Membrane-Bound DD-Carboxypeptidases from *Bacillus megaterium* KM

GENERAL PROPERTIES, SUBSTRATE SPECIFICITY AND SENSITIVITY TO
PENICILLINS, CEPHALOSPORINS AND PEPTIDE INHIBITORS OF THE
ACTIVITY AT pH 5

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1. The membrane from *Bacillus megaterium* KM contained a DD-carboxypeptidase with optimum activity under the following conditions: pH 5.2, bivalent cation, 3 mM; ionic strength, 40 mM; temperature, 35°C. It was inactivated by treatment with *p*-chloromercuribenzoate but was fairly insensitive to 2-mercaptoethanol. 2. The enzyme was inhibited by penicillins and cephalosporins. The inhibition of this enzyme was partially reversed on dialysis but 0.2 M 2-mercaptoethanol could neither prevent nor reverse the inhibition. 3. The enzyme was extremely sensitive to changes in the configuration and size of the side chain of the C-terminal dipeptide of the substrate. An aliphatic side chain of a well-defined length and polarity was required in the residue that precedes the C-terminal dipeptide. 4. The enzyme was inhibited by a wide range of analogues of the peptidic portion of the natural substrate.

DD-Carboxypeptidases and transpeptidases are enzymic activities involved in the last stages of the biosynthesis of the bacterial cell wall peptidoglycan. The transpeptidases are currently accepted as the killing target of the penicillin and cephalosporin families of antibiotics (Wise & Park, 1965; Tipper & Strominger, 1965). It has also been proposed that carboxypeptidase and transpeptidase activities might be different enzymic manifestations of the same protein depending on its environment and the nucleophilic groups available (Leyh-Bouille *et al.*, 1970, 1971). This has been shown to be correct for the DD-carboxypeptidases-transpeptidases from *Streptomyces* strains R61, K11 and R39 (Pollock *et al.*, 1972), and their specificity, kinetics and mechanism of inhibition by penicillin have been studied in detail (Perkins *et al.*, 1973; Frère *et al.*, 1973; Ghuysen *et al.*, 1973; Nieto *et al.*, 1973b). However, these enzymes were soluble proteins, excreted by the bacteria during growth and it could be argued that they were not representative of the membrane-bound enzymes of the same bacteria. The availability of synthetic substrates makes it possible to study the carboxypeptidase and transpeptidase activities while they are still bound to the membrane, independently of the remaining activities involved in the biosynthesis of the peptidoglycan. Reynolds (1971a,b) has described the peptidoglycan biosynthetic system from *Bacillus megaterium* KM. His results showed the

presence of an active DD-carboxypeptidase and suggested that transpeptidation was also taking place. In the present paper we study in more detail the DD-carboxypeptidase activity from *B. megaterium* KM that has a pH optimum near 5.1. For a convenient description of the specificity, the amino acid residues in the substrate will be numbered H. 5-4-3-2-1-OH.

Materials and Methods

Non-radioactive peptides

The synthesis and characterization of the peptides used in this work have been described previously (Nieto & Perkins, 1971; Nieto *et al.*, 1973a).

Non-radioactive nucleotide precursor

The precursor UDP-MurNAc-L-alanyl-D-isoglutamyl-meso-diaminopimelyl-D-alanyl-D-alanine was obtained from *Bacillus licheniformis* lyt⁻ 3f (Forsberg & Rogers, 1971) by accumulation in the presence of vancomycin. This mutant accumulated comparatively high amounts of nucleotide either by the Mg²⁺-starvation method (Garrett, 1969) or in the presence of vancomycin. The second method more consistently gave high yields of nucleotide precursor. *B. licheniformis* lyt⁻ 3f was grown at 35°C in CY medium (Novick, 1963) to mid-exponential phase. The cells were harvested and resuspended (1.5–2.0 mg dry wt./ml) in the buffered glucose-amino acid-chloramphenicol medium described by Bordet & Perkins

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(1970), except that EDTA was replaced by 1 mM-MgCl₂, and vancomycin was added to a final concentration of 12 µg/ml. The bacteria were incubated at 35°C on a reciprocal shaker, harvested after various periods of contact with antibiotic and the nucleotide was extracted at 0°C with ice-cold 10% (w/v) trichloroacetic acid. After removal of trichloroacetic acid by extraction into ether, total *N*-acetylhexosamine was measured by the method of Reissig *et al.* (1955) after hydrolysis in 0.1 M-HCl at 100°C for 5 min. Maximum accumulation occurred after 2 h contact with the antibiotic, and this incubation period was used as a routine. The nucleotide-pentapeptide was purified as described by Bordet & Perkins (1970). An average yield of 19–20 µmol/g dry wt. of bacteria was obtained.

Radioactive peptides

The synthesis of D-Ala-D-[U-¹⁴C]Ala and Ac₂-L-Lys-D-Ala-D-[¹⁴C]Ala has been previously described (Nieto *et al.*, 1973a). α-Ac-L-Lys-D-Ala-D-[¹⁴C]Ala and ε-Ac-L-Lys-D-Ala-D-[¹⁴C]Ala were obtained as by-products in the synthesis of Ac₂-L-Lys-D-Ala-D-[¹⁴C]Ala (Nieto *et al.*, 1973a). They were separated by paper electrophoresis in 0.25 M-formic acid, pH 1.9, (10 V/cm, 3 h). Under these conditions the ε-acetyl peptide travelled 11.5 cm and the α-acetyl peptide, 12.5 cm. After radioautography the bands were cut out and eluted with water. The specific radioactivity of all acetylated tripeptides was 30 mCi/mmol (Nieto *et al.*, 1973a). The identity of these peptides was ascertained by comparing their electrophoretic mobilities with those of non-radioactive standards synthesized and characterized as previously described (Nieto & Perkins, 1971). The synthesis of DL-[³H]Ala-D-Ala was carried out in the following stages.

Benzoyloxycarbonyl-DL-[³H]alanine. To DL-[³H]-alanine (1.326 µmol, 1510 mCi/mmol, The Radiochemical Centre, Amersham, Bucks., U.K.) in water (300 µl) were added NaHCO₃ (3.0 mg) and benzoyloxycarbonyl chloride (2.0 µl; Sigma Chemical Co., St. Louis, Mo., U.S.A.) while cooling in an ice-bath and stirring vigorously with a magnetic stirrer. These conditions were kept for 4 h at the end of which the reaction mixture was acidified to pH 1 in the cold and the benzoyloxycarbonyl derivative was extracted into five 1 ml portions of ethyl acetate. Measurements of radioactivity in the aqueous phase and the ethyl acetate extract afforded a yield of 95% for the conversion into the *N*-protected amino acid. The compound was further purified by ascending paper chromatography (washed Whatman 3MM paper) in butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) as solvents, and the radioactive intermediate was eluted from the paper with ethanol-1% aq. NH₃ (1:1, v/v).

