final concentration, for 15 min at 37 °C followed by non-radioactive benzylpenicillin (10 mM final concentration) and denaturing buffer. The resulting solutions (at the most 50 μl) containing 1% sodium dodecyl sulfate, 10% glycerol, 60 mM Tris-HCl pH 6.8, 5% mercaptoethanol and 0.001% bromophenol blue, were boiled for 1 min.

Samples larger than 20 μl were treated with [^{14}C]-benzylpenicillin and non-radioactive benzylpenicillin as above, and were precipitated with 4 vol. of acetone at 4 °C. The precipitates were collected by centrifugation, dried *in vacuo*, redissolved in the denaturing buffer containing the same reagents at the same final concentrations as above and the solutions were boiled for 1 min. Precipitation with acetone eliminated Gena-

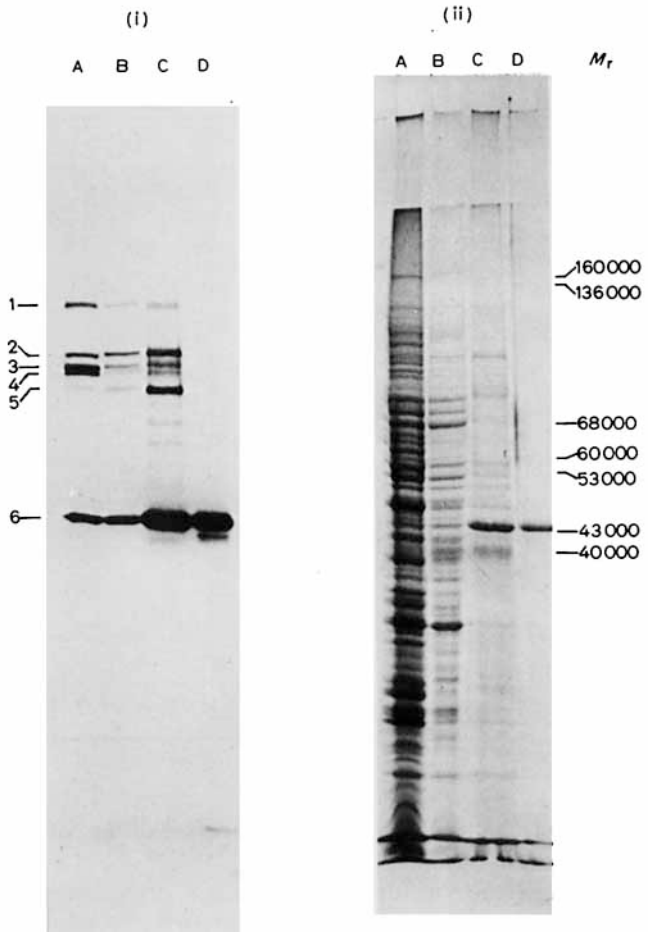

Fig. 1. *Isolation of the* DD*-carboxypeptidase and penicillin-binding protein 6 by affinity chromatography and polyacrylamide gel electrophoresis in Genapol X-100.* Analysis of the isolation procedure by dodecyl sulfate/polyacrylamide gel electrophoresis. (i) Penicillin-binding proteins as detected by fluorography (numbers on the left). (ii) Proteins as detected by staining with Coomassie brilliant blue R-250. Tracks: (A) isolated membranes; (B) after solubilization with Genapol X-100 (step 1); (C) after adsorption and elution from ampicillin-bound CH-Sepharose 4B (step 2); (D) final enzyme preparation after polyacrylamide gel electrophoresis in Genapol X-100 (step 3). The M_r values are those of proteins of known molecular weight

pol X-100 from the samples. When present at too high concentrations, this detergent alters proteins migration.

After polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate, the [^{14}C]-benzylpenicillin binding proteins were detected by fluorography [8,9]. Kodak X-omat L films XL-1 were used (without preflash) and the time of exposure was of 15 days at -70 °C.

RESULTS

Membrane-Bound Penicillin-Binding Proteins

The isolated membranes of *S. faecalis* contain six penicillin-binding proteins exhibiting M_r values of 122000 (1), 97000 (2), 92000 (3), 91000 (4), 82000 (5) and 43000 (6) (Fig. 1, track A). Number 6 is the major one and is most likely the DD-carboxypeptidase since this enzyme is known to represent one third of the total penicillin-binding sites of the membranes [3]. Solubilization and purification of the membrane-bound DD-carboxypeptidase were monitored on the basis of both enzymic assays and [^{14}C]benzylpenicillin binding experiments.

