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Substrate specificity of *Escherichia coli* LD-carboxypeptidase on biosynthetically modified muropeptides

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Abstract

Escherichia coli murein can be biosynthetically modified. Amino acids at positions 3 and 4 (*m*-diaminopimelic acid and D-alanine, respectively) on the peptide moieties can be changed under appropriate growth conditions. The activity of *E. coli* LD-carboxypeptidase on biosynthetically modified substrates has been studied in vitro. The enzyme hydrolysed all tested disaccharide-tetrapeptide monomeric muropeptides modified at position 4. Monomers with *m*-lanthionine, but not with L-ornithine, instead of *m*-diaminopimelic acid at position 3 were accepted. However, neither cross-linked muropeptides nor macromolecular murein were substrates for the reaction. Our observations argue against a direct effect of LD-carboxypeptidase on macromolecular murein metabolism.

Key words: Escherichia coli; Carboxypeptidase; Murein; Peptidoglycan, D-Amino acid

1. Introduction

Metabolism of the peptidoglycan (murein) bacterial sacculus requires a large number of dedicated enzymes. Murein hydrolases split specific bonds in murein, murein precursors and murein degradation products [1]. Carboxypeptidases specifically remove the D-Ala residues in the peptide side chain (*R*-L-Ala-D-Glu-(γ)-*m*A2pm-D-Ala-D-Ala) of muropeptides. DD-carboxypeptidase I releases the C-terminal D-Ala from pentapeptides (MUR-5) producing tetrapeptides (MUR-4) [2,3]. LD-Carboxypeptidases (LD-CPase) cleave the LD peptide bond binding the L center of *m*A2pm to D-Ala in MUR-4, yielding tripeptides (MUR-3). A periplasmic protein (M_r = 32,000) with LD-CPase activity has been recently purified in *Escherichia coli* [4,5].

The function of LD-CPase is under discussion [1,5,6]. LD-CPase activity apparently oscillates along the cell cycle with a maximum at the time of septation [7]. Cell division models in which cyclic oscillations of MUR-3 play a key role have been postulated [8,9]. However, the possibility of a biosynthetic origin for MUR-3 has been proposed [10].

Natural amino acids at positions 3 and 4 in the side chain of muropeptides can be biosynthetically replaced by other compounds in *E. coli*. Lanthionine can substitute for *m*A_2pm at position 3 by growing *m*A_2pm auxotrophs in the presence of the analogue [11]. Cells grown in the presence of certain D-amino acids (D-Met, D-Phe, D-Trp, etc.) accumulate new muropeptides in which the D-Ala at position 4 is replaced by a residue of the D-amino acid [12]. Modification-prone residues are those connected by the LD-CPase-sensitive bond. Hence, modification of murein at the indicated positions might alter LD-CPase activity in vivo. Regular composition of murein is quickly recovered upon transfer of cells to normal media [13]. A direct involvement of LD-CPase in the recovery of normal composition appears to be likely a priori. Therefore we investigated the activity of LD-CPase on modified muropeptides and murein in vitro, to obtain further information on the enzyme function.

2. Materials and methods

2.1. Preparation of substrates and analysis of peptidoglycan composition

Native and D-amino acid-modified mureins were prepared as described [12,14] from cultures of *E. coli* MC6RP1 (K12, F⁻, *proA leuA thr dra drm lysA thi*) [15] grown for four generations in LB medium [16] and LB medium plus the D-amino acid (3 mg/ml), respectively. Lanthionine-modified murein was prepared from *E. coli* W7 (*dapA lysA*) [17] cultures grown in LB medium supplemented with lanthionine (40 μg/ml) for ten generations. Ornithine-containing muropeptides were purified from murein of *Thermus thermophilus* ([18]; manuscript in preparation). Muramidase (Cellosyl, Hoecht, Germany) digests of normal and modified mureins were fractionated by HPLC on an Hypersil RP18 column (3 μm particle size, 250 × 4 mm) (Teknochroma, Barcelona, Spain) as described [14,19]. For analytical purposes quantification of individual muropeptides was performed by automated integration of the A_{204} peak areas. For preparative purposes the muropeptides of interest were collected, desalted by HPLC [20], lyophilized, dissolved in water, and quantified by chemical determination of the amount of the di-amino acid [21]. It is important to note that muropeptides purified by this method have the *N*-acetylmuramic acid residues reduced to *N*-acetyl muramitol due to the preparation procedure for HPLC.