Benzoyloxycarbonyl-DL-[³H]Ala-D-Ala benzyl ester. Benzoyloxycarbonyl-DL-[³H]alanine (1.245 µmol, 1510 µCi/µmol) in dichloromethane (200 µl) was mixed with a solution of D-alanine benzyl ester free base (Nieto & Perkins, 1971) (15 µmol) in the same solvent (200 µl). The mixture was cooled in an ice-bath and a solution of dicyclohexylcarbodi-imide (4 µmol; BDH Chemicals Ltd., Poole, Dorset, U.K.) in dichloromethane (200 µl) was added with stirring. After 2 h stirring in the ice-bath, the reaction mixture was stored at 4°C overnight, then concentrated to dryness *in vacuo* at room temperature.

DL-[³H]Ala-D-Ala. The above reaction mixture was dissolved in anhydrous trifluoroacetic acid (1 ml; BDH Chemicals Ltd.), and dry HBr (Matheson Gas Products, East Rutherford, N.J., U.S.A.) bubbled through the solution for 90 min, with precautions to exclude moisture. The reaction mixture was concentrated to dryness under vacuum at room temperature and kept in a vacuum desiccator over KOH and P₂O₅ overnight. It was then redissolved in 0.2 M-formic acid and purified by paper electrophoresis in the same solvent (pH 1.9) as described by Nieto *et al.* (1973a) for D-Ala-D-[U-¹⁴C]Ala. The final yield of dipeptide as judged by radioactivity measurements was 20%, and the specific radioactivity of the dipeptide should be that of the starting DL-[³H]alanine.

Preparation of radioactive nucleotide precursors.

The peptidoglycan precursors UDP-MurNAc-L-Ala-D-isoGlu-*meso*-A₂pm-D-Ala-D-[U-¹⁴C]Ala and UDP-MurNAc-L-Ala-D-isoGlu-*meso*-A₂pm-D-[³H]-Ala-D-Ala were prepared by Dr. J. B. Ward by enzymic condensation between UDP-MurNAc-L-Ala-D-isoGlu-*meso*-A₂pm and the corresponding radioactive dipeptide (Ward, 1974). The yield of the enzymic coupling was higher than 90% for the peptide D-Ala-D-[U-¹⁴C]Ala and near 50% for DL-[³H]Ala-D-Ala indicating that, as expected, only the D-D sequence of configurations participated in the reaction. Their final specific radioactivities were those of the starting labelled amino acid, i.e. 1510 mCi/mmol for the ³H-labelled precursor and 30 mCi/mmol for the ¹⁴C-labelled compound. Digestion of the nucleotide precursors with the purified DD-carboxypeptidase from *Streptomyces albus* G (Ghuysen *et al.*, 1970), of which the specific requirement for a C-terminal D-D sequence of configurations as well as its lack of carboxypeptidase II activity are well established (Leyh-Bouille *et al.*, 1970), showed that the label was in the positions expected. The experiment was as follows: the radioactive nucleotides (1 µl; 15700 d.p.m./µl for the ¹⁴C-labelled nucleotide, and 213 200 d.p.m./µl for the ³H-labelled nucleotide), considered as carrier-free, and non-radioactive nucleotide precursor (20 nmol) were digested for 4 h at 37°C with

about 400 units (nmol/h) of *S. albus* G enzyme in a final volume of 30 μ l of 20 mM-Tris-HCl buffer-2 mM-MgCl₂, pH 7.5. The D-alanine liberated was converted into the dinitrophenyl derivative as described previously (Ghuysen *et al.*, 1970), and the $E_{360}^{1\text{cm}}$ and radioactivity of the Dnp-alanine was measured. Similar amounts of D-alanine were cleaved from both nucleotides (60–70% hydrolysis), but only the Dnp-alanine from the ¹⁴C-labelled precursor was radioactive and gave an amount of radioactivity corresponding to the $E_{360}^{1\text{cm}}$. In the tritiated precursor virtually all the radioactivity remained in the aqueous phase, i.e. could not be extracted into ether as Dnp-alanine.

Antibiotics

Vancomycin hydrochloride (Vancocin-HCl) was kindly given by Eli Lilly and Co. Ltd. (Basingstoke, Hants., U.K.). Penicillin G was purchased from the Compañía Española de Penicilina, S.A. (Madrid, Spain), cephaloridine from Lilly Indiana de España, S.A. (Madrid, Spain) and carbenicillin from Beecham Research Laboratories Ltd., Brentford, U.K. Dicloxacillin, ampicillin, methicillin and cephalixin were gifts from Dr. J. E. Acosta (Landerlan, S.A., Madrid, Spain).

DD-Carboxypeptidase activity

The standard incubation mixture contained (final concns.) sodium acetate buffer, pH 5.0, (40 mM), MgCl₂ (3 mM), particulate enzyme and substrate in a final volume of 30 μ l. Reference blanks contained the complete incubation mixture except substrate, which was added to the blank after inactivation of the enzyme. All incubation mixtures were set up in duplicate. Incubation was for 30–60 min at 35°C and the reaction was stopped by heating for 1–2 min in a boiling-water bath. The terminal amino acid liberated was measured as previously described (Nieto *et al.*, 1973a). Frequently when substrate with a radioactive C-terminal D-alanine was used, the D-alanine cleaved was dinitrophenylated and the Dnp-D-alanine spot on t.l.c. was removed, eluted, and both its $E_{360}^{1\text{cm}}$ and radioactivity were measured. The molar extinction coefficient of Dnp-alanine (Sigma Chemical Co.) in methanol-0.01 M-NH₃ (1:1, v/v) was determined as $\epsilon = 17400$ at 360 nm. Activity was expressed in nmol of C-terminal amino acid cleaved/h per mg of protein. All the assay mixtures contained 0.1–0.5 mM-2-mercaptoethanol depending on the volume of particulate enzyme used, although in fact it did not appear to affect the activity of the enzyme. The enzyme readily attacked Ac₂-L-Lys-D-Ala-D-Ala and this peptide will be referred to as 'standard substrate'.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard.