Solubilization of the Membrane-Bound DD*-Carboxypeptidase*

Preliminary attempts to solubilize the enzyme with 1–2 M NaCl, 2–8 M urea with and without EDTA and 4–6 M guanidinium HCl either failed or yielded poor yields. In these assays, 100–200 µl membrane samples were centrifuged, the pellets resuspended in 100–200 µl of the reagent solutions and the enzyme activity estimated in the dialysed supernatant fractions obtained after additional centrifugation. Cetyltrimethylammonium bromide inactivated

Table 1. *Solubilization of the membrane-bound DD-carboxypeptidase*

Expt	Phosphate buffer		Membranes	Genapol X-100	Yield of the extraction		Specific activity
	concn	pH			total protein	enzyme activity	
	mM		mg protein/ml	% (v/v)	%		μmol min^{-1} mg^{-1}
1	0 (H_2O)		10	1	5	11	0.043
	10	6	10	1	23	93	0.065
	10	7	10	1	34	90	0.043
	10	8	10	1	39	90	0.040
	10[a]	9[a]	10	1	45	100	0.038
	10[a]	10[a]	10	1	64	100	0.027
2	5	8	10	1	29	87	0.050
	10	8	10	1	30	87	0.047
	20	8	10	1	33	93	0.043
	50	8	10	1	38	91	0.038
3	10	8	10	0.1	11	38	0.052
	10	8	10	0.2	17	69	0.060
	10	8	10	0.5	21	87	0.062
	10	8	10	1	23	91	0.058
	10	8	10	2	28	98	0.052
	10	8	10	5	36	100	0.042
4	10	8	5	1	38	100	0.038
	10	8	10	1	36	97	0.040
	10	8	20	1	32	79	0.037

[a] Carbonate buffer.

the enzyme. Both Nonidet P-40 and Genapol X-100 solubilized it quantitatively. Genapol X-100 was selected because of its negligible absorbance in ultraviolet light.

The effects of pH (expt 1), phosphate concentrations (expt 2), Genapol X-100 concentrations (expt 3) and membrane protein concentrations (expt 4) were studied separately (Table 1). In all cases, membrane suspensions were incubated under the conditions indicated for 15 min at 37 °C and the supernatant fractions obtained after centrifugation at $150000 \times g$ for 60 min at 4 °C, were analyzed. In terms of the amount of enzyme activity solubilized, an alkaline pH (8–10) was most favorable, the phosphate buffer concentration (at pH 8) had little influence and a Genapol X-100 concentration at least equal to 0.1% and a membrane concentration not exceeding 10 mg protein/ml should be used. The procedure finally selected was to treat a membrane suspension (10 mg protein/ml) in 10 mM phosphate pH 8 with 1% (final volume) Genapol X-100. As illustrated in Table 1 the yields were 90–97% of enzymic activity solubilized and 25–40% of total proteins solubilized. The supernatant fraction thus obtained had a 2.5–3.5-fold increased specific activity. Penicillin-binding and protein analyses by polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate (Fig. 1, track B) confirmed the relatively high selectivity of the procedure as a means for solubilizing penicillin-binding protein 6 (as well as 2 and 5).

Purification of the Solubilized DD-Carboxypeptidase

Step 1. A membrane suspension containing 860 mg of protein in 86 ml of 10 mM phosphate pH 8 was treated with 1% Genapol X-100 (final concentration) for 30 min at 37 °C. After centrifugation at 4 °C and $150000 \times g$ for 100 min, the supernatant fraction was supplemented with 4 g of ampicillin-bound CH-Sepharose 4B and the suspension was slowly shaken for 30 min at 37 °C (with the help of a Büchler rotavapor). The ampicillin-bound Sepharose with the enzyme fixed on it was collected by filtration in a small chromatography column (2.3-cm diameter) and washed with 10 mM phosphate pH 8, 1 M NaCl, 0.1% Genapol X-100 until the effluent had a negligible absorbance at 280 nm. About 250 ml of buffer were used.

