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Purification of a Nocardicin A-Sensitive LD-Carboxypeptidase from *Escherichia coli* by Affinity Chromatography

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An LD-carboxypeptidase releasing the terminal D-Ala from UDP–MurNAc–L-Ala–D-Glu–m–A_2pm–D-Ala (UDP-MurNAc-tetrapeptide) was purified from *Escherichia coli* to biochemical homogeneity and characterized biochemically. Final purification was achieved by nocardicin A-Sepharose affinity chromatography. An apparent molecular weight of 32,000 was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the enzyme, which seems to be a monomeric protein as indicated by gel filtration. The optimum pH of the enzyme was 8.4, and the pI was 5.5. The K_m for UDP-MurNAc-tetrapeptide was 1.5×10^{-4} M, and the V_{max} was 0.4 nmol/min. Nocardicin A inhibited the enzyme competitively, with a K_i of 5×10^{-5} M. Benzylpenicillin, cephalosporin C, thienamycin, and D-alanyl-D-alanine did not affect the enzyme activity. Possible functions of the enzyme for growth and division of the murein sacculus are discussed.

A wide range of bacterial carboxypeptidases cleaving off terminal alanine residues from the peptide substituents of murein have been described (28). Three DD-carboxypeptidase activities in *Escherichia coli*, are known to specifically hydrolyze the D-alanyl-D-alanine terminus of the pentapeptidyl sidechain –L-Ala–D-Glu–m–A_2pm–D-Ala–D-Ala (13). Being penicillin-sensitive enzymes, they belong to the family of penicillin-binding proteins (PBPs). Whereas PBP 5 and PBP 6 are specific carboxypeptidases (29), PBP 4 has been found, in addition to its DD-carboxypeptidase activity, to hydrolyze the murein-cross-linking DD-peptide bond between D-Ala and m-A_2pm, thus also showing endopeptidase activity (19).

The DD-carboxypeptidases have been studied in considerable detail as a model system for the interaction of β-lactams with the active sites of penicillin-sensitive enzymes (9, 16). However, the specific function of these carboxypeptidases in the process of growth and division of the murein sacculus is still not understood. A regulatory role is indicated by their interference with the levels of pentapeptidyl residues, which represent the donors for the crucial murein cross-linking reaction catalyzed by DD-transpeptidases (14). Experimental evidence seems to support this proposal (21, 25), and interestingly mutants lacking all three DD-carboxypeptidases have not been obtained yet.

Penicillin-insensitive LD-carboxypeptidases that remove the terminal D-Ala from the tetrapeptidyl moiety -L-Ala–D-Glu–m–A_2pm–D-Ala have also been identified in *E. coli* (1, 15, 23, 24). One LD-carboxypeptidase present in *E. coli* has been purified and determined to be a monomeric protein with a molecular mass of 12 kDa (24). Another LD-carboxypeptidase has been partially purified and reported to be an 86-kDa protein consisting of two 43-kDa polypeptides (2). This enzyme can easily be extracted from cells by Tris-EDTA, suggesting a localization in the periplasm (1). In synchronized cultures, the enzyme was found to be most active at the time of cell division. The activity of the enzyme has been proposed to be controlled by a still unidentified inhibitor (2).

Recently, an attractive model in which the LD-carboxypeptidase determines whether the cell elongates or divides by controlling the level of tripeptidyl murein precursors has been put forward (3). Therefore, we decided to purify the enzyme to biochemical homogeneity as a prerequisite for the attempt to clone and map the gene and ultimately to construct proper mutants.

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MATERIALS AND METHODS

Strain and growth conditions. *Escherichia coli* W3110 was grown in Luria broth with aeration at 37°C and harvested during the logarithmic growth phase.

LD-carboxypeptidase assay. To assay for LD-carboxypeptidase activity, we used as a substrate UDP–MurNAc–L-Ala–D-Glu–m–A_2pm–D-Ala (UDP-MurNAc-tetrapeptide), prepared as described previously (18). Samples were incubated in the presence of 2.5 mM UDP-MurNAc-tetrapeptide in 0.1 M Tris-HCl buffer, pH 8.0, in a total volume of 100 μl for 20 min at 37°C. The reaction was terminated by heating the samples in a boiling water bath for 3 min.