Preparation of particulate DD-carboxypeptidase from *B. megaterium* KM

B. megaterium KM was grown at 36°C as described by Reynolds (1971a,b). All subsequent operations were carried out at 0–4°C. The bacteria were harvested by centrifugation and washed once with 0.05 M-Tris-HCl buffer, pH 7.8, containing 10 mM-MgCl₂. The cells were broken by grinding with alumina (levigated alumina from Norton, Welwyn Garden City, Herts., U.K.) as described by Bordet & Perkins (1970) and the broken cells-alumina mixture was suspended in 50 mM-Tris-HCl buffer, pH 7.8, containing 10 mM-MgCl₂ and 1 mM-2-mercaptoethanol. The volume of buffer used (in ml) was four times the weight (in grams) of the cells-alumina mixture. The suspension was centrifuged at 3000g for 10 min and the pellet discarded. The supernatant was then submitted to successive differential centrifugations as follows: 6000g for 10 min; 38000g for 20 min; 100000g for 30 min. The respective pellets were collected, washed once with 50 mM-Tris-HCl buffer, pH 7.8, containing 10 mM-MgCl₂ and 1 mM-2-mercaptoethanol, and finally suspended in the same buffer to a final protein concentration of 10–20 mg/ml. The results obtained are summarized in Table 1. Most of the total activity assayed at pH 5 was in the 6000–38000g fraction. Therefore in routine preparations, after grinding with alumina, the 3000g centrifugation was eliminated and the 6000–38000g 20 min pellet was used throughout all the work. If after the grinding with alumina, MgCl₂ was omitted from the buffer during the following centrifugations, the final preparation yielded only 15–20% of the protein obtained by the standard method and the specific activity was only 50%. A similar result was obtained if sodium acetate buffer, pH 5, was used instead of Tris-HCl buffer, pH 7.8.

Penicillinase activity

This was measured as described by Novick (1962).

Paper electrophoresis

Whatman 3MM paper was used and if required for preparative purposes washed as described before (Nieto & Perkins, 1971). Electrophoresis was usually carried out at 10 V/cm, 2–3 h in 0.25 M-formic acid, pH 1.9. The peptides α -Ac-L-Lys-D-Ala-D-[¹⁴C]Ala and ϵ -Ac-L-Lys-D-Ala-D-[¹⁴C]Ala did not separate from alanine in this system. They were separated at

Table 1. Protein contents and DD-carboxypeptidase activity in the different fractions obtained by differential centrifugation

DD-Carboxypeptidase activity was determined as described in the standard assay with UDP-MurNAc-L-Ala-D-isoGlu-meso-A₂pm-D-Ala-D-[¹⁴C]Ala (0.33 mM) as substrate and 20–30 µg of protein. Incubation time was 30 min.

Fraction	Protein content (mg of protein/g dry wt. of cells)	DD-Carboxy- peptidase activity (nmol/h per mg of protein)
3000–6000g, 10 min	4.8	30
6000–38000g, 20 min	9.3	163
38000–100000g, 30 min	2.2	140

higher voltages (60 V/cm) in pyridine–acetic acid–water (1:10:1000, by vol.) buffer, pH 3.5.

Electron microscopy

The microscope was a JEM 100 B (Jeol Ltd., Tokyo, Japan). Copper grids coated with a thin carbon film [5–7 nm (50–70 Å)] were used and the preparation was negatively stained with 1% ammonium molybdate in 2% ammonium acetate, pH 7.2.

Results

Particulate enzyme preparation

A membrane fraction that contained DD-carboxypeptidase activity was prepared from cultures of *B. megaterium* KM. The standard preparation (obtained as described in the Materials and Methods section) was the fraction sedimenting between 6000 and 38000g for 20 min. Electron-microscope examination of this preparation showed it to consist predominantly of membrane fragments and vesicles of sizes ranging from about 100 to 500 nm, and a few flagella and lipid-like droplets, probably poly-β-hydroxybutyrate granules. It thus seems correct to refer to the particulate DD-carboxypeptidase as membrane-bound activity. Washing the 6000–38000g fraction once with 50 mM-Tris-HCl buffer, pH 7.8, containing 10 mM-MgCl₂ and 1 mM-2-mercaptoethanol removed non-active protein preferentially without loss of enzyme so that the specific activity was increased slightly, but further washing was without effect. Therefore the standard particulate preparation used in this work was washed only once with the above buffer before being resuspended in the same buffer, pH 7.8, or in the sodium acetate buffer, pH 5, used in the DD-carboxypeptidase assay. Three successive washes with 50 mM-sodium acetate

buffer, pH 5, removed only 1.5% of the membrane-bound protein and 0.4% of the activity. The standard membrane-bound enzyme preparation had a specific activity of 450 ± 50 nmol of substrate hydrolysed/h per mg of protein (in eight independent preparations) when 30–80 µg of protein were incubated under standard assay conditions with Ac₂-L-Lys-D-Ala-D-Ala (1.7 mM), for 1 h, and 170 ± 20 nmol/h per mg when UDP-MurNAc-pentapeptide (0.33 mM) was incubated for 30 min with 20–30 µg of membrane protein under the same conditions. The membrane preparation was stored at –20°C. Under these conditions the enzymic activity remained constant for more than 1 year whether stored in buffer at pH 7.8 or pH 5.0. However, frequent freezing and thawing tended to decrease the stability of the enzymic activity with concomitant onset of proteolysis, as judged by the appearance of free amino acids and peptides which were absent in the original standard membrane preparation. Therefore after preparation of the membrane it was distributed in many tubes in the quantities required for DD-carboxypeptidase assay, frozen and then thawed only once.

The specific activity of DD-carboxypeptidase in membrane preparations obtained from cells from cultures varying from early exponential phase to stationary phase was the same within experimental error. However, cultures at a concentration of 0.5–0.8 mg dry wt./ml were convenient to use because cells from older cultures were more difficult to break by alumina grinding.

pH-activity profile

The pH-activity profile for the membrane-bound DD-carboxypeptidase activity is shown in Fig. 1. At low ionic strength (*I* = 0.04) and in the presence of 3 mM-Mg²⁺ the optimum activity was at pH 5.2. Without Mg²⁺ a much flatter curve was obtained, with a maximum at pH 6, which could be somewhat increased to a maximum at pH 7 by raising the ionic strength to *I* = 0.79.