Step 2. The enzyme · ampicillin · Sepharose complex was resuspended in 15 ml of 10 mM phosphate pH 8 containing 0.8 M neutral hydroxylamine and 0.1% Genapol X-100. After 5 min at 22 °C, the extract was collected by filtration (extract 1). The hydroxylamine treatment was repeated three times, each time by maintaining the suspension for 30 min at 22 °C (extracts 2–4). Washing with the above phosphate buffer + 0.1% Genapol X-100 (without hydroxylamine) until the effluent was virtually free of materials absorbing at 280 nm yielded the extract 5. Extracts 1–5 were dialysed separately for 30 h at 4 °C against the phosphate/Genapol X-100 buffer. They

Table 2. *Solubilization and purification of the membrane-bound* DD-*carboxypeptidase*
Enzyme activity was measured at a 3 mM Ac_2-LLys-DAla-DAla concentration and in 50 mM carbonate buffer pH 10, as μmol tripeptide hydrolyzed

Step	Total protein	Total enzyme activity	Specific activity	Yield	Purification
	mg	μmol/min	μmol × min^{-1} × (mg protein)$^{-1}$	%	-fold
Isolated membranes	860	9.6	0.011		1
1	241	8.1	0.034	84	3
2	14	3	0.22	31	20
3	0.8	1.17	1.45	12	130

exhibited a specific DD-carboxypeptidase activity which was 15–30-fold higher than that of the membranes. These extracts were pooled together and concentrated to 3.3 ml by ultrafiltration on Amicon UM 10 membranes. Fig. 1 (track C) shows the protein and penicillin-binding patterns of the preparation thus obtained. Note that under the above conditions proteins 2 and 5 behaved exactly like protein 6. A 6th extract obtained after an additional hydroxylamine treatment for 15 h at 22 °C and dialysis as above gave rise to a preparation exhibiting the same specific enzyme activity as the membranes; this extract was discarded.

Step 3. The enzyme preparation after step 2 (14 mg) was divided into 32 samples (100 μl each) and the samples (4 runs of 8 samples) were submitted to polyacrylamide gel electrophoresis in Genapol X-100. After each run, the gels were cut into 2-mm slices and those slices originating from identical positions on the gels were mixed together and extracted, fives times successively, with 800 μl of 10 mM phosphate pH 8 + 0.01% Genapol X-100 for 16 h at 4 °C. The extracts were dialysed for 40 h at 4 °C against 10 mM phosphate pH 8 + 0.01% Genapol X-100 and concentrated to 1 ml by ultrafiltration. Protein estimation, measurement of enzyme activity and protein and penicillin-binding analyses by polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed that altogether the extracts originating from 4 or 5 contiguous slices of the gels contained all the enzyme activity but that two of them (corresponding to a migration of about 4.9–5 cm from the top of the stacking gel toward the anode) (a) had most of the enzyme, (b) exhibited the same high specific enzyme activity and (c) gave rise by polyacrylamide gel electrophoresis in sodium dodecyl sulfate to one single band whose migration coincided with that of penicillin-binding protein 6 (M_r 43000). The extracts containing the purified enzyme and originating from the four different electrophoretic runs were pooled together and concentrated to 3 ml by ultrafiltration, yielding the final enzyme preparation (Fig. 1, track D). Table 2 gives the total recoveries and enrichments in specific DD-carboxypeptidase activity after each step of the purification procedure. Note that the ratio of DD-carboxypeptidase activity to exchange activity (not shown in Table 2) remained unchanged throughout the purification.

Interaction between Purified Enzyme and Peptide Donor Ac_2-LLys-DAla-DAla

From Lineweaver-Burk plots obtained with the purified enzyme, the K_m value for the tripeptide (in 50 mM carbonate buffer pH 10) was 6 mM and V was 4.8 μmol of tripeptide hydrolyzed × min^{-1} × (mg protein)$^{-1}$. The corresponding values with the membranes were K_m = 11 mM and V = 33 nmol × min^{-1} × (mg protein)$^{-1}$. *p*-Chloromercuribenzoate (1 mM), EDTA (5 mM) and dithiothreitol, mercaptoethanol or iodoacetate (10 mM) had no effects on the hydrolytic reaction and/or the exchange reaction (in 50 mM carbonate buffer pH 10).