UDP-MurNAc-tripeptide and UDP-MurNAc-tetrapeptide, the product and uncleaved substrate of the enzyme reaction, respectively, were separated by reversed-phase high-pressure liquid chromatography (HPLC) (18). Before HPLC fractionation, 5 μl of 20% H_3PO_4 and 30 μl of 0.05% trifluoroacetic acid were added to the samples, and precipitated material was removed by centrifugation. An aliquot (120 μl) of the supernatant was applied to a column (12.5 by 4.6 cm) prepacked with 5-μm Hypersil ODS (Bischoff, Leonberg, Germany). The column was eluted at room temperature with a flow rate of 1 ml/min by using a linear gradient of 1.2 to 12% acetonitrile in 0.05% trifluoroacetic acid in H_2O that was built up in 20 min. This was followed by washing the column with 60% acetonitrile in 0.05% trifluoroacetic acid for 7.5 min and by reequilibrating with starting buffer for 17.5 min. The eluent was monitored by UV A_{205} readings.

One unit of enzyme activity was defined as the amount of LD-carboxypeptidase required to release 1 nmol of D-alanine per min from UDP-MurNAc-tetrapeptide under the standard conditions of the assay described above.

Preparation of nocardicin A-Sepharose. For the prepara-

tion of nocardicin A-Sepharose, CNBr-activated Sepharose 4B (4.5 g; Pharmacia-LKB, Freiburg, Germany) was swollen and washed according to the recommendations of the manufacturer, first in 1 mM HCl and then with coupling buffer (0.1 M $NaHCO_3$, pH 8.0, containing 0.5 M NaCl). Nocardicin A (190 mg; Fujisawa Pharmaceutical Co., Osaka, Japan), dissolved in 22 ml of coupling buffer containing 0.5 M NaCl, was gently shaken with the Sepharose beads for 6 h at room temperature. The efficiency of the reaction was controlled by recording the UV absorbance of the supernatant between 350 and 190 nm before and after the coupling step. The remaining active groups were blocked in 0.1 M Tris-HCl, pH 8.0, overnight. The nocardicin-Sepharose conjugate was ready for column chromatography after three washings with 0.1 M sodium acetate buffer (pH 4.0) containing 0.5 M NaCl, alternating with 0.1 M Tris-HCl buffer (pH 8.0) with 0.5 M NaCl, and a final wash with 10 mM potassium phosphate buffer (pH 7.2).

Enzyme purification. To isolate the enzyme, the following purification scheme was established. All procedures were performed at 4°C.

(i) Preparation of a crude cell extract. A cell extract was prepared from 75 g (wet weight) of *E. coli* W3110 cells resuspended in 150 ml of 2 mM potassium phosphate buffer, pH 7.2, containing 2 mM dithioerythritol (DTE). After the addition of DNase I (5 μg/ml) the cells were disrupted in a precooled French pressure cell at 15,000 lb/in^2. Cells and cellular debris were spun down at 200,000 × *g* for 45 min, and the supernatant was dialyzed against the buffer mentioned above.

(ii) DEAE-cellulose chromatography. The crude extract (2.6 g of protein at a concentration of 6.5 mg/ml) was loaded on a DEAE-cellulose (DE52; Whatman) column (2.4 by 26 cm) equilibrated with 2 mM potassium phosphate buffer, pH 7.2, containing 2 mM DTE. The column was washed first with 5.5 column volumes of equilibration buffer and then with 2 times the column volume of 20 mM potassium phosphate buffer, pH 7.2, containing 2 mM DTE (starting buffer). The enzyme was eluted at a flow rate of 105 ml/h with a linear gradient ranging from 600 ml of starting buffer to 600 ml of 80 mM potassium phosphate, pH 7.2, containing 2 mM DTE. The enzyme eluted at about 38 mM phosphate buffer. The pooled active fractions were filled into a dialysis bag and kept embedded in polyethylene glycol 20000 until the preparation was concentrated to 32 ml. The enzyme was finally dialyzed against 25 mM histidine-HCl buffer, pH 6.2.