Ionic-strength requirements

The dependence of the enzymic activity on the concentration of sodium acetate buffer, pH 5.0, is shown in Fig. 2. The activity showed an optimum at around 40 mM and decayed slowly at higher concentrations of buffer. The result was identical regardless of whether nucleotide precursor or Ac₂-L-Lys-D-Ala-D-Ala was used as substrate.

Effect of bivalent cations at pH 5

The standard membrane was prepared and stored in 10 mM-MgCl₂. To study the effect of bivalent cations on the DD-carboxypeptidase activity, those

Fig. 1. Dependence on pH of the DD-carboxypeptidase activity

Incubation mixtures contained, in a final volume of 30 μ l, the following (final concns.): (A) (low ionic strength with Mg^{2+}), buffer (40mM) $Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala$ (1.44mM), $MgCl_2$ (3mM) and membranes (124 μ g of protein); $\bullet$, sodium acetate; $\circ$, sodium cacodylate; $\square$, Tris-HCl. (B) (Cation-deprived membranes at low ionic strength). Membranes were incubated with EDTA and dialysed as described in the text. The incubation mixtures were as in (A), except that $MgCl_2$ was omitted. Symbols as for (A). (C) (Cation-deprived membranes at high ionic strength). Membranes, treated as in (B), were incubated in buffers of final ionic strength 0.79, with no added $MgCl_2$. $\bullet$, Sodium acetate; Δ , sodium phosphate. Incubations were for 15 min at 35°C. All activities shown are as a percentage of the activity found at pH 5.2 at low ionic strength and in the presence of 3 mM- Mg^{2+} (condition A).

already present in the membrane preparation had to be removed. This was carried out by treating the membranes with EDTA (40mM) followed by dialysis against a large volume of 50mM-Tris-HCl buffer, pH 7.8, containing EDTA (2mM). Finally the membranes were dialysed against 50mM-Tris-HCl buffer, pH 7.8, to eliminate EDTA. Table 2 shows the effect of these treatments on the DD-carboxypeptidase activity. Deprivation of bivalent cations either by performing the assay in the presence of EDTA, or by preincubating with EDTA for 30min or overnight, or by dialysis as indicated above decreased the activity at pH 5 to about 20% of the original. If $MgCl_2$ was reintroduced the activity was almost completely restored (Table 2).

The effect of several bivalent cations on the activity of the enzyme was examined by using bivalent cation-deprived membranes as described above. The results are shown in Table 3.

Fig. 2. Ionic strength-dependence of the DD-carboxypeptidase activity at pH 5

Incubation mixtures contained, in a final volume of 30 μ l (final concns.), sodium acetate buffer, pH 5.0; $MgCl_2$ (4mM), and ($\circ$) UDP-MurNAc-L-Ala-D-isoGlu-meso-A₂-p₂-D-Ala-D-[^{14}C]Ala (0.33mM) and membrane protein (28 μ g), or ($\bullet$) $Ac_2-L-Lys-D-Ala-D-Ala$ (1.8mM) and membrane protein (82.3 μ g). Incubations were at 35°C for 30 min in the case of the labelled nucleotide precursor or 1 h for the standard substrate. Activity is expressed as a percentage of the specific activity at a buffer concentration of 40mM. This was 180nmol/h per mg of protein for the nucleotide and 350nmol/h per mg for the standard substrate and corresponded to an overall hydrolysis of 25% and 53% of the total substrate respectively.

The best activators were Mg^{2+} , Ca^{2+} , Mn^{2+} and Co^{2+} . These were equally effective in increasing the activity. Zn^{2+} was only about 50% as effective and Cu^{2+} and Hg^{2+} were poisons. Fig. 3 shows the dependence of the activity of the enzyme on the concentration of $MgCl_2$. A plateau of activity was reached beyond a concentration of about 3mM.

Linearity of enzymic activity at pH 5

The DD-carboxypeptidase activity assayed at pH 5 was linear with membrane protein concentration up to at least a value of 3.0mg/ml and with time up to about 2h. The conditions for the assay were as described in the Materials and Methods section, with standard substrate (1.7mM).

Effect of 2-mercaptoethanol and p-chloromercuribenzoate

The presence of up to 200mM-2-mercaptoethanol in the DD-carboxypeptidase standard assay mixtures did not have any effect on the activity of the enzyme. However, preincubation of the membrane for 2h in the same buffer with 200mM-2-mercaptoethanol at 25°C decreased the DD-carboxypeptidase activity by about 48% relative to a control without added thiol. Preincubation of the membrane preparation with

Table 2. *Inhibition of DD-carboxypeptidase activity at pH 5 by Mg²⁺ deprivation, and reversal of the inhibition*

Expt. 1. The control contained (final concns.) sodium acetate buffer, pH 5 (40 mM), MgCl₂ (10 mM), standard substrate (1.7 mM) and standard membrane protein (48 µg) in a final volume of 30 µl. The experiment was identical except that it contained only the MgCl₂ added with the membrane protein (final concn. 0.4 mM) and EDTA was added to a final concn. of 2–30 mM. Incubation was for 1 h at 35°C. Under these conditions increasing the EDTA concentration beyond 2 mM or preincubating the membrane with EDTA for 30 min or overnight at 4°C or 25°C did not make any difference. Expt. 2. To a suspension of standard membranes in 50 mM-Tris-HCl buffer, pH 7.8–10 mM-MgCl₂ (200 µl; 24.1 mg of protein/ml), EDTA (7.9 µmol), was added and the mixture left at room temperature for 1 h. It was then dialysed against 1 litre of 50 mM-Tris-HCl buffer (pH 7.8)–2 mM-EDTA, at 4°C overnight. Protein concentration in the dialysis bag became 13 mg/ml. The membrane suspension was then dialysed against 1 litre of the same buffer without EDTA. After the second dialysis the protein concentration of the membrane in the dialysis bag was 7 mg/ml. The control was untreated standard membrane as in Expt. 1. Incubation mixture 1 was as the control, but no Mg²⁺ was added and dialysed membrane (35 µg protein) was used instead of the standard membrane. Incubation 2 was as incubation 1 but MgCl₂ was added to a final concn. of 10 mM. Final volume was 30 µl in all cases and incubations were for 1 h at 35°C.

	Specific activity (nmol/h per mg)	Activity (%)
Expt. 1		
Control	390	100
Experiment	68	17.4
Expt. 2		
Control	395	100
Incubation 1	75	19
Incubation 2	373	94.5

sodium *p*-chloromercuribenzoate (0.5 mM) for 30 min or longer totally abolished the activity of the enzyme.