Interaction between Purified Enzyme and β-Lactam Antibiotics

As observed with the membrane-bound enzyme, the solubilized enzyme (E) reacted with β-lactams (I) to form enzyme · antibiotic complexes (EI*) devoid of DD-carboxypeptidase-exchange activity and exhibiting rather high stability. Breakdown of complex EI* caused reactivation of the enzyme and the release of the antibiotic molecule as inactive metabolites. The general equation for the interaction is thus

$$E + I \xrightarrow{k_f} EI^* \xrightarrow{k_b} E + \text{antibiotic metabolites}$$

($k_f = k_{formation}$ and $k_b = k_{breakdown}$).

*Breakdown of Complex EI**

Enzyme (20 μl of the final preparation containing 5.2 μg of protein) and 20 mM β-lactam (previously lyophilized in the test tube) were incubated together for 15 min at 37 °C. The excess of β-lactam was destroyed by treatment with 5 μl penicillinase for 5 min at 22 °C. The solution containing the complex EI* was diluted to 200 μl with 5 mM phosphate pH 7.5 + 3 mM NaN_3 and 0.01% Genapol X-100 and in-

Table 3. *Effects of β-lactam antibiotics on the* S. faecalis *DD-carboxypeptidase*
k_b is the rate constant for the breakdown of the enzyme · antibiotic complex EI* at 37 °C, k_f is the rate constant for formation of the enzyme · antibiotic complex EI* at 37 °C

Antibiotic	$10^5 \times k_b$ for		Half-life of		k_f for	
	membrane-bound enzyme	purified enzyme	membrane-bound enzyme	purified enzyme	membrane-bound enzyme	purified enzyme
	s^{-1}		min		$M^{-1} s^{-1}$	
Phenoxymethylpenicillin	9.6	8.6	130	135	560	1220
Benzylpenicillin	4.4	2.8	265	410	445	1045[a]
Ampicillin	1.5	1.3	820	912	230	600
Carbenicillin	3.7	3.5	310	330	19	27
Oxacillin	23.5	15	50	75	4.5	4.6
Cloxacillin	6	8	205	145	0.8	0.8
Methicillin	6.3	7	185	165	1.85	3.6

[a] $K = 24\ \mu M, k_3 = 2.5 \times 10^{-2}\ s^{-1}$ ($k_f = k_3/K$).

cubated at 37 °C. Samples (20 µl) were removed after increasing times and the extent of enzyme reactivation was determined. After correction as indicated elsewhere [3], plots of ln $[1 - (E_t/E_0)]$ versus time (where E_t = the concentration of active enzyme present at time t and E_0 = the total, active + inhibited, enzyme concentration) showed that reappearance of enzyme activity was a first-order reaction (k_b in s^{-1}) at least when enzyme recovery did not exceed 60 – 80% (depending upon the antibiotic used). The k_b values thus obtained ranged from $1.3 \times 10^{-5}\ s^{-1}$ (i.e. a half-life of 910 min) to $15 \times 10^{-5}\ s^{-1}$ (i.e. a half-life of 75 min) (Table 3). They were very similar to the k_b values obtained previously with the membrane-bound enzyme.

An enzyme sample, previously filtered on Sephadex G-75 in 10 mM phosphate pH 8 + 0.01% Genapol X-100 ($K_D = 0$) was used to form complex EI* with an excess of [^{14}C]benzylpenicillin. The enzyme · antibiotic complex was reisolated by filtration on Sephadex G-75. Spontaneous breakdown gave rise to [^{14}C]-phenylacetylglycine as observed with the membrane-bound enzyme [3].

*Formation of Complex EI**

Enzyme (20 µl of the final preparation containing 5.2 µg protein) and various β-lactam antibiotic concentrations were incubated together at 37 °C (in 100-µl final volumes). The molar ratios of antibiotic to enzyme were at least equal to and most often higher than 10. After increasing times (0 – 10 min), 20-µl samples were removed from the reaction mixtures, supplemented with 2 µl penicillinase to destroy the excess of the antibiotic and the residual activity was estimated. After correction of the data as described elsewhere [3], the apparent rate constants k_a for formation of complex EI* at each β-lactam concentration used were estimated from the slopes of the straight lines obtained by plotting ln (A_t/A_0) versus time.