(iii) Chromatofocusing. The enzyme preparation obtained by DEAE-cellulose chromatography (286.7 mg of protein at a concentration of 8.96 mg/ml) was fractionated further on a polybuffer exchanger 94 column (Pharmacia LKB, Uppsala, Sweden). The column (0.9 by 38.5 cm) was washed with 25 mM histidine-HCl buffer (pH 6.2), and then before the sample was applied, 5 ml of polybuffer 74 (pH 4.0), diluted 1:10, was applied to the column. The enzyme was eluted at flow rate of 18.9 ml/h with 495 ml of polybuffer 74 (pH 4.0) diluted 1:10. Fractions (6.3 ml) were collected. The LD-carboxypeptidase eluted at a pH of 5.5. The active fractions were pooled, concentrated with polyethylene glycol 20000 as described above, and dialyzed against 10 mM potassium phosphate buffer (pH 7.2) containing 2 mM DTE.

(iv) Nocardicin A-Sepharose affinity chromatography. Nocardicin A-Sepharose, prepared as described above, was packed into a column (18 by 0.9 cm) and equilibrated with 10 mM potassium phosphate buffer, pH 7.2, containing 2 mM DTE. The dialyzed enzyme pool from the chromatofocusing step was applied to the column and eluted at a flow rate of 4.8 ml/h. Fractions (1.2 ml) were collected. The fractions containing LD-carboxypeptidase activity (Fig. 1) were pooled, concentrated with polyethylene glycol 20000 to about 2.5 ml, and purified further by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

(v) Preparative SDS-PAGE. Final purification was obtained by preparative SDS-PAGE (20) on a 10 to 18% polyacrylamide gradient gel (1.5 mm thick; 16 by 18 cm). About 200 μg of protein was applied, and electrophoresis was performed at a constant current of 25 mA until the tracking dye was 0.5 cm from the bottom of the gel. After the gel was stained with Coomassie blue, the 32-kDa band was eluted electrophoretically with a high-salt-barrier electroelution device (HSB-Elutor; Biometra, Göttingen, Germany). The protein turned out to be homogeneous by analytical SDS–10 to 18% polyacrylamide gradient gel electrophoresis, showing a single band after silver staining (26) (Fig. 2).

RESULTS AND DISCUSSION

Purification of LD-carboxypeptidase. The purification of LD-carboxypeptidase was hampered by the fact that the enzyme is extremely labile when diluted in the course of purification, even in the presence of 2 mM DTE. Furthermore, the enzyme is a minor component of the cell proteins, making up less than 0.1% of the extracted soluble proteins. The specific release of the enzyme by Tris-EDTA treatment of whole cells (2) turned out to be of no advantage for enzyme purification on a preparative scale, since the yield was rather low (less than 10%) compared with that of a total crude extract obtained after mechanical breakage of the cells in a French pressure cell. All attempts to purify the enzyme by standard protein purification methods failed. Since it has been shown that nocardicin A, a monocyclic β-lactam antibiotic, inhibits LD-carboxypeptidase activity in *Gaffkya homari* (12) as well as in *E. coli* (7, 23, 24), we finally tried to purify the enzyme with immobilized nocardicin A. Indeed, by affinity chromatography on nocardicin A-Sepharose, sufficient enrichment of the LD-carboxypeptidase was obtained, allowing a clear-cut identification of the enzyme by SDS-PAGE despite the presence of an additional protein (Fig. 1A). The protein migrating with an apparent molecular mass of 32 kDa coelutes from the column with LD-carboxypeptidase, as shown in Fig. 1. It is interesting to note that a second protein of about 36 kDa (Fig. 1B) was copurified with the carboxypeptidase by nocardicin affinity chromatography. By raising the salt concentration of the elution buffer, a third protein with an apparent molecular mass of approximately 34 kDa could be eluted from the column (data not shown). Although these proteins were inactive in the LD-carboxypeptidase assay when UDP-MurNAc-tetrapeptide was used as a substrate, their interaction with nocardicin A may indicate that they are related to LD-carboxypeptidases.