Temperature inactivation of the pH 5 enzyme

To test the stability of the enzyme with the temperature the membrane preparation suspended in the assay mixture was preincubated for 30 min without substrate in a thermostated water bath at the desired temperatures. Substrate was then added and the reaction mixtures were incubated at 35°C for 1 h. Fig. 4 shows that the activity was stable up to about 35°C, then decayed quite sharply and was totally inactivated by preincubation at 35°C.

Activity of the enzyme as a function of the temperature

The result of carrying out the incubation with the substrate at different temperatures is shown in Fig. 5.

The enzyme increased its activity with temperature until that at which thermal inactivation started (Fig. 4) and then decayed very rapidly.

Table 3. *Effect of the nature of the bivalent cation on the membrane-bound DD-carboxypeptidase activity at pH 5*

Control was as in Table 4. In all other cases bivalent-cation-depleted membrane, obtained as described in Table 4, Expt. 2, was used. Incubation mixtures contained (final concns.) sodium acetate buffer, pH 5.0, (40 mM), membrane protein (42 µg), MgCl₂ (10 mM) and standard substrate (1.7 mM) in a final volume of 30 µl. Incubations were for 60 min at 35°C. Less than 30% of the substrate was used in all cases. M stands for bivalent cation.

M	Specific activity (nmol/h per mg)	Activity (%)
Control	386	100
Depleted membrane	84	22
Mg ²⁺	345	90
Ca ²⁺	355	92
Mn ²⁺	320	83
Co ²⁺	340	88
Zn ²⁺	195	50
Fe ²⁺	66	17
Pb ²⁺	63	16
Cu ²⁺	9	2
Hg ²⁺	0	0

Fig. 3. *Dependence of the enzyme activity at pH 5 on the MgCl₂ concentration*

Incubations contained (final concns.) sodium acetate buffer, pH 5, (40 mM), UDP-MurNAc-L-Ala-D-isoGlu-meso-A₂pm-D-Ala-D-[¹⁴C]Ala (0.33 mM), bivalent-cation-depleted-membrane protein (28 µg) and MgCl₂ at the concentration shown in a final volume of 30 µl. Incubations were for 30 min at 35°C.

Fig. 4. Thermal inactivation of the DD-carboxypeptidase. Membrane protein (60 μ g), in a final volume of 30 μ l of 40 mM-sodium acetate buffer-3 mM-MgCl₂, was pre-incubated at the desired temperatures for 30 min. Standard substrate (50.7 nmol) was then added and the mixture incubated at 35°C for 1 h. Final volume was 33 μ l. The D-alanine liberated was determined by dinitrophenylation, and the activity expressed as a percentage of a control that was not preincubated.

Fig. 5. Activity of the enzyme as a function of the temperature. Membrane protein (75 μ g) and Ac₂-L-Lys-D-Ala-D-[¹⁴C]-Ala (18 nmol) in a final volume of 30 μ l of 40 mM-sodium acetate buffer (pH 5.0)-3 mM-MgCl₂ were incubated for 30 min at the desired temperature. The D-[¹⁴C]alanine liberated was determined as described in the Materials and Methods section.

Fig. 6. Carboxypeptidase II and DD-carboxypeptidase activity at pH 5

Assay mixtures contained, in a final volume of 30 μ l, the following (final concns.): sodium acetate buffer, pH 5.0, (40 mM), MgCl₂ (3 mM), membrane protein (73 μ g) and (○) UDP-MurNac-L-Ala-D-isoGlu-meso-A₂pm-D-Ala-D-[¹⁴C]Ala or (●) UDP-MurNac-L-Ala-D-isoGlu-meso-A₂pm-D-[³H]Ala-D-Ala (both 2.2 mM). Incubations were at 35°C. The release of radioactive D-alanine was measured as described in the Materials and Methods section.

Carboxypeptidase II activity

An enzyme that hydrolysed a C-terminal LD linkage in a peptidoglycan precursor that had lost its terminal D-alanine residue was originally observed in *Escherichia coli* and termed carboxypeptidase II (Izaki & Strominger, 1968). Similar activity was sought in the membrane preparations from *B. megaterium*, by using Ac₂-L-Lys-D-Ala (2.2 mM) as substrate and other conditions as for the DD-carboxypeptidase activity, but no activity was found. When DD-carboxypeptidase and carboxypeptidase II were assayed simultaneously at pH 5 under identical conditions with UDP-MurNac-L-Ala-D-isoGlu-meso-A₂pm-D-Ala-D-[¹⁴C]Ala as substrate and the same nucleotide precursor ending in D-[³H]Ala-D-Ala the carboxypeptidase II activity was extremely weak compared with the DD-carboxypeptidase activity and did not need to be taken into account when the DD-carboxypeptidase was assayed by the dinitrophenylation method (Fig. 6).

Kinetics of the DD-carboxypeptidase at pH 5.0

The values of K_m and V_{max} were determined from initial-velocity studies by means of double-reciprocal plots, by using substrate concentrations ranging from 0.2 to 20 mM. Assay conditions were standard (see the Materials and Methods section) and the values obtained were $K_m = 1.6$ mM; $V_{max} = 500$ nmol/h per

Table 4. *Specificity profile of the DD-carboxypeptidase (pH5): influence of the side chains in the C-terminal dipeptide*

Assays were carried out as indicated in the text. The average specific activity on the standard substrate, Ac₂-L-Lys-D-Ala-D-Ala was 470 nmol/h per mg of protein. Under the conditions used activity was linear with time. In some experiments Triton X-100 (1% final concn.) was added to the assay mixtures. Under these conditions 30% of the protein and 50–60% of the activity could not be sedimented at 150000 g for 2 h. The values given represent the percentage of the velocity at which peptide 1 was hydrolysed in the absence of detergent.

Peptide	Specific activity (%)	
	Buffer	1% Triton X-100
1. Ac ₂ -L-Lys-D-Ala-D-Ala	100	117
2. Ac ₂ -L-Lys-D-Ala-Gly	74	
3. Ac ₂ -L-Lys-D-Ala-D-Leu*	0	0
4. Ac ₂ -L-Lys-D-Ala-L-Ala	0	
5. Ac ₂ -L-Lys-D-Ala-D-Lys	0	
6. Ac ₂ -L-Lys-D-Ala-D-Glu	0	
7. Ac ₂ -L-Lys-L-Ala-D-Ala	0	0
8. Ac ₂ -L-Lys-D-Leu-D-Ala	0	
9. Ac ₂ -L-Lys-Gly-D-Ala	6.7	14
10. Ac ₂ -L-Lys-D-Glu-D-Ala	0	
11. Ac ₂ -L-Lys-D-Leu-D-Leu	0	

* In the t.l.c. of Dnp-Leu the solvent used was benzene-ethyl acetate (10:1, v/v).