With benzylpenicillin, the secondary plots of k_a versus [I] were straight lines passing through the origin of the coordinates at low [I] values and showing deviation from linearity at high [I] values (Fig. 2A). This behaviour is compatible with a two-step process for the formation of complex EI* : $E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^*$ where EI is an intermediate enzyme · β-lactam complex characterized by a dissociation constant K (in M) and k_3 the first-order rate constant for the transformation of complex EI into complex EI*. Such a mechanism was found previously for the interaction between β-lactams and both exocellular DD-carboxypeptidases, transpeptidases R61 and R39 [11,12]. From the reciprocal plot $1/k_a$ versus $1/[I]$ (Fig. 2B), a value of $K = 24\ \mu M$ for the complex formed between benzylpenicillin and the *S. faecalis* enzyme and a value of $k_3 = 2.5 \times 10^{-2}\ s^{-1}$ were obtained (Table 3).

With the other β-lactams and within the limits of the concentrations used, the secondary plots k_a versus [I] did not show deviation from linearity. The second-order rate constant k_f for formation of complex EI* was estimated from the slopes of the lines (k_f in $M^{-1} s^{-1}$ is equivalent to k_3/K). The k_f values thus obtained ranged from 0.8 to $1200\ M^{-1} s^{-1}$ (Table 3). They were either similar to or at least of the same order of magnitude as those obtained with the membrane-bound enzyme.

With all the antibiotics tested, the half-lives of the complexes EI* formed with the DD-carboxypeptidase were longer than the generation time of *S. faecalis*. Hence, if one assumes that the inactivation of the DD-carboxypeptidase is lethal for the cell, the effectiveness of an antibiotic as a growth inhibitor should be related to its k_f value. With six of the seven antibiotics tested, the higher the k_f value, the lower was the minimum antibiotic concentration required to

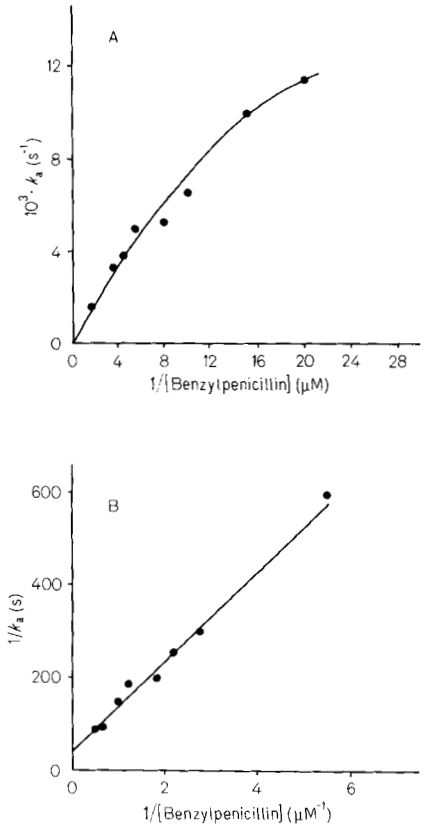

Fig. 2. *Formation of complex EI* between benzylpenicillin and the purified* S. faecalis *DD-carboxypeptidase.* (A) Plots of apparent rate constant k_a for formation of complex EI* versus increasing concentrations of benzylpenicillin. (B) Reciprocal plots $1/k_a$ versus 1/[benzylpenicillin]

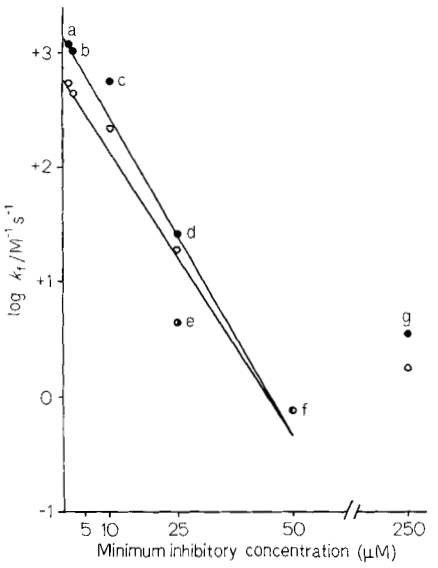

Fig. 3. *Relationship between the minimum antibiotic concentration required to inhibit cell growth and the second-order constant* k_f *for the formation of complex EI* between antibiotic and the* S. faecalis *DD-carboxypeptidase.* (○) Membrane-bound enzyme; (●) purified enzyme. (a) Phenoxymethylpenicillin, (b) benzylpenicillin, (c) ampicillin, (d) carbenicillin, (e) oxacillin, (f) cloxacillin and (g) methicillin. The minimum inhibitory concentrations are those published previously [3]. The lines were drawn according to a curve-fitting linear regression program. Coefficients of determination: $r^2 = 0.95$ with the membrane-bound enzyme and $r^2 = 0.92$ for the purified enzyme