A summary of the purification procedure is given in Table 1, and results are shown in Fig. 2. Active enzyme protein was enriched by 1,500-fold, and the final yield was approximately 15%. Electroelution of the band corresponding to LD-carboxypeptidase (Fig. 1) resulted in a homogenous protein preparation, yielding a single band on an SDS-polyacrylamide gel when stained with silver nitrate (Fig. 2B).

Properties of purified LD-carboxypeptidase. The apparent molecular mass of the homogeneous native enzyme, as determined by gel filtration on Sephadex G-100 Superfine, is approximately 39 kDa (Fig. 3). In analytical SDS-PAGE

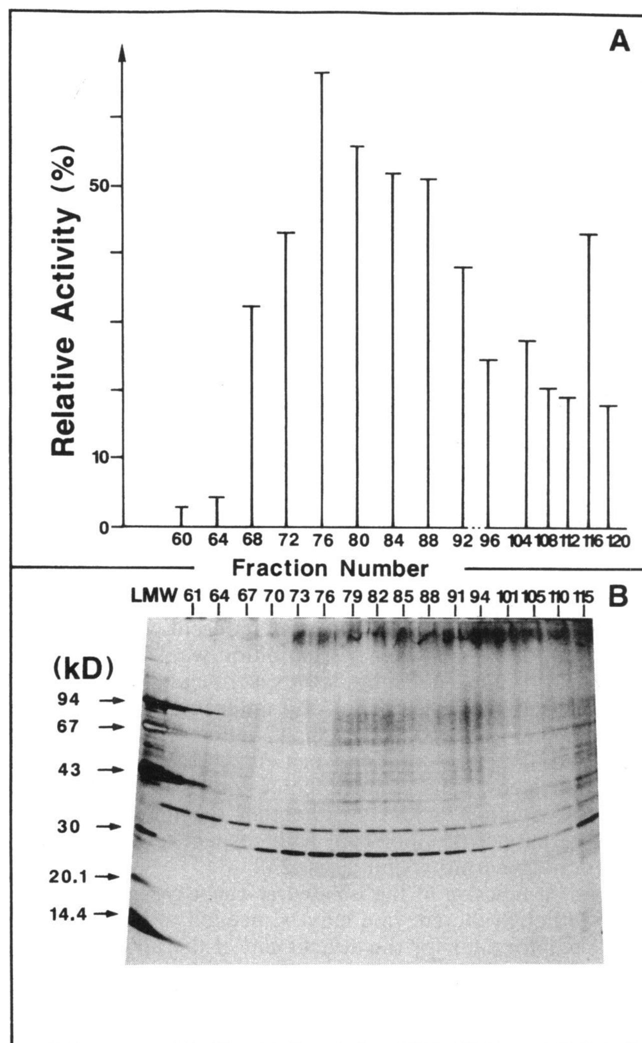

FIG. 1. Protein composition and LD-carboxypeptidase activity in the fractions eluting from nocardicin A-Sepharose. (A) LD-carboxypeptidase activity in aliquots of the fractions. (B) Protein analysis of aliquots of the fractions by SDS-PAGE (10 to 18% polyacrylamide gradient) with silver nitrate staining. Low-molecular-weight markers (LMW) were as described in the legend to Fig. 2.

(Fig. 2B) calibrated with a set of low-molecular-weight standard proteins, the purified, denatured protein showed a single band at a position corresponding to a molecular mass of 32 kDa (Fig. 3). We conclude that the native enzyme is a monomeric protein.