Table 5. *Specificity profile of the DD-carboxypeptidase (pH5): influence of the residue that precedes the C-terminal dipeptide D-Ala-D-Ala*

Experimental conditions as in Table 4.

	Specific activity (%)	
	Buffer	1% Triton X-100
1. Ac ₂ -L-Lys-D-Ala-D-Ala	100	117
12. α -Ac-L-Lys-D-Ala-D-[¹⁴ C]Ala	12	
13. ϵ -Ac-L-Lys-D-Ala-D-[¹⁴ C]Ala	2.8	
14. L-Lys-D-Ala-D-[¹⁴ C]Ala	0	
15. Ac-Gly-D-Ala-D-Ala	0	
16. Ac ₂ -L-A ₂ bu-D-Ala-D-Ala	34	53
17. Ac ₂ -L-Orn-D-Ala-D-Ala	1.4	5.4
18. N-Ac-L-Tyr-D-Ala-D-Ala	49	
19. R- <i>meso</i> -A ₂ pm-D-Ala-D-Ala*	85	
20. D-Glu- γ -L-Lys-D-Ala-D-Ala	15	
21. L-Ala-D-Glu- γ -L-Lys-D-Ala-D-Ala	15	
22. α -Ac-L-Lys-D-Ala-D-Ala-NH ₂	0	

* R, UDP-MurNAc-L-Ala-D-isoGlu-.

mg of protein for the natural precursor UDP-MurNAc-L-Ala-D-isoGlu-*meso*-A₂pm-D-Ala-D-Ala, and $K_m = 0.8$ mM, $V_{max} = 590$ nmol/h per mg of protein for the standard substrate Ac₂-L-Lys-D-Ala-D-Ala.

Substrate specificity

To study the specificity profile, the enzyme (70 μ g of protein) was incubated with the peptide at a final concentration of 1.4–1.8 mM as indicated in the Materials and Methods section. Incubations were for 60 min except when radioactive peptides were used. In the latter case incubations were for 30 min. For all peptides parallel incubations under identical conditions with the standard substrate were carried out and the extent of cleavage of the C-terminal peptide bond was expressed as a percentage of that for Ac₂-L-Lys-D-Ala-D-Ala. Except when radioactive peptides were used, the extent of cleavage of the terminal amino acid was measured by the dinitrophenylation technique. The results are shown in Tables 4 and 5. The enzyme required a free carboxyl group (peptide 22) and showed considerable specificity with regard to the side chains of residues 1 and 2 (Table 4). When D-alanine in the terminal position was replaced by glycine, L-alanine, D-leucine, D-lysine or D-glutamic acid, only the peptide containing glycine served as a substrate (peptides 2–6). Substitutions at the second residue again showed that the peptide containing glycine was a substrate, though a poor one, whereas peptides with D-leucine, D-glutamic acid or L-alanine were not (peptides 7–10).

The residue preceding the C-terminal dipeptide also had a strong influence on the enzymic activity. When the amino groups of the lysine residue were left unsubstituted the peptide became a poor substrate (peptides 12, 13 and 14). A free α -amino group (peptide 13) was more effective in preventing enzymic activity than the same group in the side chain (peptide 12). A comparable substrate in which the free amino group in the side chain was in the α -position to a carboxyl group (peptide 19) was quite readily attacked by the enzyme. For peptides with no free amino groups on the third residue (peptides 1, 15, 16, 17 and 18) the length of the side chain was critical. Thus peptide 15 with no side chain was not a substrate, but when the side chain was AcNH(CH₂)₂– enzyme activity was quite good (peptide 16). Increasing this side chain to AcNH(CH₂)₃– made the substrate an extremely poor one (peptide 17) whereas a further increase to AcNH(CH₂)₄– produced the best substrate tested (peptide 1). Surprisingly perhaps, a peptide with an aromatic side chain in residue 3 (peptide 18) was a fairly good substrate. So far as one can tell from this limited series, amino acids beyond residue 3 had little or no influence on the enzymic activity (compare peptides 12, 20 and 21).

Inhibition by peptides

The DD-carboxypeptidase activity at pH5 was inhibited by a wide range of synthetic peptides as shown in Table 6. Although dipeptides with a free amino group were sometimes better inhibitors than the same peptides acetylated (compare peptides 23 and 26), a tripeptide with two free amino groups at residue 3 was neither a substrate nor an inhibitor (Tables 5 and 6, peptide 14). An increase in the size of D side chains in residues 1 and 2 prevented the peptides from being substrates (Table 5), but whereas a larger side chain than methyl in residue 2 also prevented binding of the peptide to the enzyme (since these peptides are not inhibitors; Table 6, peptides 8 and 10) this was not the case with residue 1 (peptides 3, 5, 6, 26, 27 and 28). If we compare peptides 27 and 28, and 6 and 26 it is clear that the addition of residue 3 improved considerably the inhibitory properties of the peptides. Probably an interaction of the peptide backbone at this position was responsible for the increased affinity for the enzyme, because although peptide 28 had no side chain at residue 3, it was a good inhibitor.

In previous work (Nieto *et al.*, 1973a) it was shown that the soluble DD-carboxypeptidases from *Streptomyces* strains R61 and *albus* G were inhibited by peptides in which the terminal D-Ala-D-Ala had been replaced by the lactam of diaminoadipic acid to make an analogue having the two methyl side chains linked covalently. Since ring formation compels the peptide bond to adopt the *cis* configuration, it was proposed that this configuration was required in the C-terminal amide bond of substrate peptides before they could combine with these enzymes. On the other hand, the enzyme from *Streptomyces* strain R39 was unaffected by the cyclic analogue peptides. To see whether such an argument might be applied to the enzyme from *B. megaterium*, peptides terminating in cyclodiaminoadipic acid were tested as inhibitors (Table 6, peptides 29 and 30). The DD-carboxypeptidase activity at pH5 was inhibited about 40% by acetyl cyclodiaminoadipic acid, when the ratio of DD-isomer to substrate was 10.7. This degree of inhibition is intermediate between those observed for the enzymes from *Streptomyces* strains *albus* G and R61 (Nieto *et al.*, 1973a).