inhibit cell growth and within the limits of experimental error, the plot of log k_f versus the minimum inhibitory concentration gave rise to a straight line (Fig. 3). This relationship, however, did not apply to methicillin which exhibited an unexpectedly high minimum inhibitory concentration (see Discussion).

Concomitant Interaction between Enzyme, Cloxacillin and Peptide Donor Ac$_2$-LLys-DAla-DAla

Cloxacillin was selected for this study because of its low k_f value (Table 3). Under these conditions, high [I]/[E] molar ratios could be used. Enzyme (0.26 μg) and tripeptide (from Reanal, see Materials and Methods) at 4.2, 6, 7.8, 9.6 and 11.4 mM (final concentrations) in 20 μl (final volume) of 10 mM carbonate buffer pH 10 were incubated in the presence of 0, 1, 2, 4 and 6 mM final concentrations of cloxacillin at 37 °C for 30 min, after which time the amounts of D-alanine liberated were estimated. Graphically, the inhibition was competitive (Fig. 4). The validity of this conclusion was examined by computer analyses using the linear regression program devised previously [13]. The weight w of each experimental value $y = 1/v$

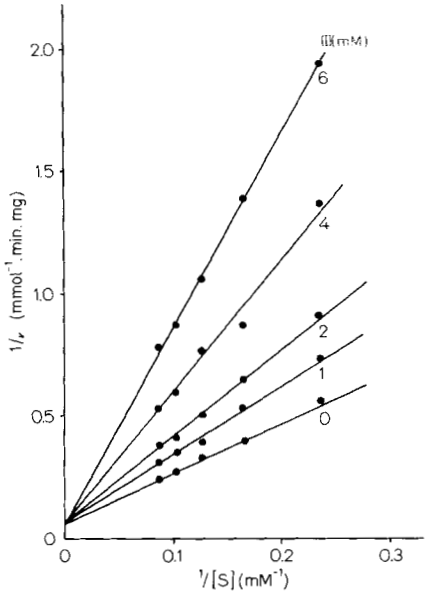

Fig. 4. *Lineweaver-Burk plots of reciprocal of initial velocity of hydrolysis of Ac$_2$-LLys-DAla-DAla versus reciprocal of Ac$_2$-LLys-DAla-DAla concentration [S] for various concentrations of cloxacillin [I]*

was estimated both as $1/y$ and $1/y^2$. The error function which was minimized was $\Sigma\, w_i^2\ (y_i - y_{th})^2$. With $w = 1/y$, the residual variance values for a competitive model $(s^2_{OR})_C$ and a non-competitive model $(s^2_{OR})_{NC}$

were 0.00389 and 0.00404, respectively, and the Fisher-Snedecor variable (F) was 0.13. With $w = 1/y^2$, the $(s^2_{OR})_C$, $(s^2_{OR})_{NC}$ and F values were 0.00106, 0.00109 and 0.39, respectively. The higher the F value, the higher is the probability that the reaction is non-competitive; in the present case F values of 2.98 and 4.32 indicate a non-competitive model with levels of confidence of 90 and 95%, respectively. Owing to the very low F values obtained, there was no indication that a ternary complex enzyme · substrate · antibiotic may be formed under the experimental conditions used. The values of the parameters involved in the reaction (with confidence intervals $t_{99} \times l_i$) were: $K_m = 29 \pm 18$ mM, $V = 14.4 \pm 6.8$ μmol of tripeptide hydrolyzed × min^{-1} × (mg protein)$^{-1}$ and $K_{i\,(app)} = 2.5 \pm 0.6$ mM. The half-life of complex EI* (145 min) is much longer than the incubation time used in the present experiments (30 min). Hence, the kinetics were not carried out at the steady state and the apparent K_i value thus obtained was necessarily different from the K_i value as defined by the equation $K_i = k_b K/(k_b + k_3)$, or if $k_b \ll k_3$, $K_i = k_b/k_f = 0.1$ mM (on the basis of the data of Table 3).