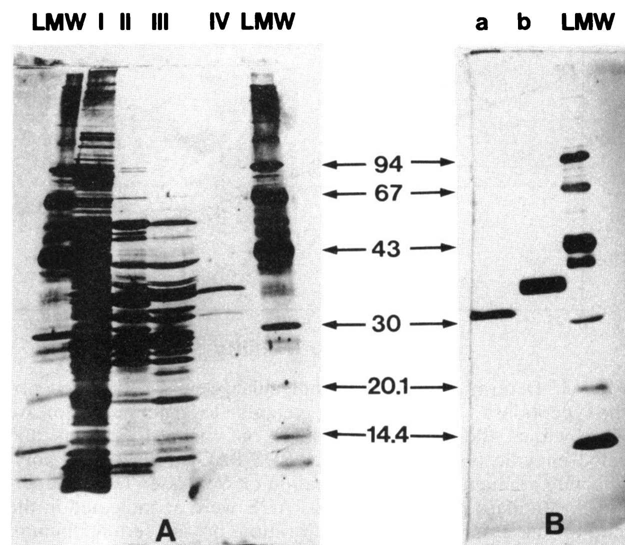

FIG. 2. Analysis of the different stages of purification of the LD-carboxypeptidase from *E. coli* by SDS-PAGE (10 to 18% polyacrylamide gradient) with silver nitrate staining. (A) Lanes: I, crude extract (50 μg); II, DEAE-cellulose (40 μg); III, chromatofocusing (20 μg); IV, nocardicin A-Sepharose (2 μg); LMW, low-molecular-weight markers (given in thousands; from top to bottom, phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin). (B) Lanes: a, LD-carboxypeptidase electroeluted by preparative SDS-PAGE (see Materials and Methods); b, unknown protein copurified with LD-carboxypeptidase by nocardicin A-Sepharose chromatography (see lane IV in panel A), electroeluted by preparative SDS-PAGE; LMW, as described for panel A.

The enzyme shows high specificity for muramyl tetrapeptides. UDP-MurNAc-pentapeptide did not act as a substrate for the enzyme at all. This is in contrast to a 34-kDa enzyme described as present in *E. coli* that releases D-alanyl-D-alanine from the activated pentapeptide precursor (11).

The enzymatic activity of the LD-carboxypeptidase was maximal at pH 8.4 in 0.1 M Tris-HCl buffer (Fig. 4). This is consistent with earlier reports by Izaki and Strominger (15) as well as by Metz et al. (24). The isolectric point of the enzyme could be estimated during the chromatofocusing step (see above). The enzyme eluted from the column at a pH of 5.5, which is identical to the isoelectric point of the LD-carboxypeptidase.

At a substrate concentration of 138 μM and an enzyme concentration of 4.8 U/ml, the LD-carboxypeptidase activity was linear with time up to 20 min. The K_m and V_{max} values were determined by measuring the initial reaction velocity with various concentrations of UDP-MurNAc-tetrapeptide.

TABLE 1. Purification of LD-carboxypeptidase from *E. coli* W3110[a]

Step	Vol (ml)	Protein (mg)	Enzyme[b]		Enrichment (fold)	Recovery (%)
			Amt (U)	Concn (U/mg)		
Crude extract	400	2,600	25,000	9.6	1	100
DEAE-cellulose	32	287	16,000	55.8	5.8	64
Chromatofocusing	15	9.86	13,125	1,331.1	138.7	52.5
Nocardicin A-Sepharose	27	0.41	3,881.3	9,466.5	986.1	15.5

[a] LD-Carboxypeptidase was purified from 75 g (wet weight) of frozen cells harvested during exponential growth with aeration in Luria broth at 37°C.
[b] Enzyme units are defined in Materials and Methods.