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2.2. LD-Carboxypeptidase assay

Assays for LD-CPase activity were as described [5] except for the substrates used. Reaction mixtures consisted of: 50 μl enzymatic extract, 20 μl 0.5 M Tris-HCl, pH 8.0, and an appropriate volume of substrate solution in distilled water. Volume was made up to 100 μl by the addition of distilled water. *N*-Acetylglucosaminyl-*N*-acetylmuramitoyl- L-Ala-D-Glu-(γ)-*m*A_2pm-D-Ala (disaccharide-tetra) was used as the substrate of reference and for detection of the enzyme at 270 μM final concentration. Samples were incubated for 60 min at 37°C. Reactions were stopped by heating the samples in a boiling water bath for 5 min. Sample pH was lowered by the addition of 7 μl 20% H_3PO_4 and 30 μl 0.05% trifluoroacetic acid. Precipitated material was removed by centrifugation and reaction products were separated and quantified by reverse-phase HPLC on a Hypersil ODS (3 μm particle size, 250 × 4 mm) (Teknochroma, Barcelona, Spain) column. The column was eluted at 40°C, 1 ml/min flow rate, with linear gradients of acetonitrile in 0.05% trifluoroacetic acid. The column was washed and equilibrated as described [5]. The exact concentrations and slopes of the gradients were optimized for each substrate–product pair using mixtures of the corresponding muropeptides.

2.3. Preparation of extracts enriched in LD-CPase activity

The method was as described by Ursinus et al. [5] except that FPLC chromatography was used as the last step instead of affinity chromatography. Frozen cells (70 g, wet weight) of *E. coli* MC6RP1 were thawed and resuspended in 110 ml of 2 mM potassium phosphate buffer, pH 7.2, containing 2 mM dithioerythritol and 10 μg/ml DNase I. Cells were broken with the help of a pre-cooled (4°C) French pressure cell at 110 MPa. The extract was subjected to DEAE-cellulose chromatography on a 2.4 × 30 cm column, as described [5]. Activity eluted between 35 and 40 mM potassium phosphate as previously reported [5]. Protein concentration in the active extract (60 ml) was 0.8 mg/ml as measured by the BCA Protein Assay Reagent method (Pierce, Ill, USA) with an enzyme activity, expressed as defined by Ursinus et al. [5], of 33.1 U/mg, and 70% yield. The extract was further enriched by FPLC chromatography on a Mono-Q HR 5/5 column (Pharmacia LKB, Uppsala, Sweden) as shown in Fig. 1. Active fractions were pooled and kept frozen (−70°C) until used. The active pool had a protein concentration of 12 μg/ml, and a specific activity of 334 U/mg. Yield was 5%.

3. Results and discussion

3.1. Digestion of modified muropeptides by LD-carboxypeptidase

The ability of LD-CPase to hydrolyze modified muropeptides was first checked in a series of tests where each purified muropeptide was digested with the enzyme for 60 min at a substrate concentration of 270 μM. The structure of assayed muropeptides as well as the results of the digestion are shown in Tabel 1. The most interesting observation was the resistance of dimeric muropeptides to LD-CPase action, irrespective of whether they were cross-linked by LD (*m*A2pm–*m*A2pm) or DD (D-Ala–*m*A2pm) peptide bridges. As expected, enzyme activity was clearly influenced by the nature of the amino acids at positions 3 and 4 in MUR-4 monomeric muropeptides. Lanthionine containing MUR-4 was more readily digested than the naturally occurring *m*A2pm-MUR-4. In contrast, L-ornithine containing MUR-4 was not accepted as a substrate at all. Substitutions at position 4 also influenced the activity of the enzyme but none of those checked impeded LD-CPase activity, suggesting that substrate recognition is more stringent at position 3 than at 4. Interestingly substitution of D-Ala by the achiral amino acid, Gly, resulted in almost complete digestion, indicating that LD-CPase does not have an absolute requirement for a D-amino acid at position 4. However, the peptide bond between *m*A2pm and the achiral ε-NH_2 group of Lys in muropeptides with the C-terminal dipeptide of lipoprotein (Arg–Lys) was not hydrolysed, arguing against an involvement of LD-CPase in lipoprotein metabolism.