Effect of 'solubilization' of enzyme by detergent

To see whether the specificity of the membrane-bound enzyme might be partly attributable to relative inaccessibility of some substrates, experiments were conducted in the presence of 1% Triton X-100. Under the conditions used, this detergent 'solubilized' about 30% of the membrane protein and 50–60% of the DD-carboxypeptidase activity. Material was taken to be 'soluble' if it could not be sedimented by centrifuging at 150000g for 2h. Results in Tables 4,

Table 6. Inhibition by peptides of the DD-carboxypeptidase activity at pH5

The assay mixtures contained, membrane protein (49 µg, 2 µl), Ac₂-L-Lys-D-Ala-D-[¹⁴C]Ala (18 nmol, 2 µl), 0.12 M-sodium acetate buffer (pH 5.0)–9 mM-MgCl₂ (10 µl), inhibitor (1–5 µl) and water to a final volume of 30 µl. Incubation was for 1 h at 35°C. Under these conditions 40% of the substrate was hydrolysed in a control without any inhibitor. I/S is the molar ratio inhibitor/substrate. None of the peptide inhibitors listed was a substrate. Inhibition is 100 (A₀–A)/A₀, A being the alanine liberated in the presence of inhibitor and A₀ that in its absence. As in Tables 4 and 5, in some experiments Triton X-100 was introduced to a final concn. of 1%.

	Inhibition (%)		
	I/S	Buffer	1% Triton X-100
3. Ac ₂ -L-Lys-D-Ala-D-Leu	11.3	12	
5. Ac ₂ -L-Lys-D-Ala-D-Lys	8.5	20	
6. Ac ₂ -L-Lys-D-Ala-D-Glu	8.3	62	
8. Ac ₂ -L-Lys-D-Leu-D-Ala	9.3 7.0	0	0
10. Ac ₂ -L-Lys-D-Glu-D-Ala	11.1	0	
14. L-Lys-D-Ala-D-Ala	10	0	
23. D-Ala-D-Glu	7.2 14.2	39 59	
24. D-Ala-D-Asp	8.9	54	
25. Ac-D-Ala-D-Ala	6.8	29	
26. Ac-D-Ala-D-Glu	10.5	15	
27. Ac-D-Ala-D-Asp	8.9	50	
28. Ac-Gly ₃ -D-Ala-D-Asp	5.5 8.3	55	70
29. Ac-racemic-cyclodiaminoadipic acid	10.7* 21.4*	17 39	
30. Ac ₂ -L-Lys-racemic-cyclodiaminoadipic acid	11.4* 22.8*	16 27	

* On the assumption that the LL configuration would be ineffective, the concentration of the inhibitory DD-isomer would be one-half the values quoted.

5 and 6 show that the detergent did not greatly influence the relative activity of the enzyme on several substrates, nor did it appear to affect the inhibitory power of Ac-Gly₃-D-Ala-D-Asp. We conclude that the results reported in Tables 4–6 are indicative of the intrinsic sensitivity of the enzyme to various substrates and inhibitors, and not to the fact that the enzyme is membrane-bound.

Inhibition of DD-carboxypeptidase (pH5) by β-lactam antibiotics

(a) Penicillinase activity of the membrane. The membrane of *B. megaterium* KM had penicillinase

activity. Its kinetic parameters under Novick's (1962) assay conditions (0.05M-potassium phosphate buffer, pH5.7) were $K_m = 0.9\text{mM}$, $V_{max} = 0.25\mu\text{mol/h per mg}$ with penicillin G as substrate. In the buffer used for the assay of the DD-carboxypeptidase (pH5) the penicillinase activity was decreased by 49% ($0.091\mu\text{mol/h per mg}$) at a penicillin concentration of 0.25mM and a membrane protein concentration of 0.123mg/ml. This activity was weak and since the DD-carboxypeptidase was inhibited by concentrations of penicillin G far below the K_m of the membrane penicillinase (see Table 7), the effect of the penicillinase was disregarded as a first approximation.

(b) *DD-Carboxypeptidase activity in the presence of antibiotics*. The effect of seven β -lactam antibiotics on the DD-carboxypeptidase assayed under standard conditions is shown in Table 7. Penicillin G was the most effective inhibitor, followed by carbenicillin; the two cephalosporins were rather poor inhibitors by comparison. If the assay at pH5 was performed with membrane deprived of bivalent cations by treatment with EDTA and subsequent dialysis as described above, the DD-carboxypeptidase activity remaining was equally as sensitive to penicillin G as that in the presence of 3mM-MgCl₂.

Table 7. *Inhibition of DD-carboxypeptidase (pH5) by β -lactam antibiotics*

The assay mixture contained, in a final volume of 30 μ l, the following (final concns.): membrane protein (73 μ g), sodium acetate buffer (40mM), MgCl₂ (3mM) and antibiotic at the concentration shown in the Table. The mixture was preincubated at 20°C for 30min then Ac₂-L-Lys-D-Ala-D-Ala (1.6mM) was added and incubation continued for 1h at 35°C. Controls without antibiotic but otherwise identical were performed side by side with the experiment and the inhibition was expressed as in Table 6.

Antibiotic	Concentration (mol/l)	Inhibition (%)
Ampicillin	10 ⁻⁵	0
	10 ⁻⁴	35
	10 ⁻³	100
Carbenicillin	10 ⁻⁶	0
	10 ⁻⁵	31
	10 ⁻⁴	90
Dicloxacillin	10 ⁻⁶	0
	10 ⁻⁵	23
	10 ⁻⁴	44
Methicillin	10 ⁻⁴	13
	10 ⁻³	80
	10 ⁻²	100
Penicillin G	10 ⁻⁶	36
	10 ⁻⁵	86
	10 ⁻³	100
Cephalexin	10 ⁻⁴	15
	10 ⁻³	23
	10 ⁻²	100
Cephaloridine	10 ⁻⁴	0
	10 ⁻³	26