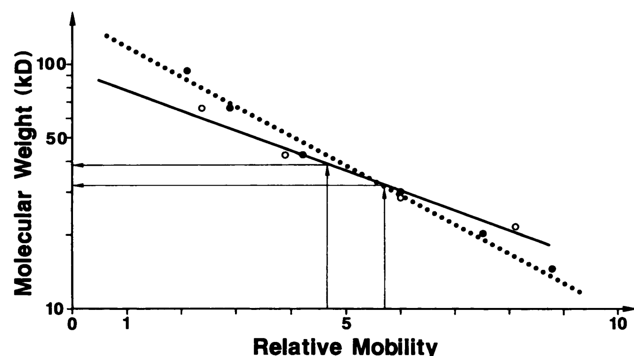

FIG. 3. Determination of the molecular weight of purified LD-carboxypeptidase. The molecular weights (logarithmic scale) are plotted versus the relative mobilities of standard proteins and purified enzyme obtained by either SDS-PAGE (10 to 18% polyacrylamide gradient) (●) or gel filtration on Sephadex G-100 Superfine (○). Standard proteins in SDS-PAGE were as indicated in the legend to Fig. 2; in the case of gel filtration, bovine serum albumin, ovalbumin, carbonic anhydrase, and trypsin inhibitor were used.

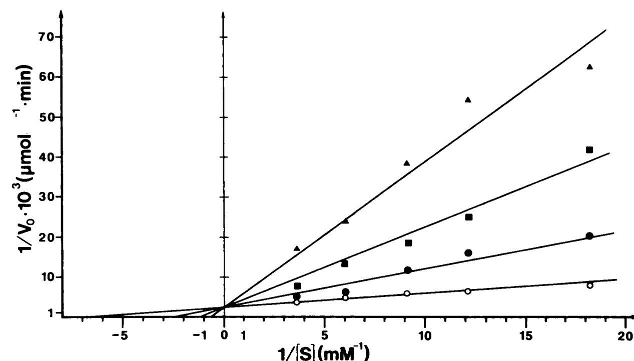

FIG. 5. Michaelis-Menten kinetics of purified LD-carboxypeptidase. The enzyme assays were performed under standard conditions as described in Materials and Methods. Lineweaver-Burk plots of the uninhibited enzyme reaction (○) and of the reaction in the presence of 50 (●), 100 (■), and 250 (▲) μg of nocardicin A per ml are shown. V_0, initial reaction velocity; [S], substrate concentration.

From a Lineweaver-Burk plot (Fig. 5), an apparent K_m of 1.5 $\times 10^{-4}$ M and a V_{max} of 0.4 nmol/min were calculated. Izaki and Strominger (15) determined a K_m of 1×10^{-4} M and Metz et al. (24) calculated a K_m of 1.08×10^{-4} M for LD-carboxypeptidase activity present in *E. coli*.

Benzylpenicillin, cephalosporin C, thienamycin, and D-alanyl-D-alanine did not inhibit the activity of the purified LD-carboxypeptidase (Table 2). The slight stimulation observed with some compounds is not considered to be of any significance. In agreement with Metz et al. and with Criegee and Hammes (7, 23, 24), inhibition of the enzyme was seen in the presence of nocardicin A, a β-lactam derivative containing a D-amino acid substituent. The antibiotic inhibited the enzyme by 60% at a 0.2 mM concentration. A K_i of 5×10^{-5} M was calculated from the Lineweaver-Burk plots of the initial reaction velocity in the presence of various concentrations of the substrate and different concentrations of nocardicin A (Fig. 5). The inhibition was shown to be a competitive one. The fact that the enzyme could be released from immobilized nocardicin A by simply eluting the column with 10 mM potassium phosphate buffer (see above) indicates that the interaction between enzyme and nocardicin A is noncovalent. This result is consistent with the proposal that the D-amino acid substituent of the antibiotic is the reactive part of the molecule with respect to its inhibitory effect on LD-carboxypeptidases (23).