DISCUSSION

Multiple membrane-bound penicillin-binding proteins have been shown to occur in a wide variety of bacteria [14,15]. Penicillin binding to one or more of them is thought to cause cessation of cell growth. The penicillin-binding proteins found in *Escherichia coli* K12 [15], in *Salmonella typhimurium* [16], in other gram-negative bacteria [17], in *Bacillus subtilis* [18,19] and in *B. megaterium* KM [20] have approximately similar profiles. Those of low molecular weight ($M_r = 40000-50000$) are the major ones; they have been identified as DD-carboxypeptidases. The DD-carboxypeptidase 1A, which presumably corresponds to penicillin-binding proteins 5/6 in *E. coli* and 5 in *S. typhimurium*, and the DD-carboxypeptidase 1B, which presumably corresponds to proteins 4 in both *E. coli* and *S. typhimurium*, react with the pentapeptide LAla-DGlu┌(L)*ms*A_2pm(L)-DAla-DAla from which they catalyse the synthesis of a cross-linked peptide dimer [16,21–24]. However, the DD-carboxypeptidases from *B. subtilis* and *B. megaterium* fail to catalyse this reaction. Curiously, no role in the physiological effects that the β-lactams produce on *E. coli* and on bacilli could be attributed to any of these DD-carboxypeptidases. Apparently, the most important penicillin targets are among the penicillin-binding proteins of high molecular weight [15,25]. Thus in *E. coli*, proteins 1B (M_r 91000), 2 (M_r 66000) and 3 (M_r 60000) play a role in cell elongation, rod shape determination and cell division, respectively. Similarly, protein 1 in *B. megaterium* [20] and protein 2 in *B. subtilis* [19] might be the targets for killing by penicillin. Quantitatively all those proteins are minor components; their enzyme properties are unknown.

S. faecalis possesses six penicillin-binding proteins with a profile similar to those observed in gram-negative bacteria and in bacilli. Protein 6, which is the one with the lowest molecular weight (M_r 43000), is the most abundant one and is a DD-carboxypeptidase. *In vitro* and at alkaline pH, the *S. faecalis* DD-carboxypeptidase is able to catalyse exchange reactions where the C-terminal D-alanine residue of Ac_2-LLys-DAla-DAla is replaced by simple amino compounds such as D-alanine or glycyl-glycine, but is unable to catalyse more complex reactions which resemble the transpeptidation reaction *in vivo* [2]. The *S. faecalis* DD-carboxypeptidase (penicillin-binding protein 6) has been solubilized from the membranes and purified to the stage where it consists of one single protein band by gel electrophoresis. The procedure does not alter the properties found previously for the membrane-bound enzyme. In particular, the k_f and k_b values which characterize the interactions between the enzyme and β-lactams are identical or at least of the same order of magnitude whether the assays are performed with the membrane-bound enzyme or with the purified enzyme. The lower k_f values observed with the membrane-bound enzyme may be due either to a more favourable environment for complex formation to occur or to the fact that the inactivation of the membrane-bound enzyme is a phenomenon in which the other five binding proteins are also involved. Formation of complex EI* between benzylpenicillin and the purified enzyme is compatible with the two-step mechanism $E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^*$ proposed previously for other model enzymes [11,12] and the interaction between purified enzyme, Ac_2-LLys-DAla-DAla peptide donor and cloxacillin is competitive at least under the conditions of inhibitor and substrate concentrations used (for further discussion, see [26,27]).

The relative effectiveness *in vivo* of six out of the seven antibiotics tested as growth inhibitors for *S. faecalis* (and as expressed by the minimum inhibitory concentration) is related to the ability of the same antibiotics to inactivate the DD-carboxypeptidase in the form of complex EI* (whether the enzyme is membrane-bound or purified and as expressed by the k_f values for the enzyme-antibiotic interaction). The higher the k_f value, the lower is the minimum inhibitory concentration. However, the relationship between the two is complex, a 50-fold decrease of the minimum inhibitory concentration being paralleled by a 1000-fold increased k_f value and furthermore, it does not apply to methicillin for which the minimum inhibitory concentration is about 8-fold higher than that expected from the corresponding k_f value. A correct interpretation of these observations must await until work with the other five penicillin-binding proteins has been undertaken.

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