Reversal of the inhibition of DD-carboxypeptidase (pH5) by penicillin G

To see whether inhibited enzyme would become active spontaneously, perhaps through hydrolysis of the antibiotics, incubation mixtures with 10 μ M-penicillin G were set up as described in Table 7, but the preincubation period was extended to 1, 2, 4 and 24h, before adding substrate and assaying the enzyme as usual. All were inhibited to the same extent (83–86%) as a control preincubated for only 30min with the antibiotic. Reversal of penicillin inhibition by 2-mercaptoethanol has been reported for a DD-carboxypeptidase from *Bacillus subtilis* (Lawrence & Strominger, 1970). We therefore examined whether 2-mercaptoethanol would prevent inhibition when added before penicillin G or reverse the inhibition when added afterwards. Incubation mixtures like those described in Table 7, (a) were made 0.2M with respect to 2-mercaptoethanol, then 10 μ M with respect to penicillin G and preincubated for 30min; substrate was then added and the enzyme assayed as usual; (b) were made 10 μ M with respect to penicillin G and after 30min at 20°C 2-mercaptoethanol (0.2M)

Fig. 7. *Reversal by dialysis of the inhibition of DD-carboxypeptidase by penicillin G at pH5*

A mixture containing (final concns.) sodium acetate buffer, pH 5.0, (40mM), MgCl₂ (3mM), membrane protein (625 μ g) and penicillin G (10mM) was incubated for 30min at 20°C. It was then transferred to a dialysis bag and dialysed against the same buffer used for incubation (1 litre) at 20°C. As a control an identical sample was treated in the same manner but without penicillin G. At the times stated in the Figure samples (30 μ l) were withdrawn from the dialysis bags for standard DD-carboxypeptidase assay (substrate Ac₂-L-Lys-D-Ala-D-Ala, 1.6mM) and protein determination (15 μ l samples). Activity is expressed as 100 $\times$ A/A₀, where A was the specific activity of a sample withdrawn from the experiment and A₀ that taken from the control after identical periods of dialysis.

was added and incubated at the same temperature for a further 2 h. Substrate was then added and the activity of the enzyme measured. The inhibition in (a) was as in a control with no thiol reagent added (i.e. 83–86%) and in experiment (b) the inhibition was even greater (95%).

Reversal of the inhibition was achieved by dialysing the penicillin G-treated membranes against a large volume of buffer at 20°C. Recovery of activity showed a sigmoid relationship with time (Fig. 7), 53% of the initial activity being recovered after dialysis overnight.

In similar experiments with two other β -lactam antibiotics, in which the recovery of activity after dialysis for 4 h was examined, the following results were obtained: 1 mM-carbenicillin produced 100% inhibition initially, which declined to 24% after dialysis; 10 mM-cephaloridine, produced 94% inhibition initially, which declined to 73% after dialysis.

Discussion

If we compare the substrate requirements of the membrane-bound DD-carboxypeptidase from *B. megaterium*, active at pH 5, with those reported for soluble DD-carboxypeptidases from *Streptomyces* species (Leyh-Bouille *et al.*, 1970, 1971, 1972) the main difference observed is the exacting requirement of the bacillary enzyme for the C-terminal dipeptide of the substrate (Table 4). Some substitutions at residue 1 allowed binding to the enzyme, because many of the non-substrate peptides in Table 4 were inhibitors (Table 6), but at residue 2 binding was prevented by increasing the size of the side chain. A side chain of L configuration in residue 3 was necessary to induce enzymic activity where the C-terminal dipeptide allowed hydrolysis (Table 5), and also a third residue enhanced the inhibitory power of appropriate peptides (Table 6). At residue 3, an aliphatic side chain without a free ω -amino group was preferred for enzyme activity but its length appeared to be critical (compare peptides 1, 16 and 17, Table 5). This tendency, but much less marked, could also be observed in the DD-carboxypeptidase from *S. albus* G (Leyh-Bouille *et al.*, 1970). Finally, as with the *Streptomyces* enzymes, residues 4 and 5 did not have any influence, positive or negative and, therefore were probably away from the enzyme surface (Table 5, peptides 12, 20 and 21). From the limited series of substrates available, it seems reasonable to conclude that favourable interactions contributing to the binding of a peptide to the enzyme were a polar interaction with the α -carboxyl group of residue 1, and interactions with the side chains of residues 1 and 2 and with the peptide bonds formed by residue 3. On the other hand, repulsive forces controlling binding probably were: steric repulsions for L side chains at residue 1 and 2 as well as for D side chains larger than a methyl group at residue 2; perhaps an

ionic repulsion of positive charges at residue 3. Once the peptide was bound to the enzyme the size, shape and charge of the side chains of residues 1 and 3 appeared to control whether the peptide would be hydrolysed or not.

The inhibition of activity caused by peptide analogues terminating in cyclodiaminoadipic acid (Table 6 peptides 29 and 30), similar to that observed with the enzymes from *Streptomyces* strains *albus* G and R61 suggests that the *B. megaterium* enzyme, too, may recognize the *cis* configuration of the C-terminal sequence. If so, then the mechanism of hydrolysis should also be similar to that proposed for the *Streptomyces* enzymes (Nieto *et al.*, 1973a).

So far as it was tested, the 'solubilization' of the enzyme from *B. megaterium* by addition of Triton X-100 did not affect the specificity of substrates or inhibitors. It seems likely therefore that the observed differences from the soluble *Streptomyces* enzymes are inherent in the enzyme itself and not attributable to the fact that the enzyme from *B. megaterium* is membrane-bound.

Inhibition by β -lactam antibiotics

The DD-carboxypeptidase activity at pH 5 was sensitive to β -lactam antibiotics. Inhibition at pH 5 did not appear to occur through penicilloylation because it was not reversed by thiol compounds but, at least for the two penicillins studied, was readily reversible on dialysis. Wickus & Strominger (1972) working with *B. megaterium* QMB 1551 found two carboxypeptidase activities one of which was irreversibly inhibited by penicillin G but was insensitive to cloxacillin, and another that was also insensitive to cloxacillin but was stimulated by penicillin G. They carried out their assays at pH 8.5 and the activities were not further characterized. Therefore a strict comparison with our work is not possible. Such striking differences between two different strains of the same micro-organism deserve further examination.

The membrane-bound DD-carboxypeptidase of *Bacillus stearothermophilus*, active at pH 6.5, was also inhibited by β -lactam antibiotics (Barnett, 1973). The inhibition of fresh enzyme was partially reversed by washing and completely reversed by treatment with β -lactamase followed by washing. Hence the results with *B. stearothermophilus* to some extent resemble the present ones with *B. megaterium*. Storage of the enzyme from *B. stearothermophilus* at -20°C caused a slow loss of catalytic activity and a rapid loss of penicillin sensitivity (Barnett, 1973). The present enzyme preserved its activity unchanged during storage at -20°C and there was no evidence for any change in penicillin sensitivity.

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