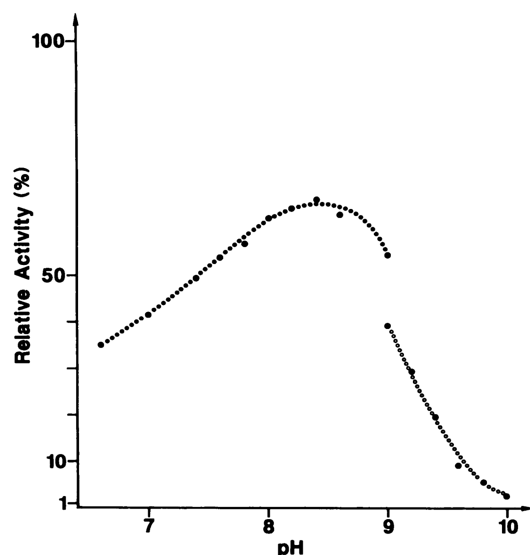

FIG. 4. Effect of pH on activity of purified LD-carboxypeptidase of *E. coli*. The buffers used were 0.1 M Tris-HCl buffer (●●●●) (pH 6.6 to 9.0) and 0.1 M $NaCO_3$ buffer (○○○○) (pH 9.0 to 10.0). Enzyme activity was assayed as described in Materials and Methods.

Possible function of the isolated LD-carboxypeptidase in *E. coli*. Although the enzyme may be needed to create tripeptide acceptor sites for the attachment of the lipoprotein (5), it seems more likely to have some regulatory function in murein metabolism. Consequently, it has been speculated that the LD-carboxypeptidase, by determining the amount of tripeptide side chains in the murein precursors, controls whether *E. coli* elongates or divides (3). This tempting model seems to be supported by earlier results indicating a fluctuation in the activity of the LD-carboxypeptidase during the cell cycle (2). An increase in activity was found at the time of cell division, as would be expected according to the model. Furthermore, it has been proposed that tripeptide side chains are the preferred acceptors for the cross-linking

TABLE 2. Effects of various compounds on the activity of LD-carboxypeptidase[a]

Compound added	Concn (μg/ml)	Relative activity (%)
None		100
D-Alanyl-D-alanine	100	116.3
	250	138.5
Cephalosporin C	100	108.5
	250	97.5
Nocardicin A	100	38.1
	250	23.3
Thienamycin	100	116.6
	250	122.9
Benzylpenicillin	100	101.0
	250	99.7

[a] Enzyme activity was assayed under standard conditions as described in Materials and Methods.

reaction catalyzed by PBP 3, the enzyme responsible for the synthesis of the septum (27). Both experimental results, however, may not be correct. The use of toluene-treated cells to determine the enzyme activity during the cell cycle could lead to differences in the measurements of LD-carboxypeptidase activity due to changes in the permeabilities of dividing cells relative to those of elongating cells. The apparent participation of UDP-MurNAc-tripeptide precursors in the in vitro murein synthesis is likely to be the result of a conversion of the tripeptide precursors to UDP-MurNAc-pentapeptides, which then become incorporated into high-molecular-weight murein. At least, according to the results of experiments performed in our laboratory under the conditions described in the literature (27), this was the case (17).

It also seems possible that the isolated enzyme that catalyzes an LD-carboxypeptidase reaction in vitro catalyzes a transpeptidase reaction in vivo. Such an activity is needed for the formation of the LD-A_2pm–A_2pm cross bridges found in the murein of *E. coli*, in particular in stationary cells (10).

Finally, the enzyme could be responsible for hooking the lipoprotein onto the murein (4, 6, 31). Despite many efforts to isolate such an enzyme, all attempts failed until now (8, 22, 31, 32). In analogy to the DD-transpeptidase reaction, in which pentapeptides play the role of the energy donor (14, 30), tetrapeptides may function as energy donors for the covalent attachment of the lipoprotein to murein. A lipoprotein-adding enzyme may therefore cleave the peptide bond between m-A_2pm and D-Ala of the tetrapeptide (donor), with a concomitant formation of a tripeptidyl-enzyme intermediate which reacts with a free ε-amino group of the carboxyl-terminal L-lysine of the lipoprotein (acceptor) (4, 6).

Any proposal for a physiological function of the LD-carboxypeptidase must be considered speculative as long as proper mutants are lacking. Amino acid sequence analysis of the LD-carboxypeptidase, purified to biochemical homogeneity, is in progress. Cloning of the gene by means of synthetic oligonucleotides may finally lead to the construction of mutants suitable for investigating the in vivo function of this enzyme in *E. coli*.

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