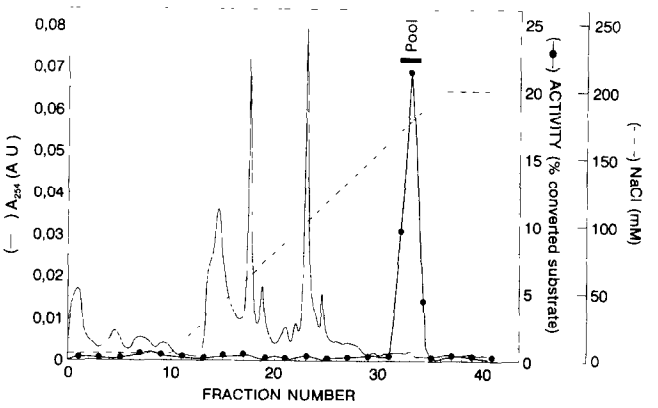

Fig. 1. Preparation of LD-carboxypeptidase enriched extracts by FPLC chromatography. Samples (5 ml) of the LD-CPase pool prepared by chromatography on DEAE-cellulose were injected into a Mono-Q HR 5/5 (Pharmacia LKB, Uppsala, Sweden) column equilibrated in 20 mM Tris-HCl, pH 7.3, at a flow rate of 0.5 ml/min. The column was washed for 10 min, adsorbed proteins were eluted with a 0–200 mM NaCl linear gradient in the same buffer (14 ml total volume) followed by 5 min under final conditions. Fractions (0.5 ml) were collected and assayed for LD-CPase activity as indicated in section 2.

The apparent K_m and V_{max} values of the enzyme for the muropeptides accepted as substrates are shown in Table 2. Digestion times were reduced to 20 min, and digestion of each muropeptide was performed at six different concentrations covering the range from 0.0125 to 0.5

Table 1
Activity of LD-carboxypeptidase on modified muropeptides

Muropeptide[a]	Substrate converted[b] (%)
R-*m*A2pm-D-Ala	56
R-*m*A2pm-D-Phe	25
R-*m*A2pm-D-Met	17
R-*m*A2pm-Gly	91
L-Arg-L-Lys-(ε)-*m*A2pm-*R*	0
R-L-Ornithine-D-Ala	0
R-*m*-Lanthionine-D-Ala	74
R-*m*-Lanthionine-D-Phe	27
R-*m*A2pm-D-Ala-*m*A2pm-*R* | D-Ala	0
R-*m*-Lanthionine-D-Ala-*m*-Lanthionine-*R* | D-Ala	0
R-*m*A2pm-*m*A2pm-*R* | D-Ala	0

[a] *R* = *N*-acetylglucosaminyl-*N*-acetyl-muramitoyl-L-Ala-D-Glu.
[b] Substrate concentration was 270 μM in all instances.

Table 2
Apparent kinetic parameters of LD-carboxypeptidase for modified muropeptides

Muropeptide[a]	K_m (mM)	V_{max}[b] (nmol · min^{-1} · μg^{-1})	r[c]
R-*m*A2pm-D-Ala	0.20	0.279	0.995
R-*m*A2pm-D-Phe	0.34	0.084	0.960
R-*m*A2pm-D-Met	0.42	0.038	0.941
R-*m*A2pm-Gly	1.02	3.191	0.940
R-*m*-Lanthionine-D-Ala	0.25	0.370	0.992
R-*m*-Lanthionine-D-Phe	0.27	0.061	0.931

[a] *R* = *N*-acetylglucosaminyl-*N*-acetyl-muramitoyl-L-Ala-D-Glu.
[b] Note that V_{max} refers to the amount of total protein as the exact amount of LD-CPase was unknown.
[c] Linear regression coefficients for the Hanes-Woolf plots of experimental data.

mM, in duplicate assays. The fraction of converted substrate was less than 15% in all instances. Values of the kinetic parameters were deduced from Hanes-Woolf plots of experimental data. The results were consistent with the experiments discussed above. The apparent K_m value found for *N*-acetyl-glucosaminyl-*N*-acetyl-muramitoyl- L-Ala-D-Glu-*m*A2pm-D-Ala (0.20 mM) was moderately higher than published values for the related compound UDP-*N*-acetyl-muramyl-L-Ala-D-Glu-*m*A2pm-D-Ala (0.1 mM) [5], in accordance with the observation that the later is a better substrate than the unreduced form of the disaccharide tetrapeptide [6].

In general the changes introduced in the peptide chain had more pronounced effects on V_{max} than on K_m, suggesting a more marked influence on peptide bond reactivity than on substrate recognition. Particularly curious was the case of the Gly-containing substrate in which a marked reduction in affinity was compensated for by a drastic increase in velocity.

3.2. *Digestion of macromolecular murein by LD-carboxypeptidase*

Once susceptibility of some soluble, modified muropeptides to LD-CPase activity was established, it was of interest to find out whether this enzyme could be involved in the elimination of D-amino acid-modified components from sacculi. The ability of LD-CPase to accept sacculi as substrate was checked by measuring the effect of the enzyme on the abundance of MUR-3 vs. MUR-4 in native and D-amino acid-modified murein. Purified sacculi (50 μg) were digested with enriched extract (100 μl) in a total volume of 200 μl for 2 h as above. Control samples without enzyme were treated in parallel. Samples were processed and analyzed as described in section 2. The paricular enzyme aliquot used in this experiment was checked in a standard assay run simultaneously. It was found to catalyze complete conversion of substrate (> 90%, for a 2 h incubation). According to the results obtained, LD-CPase did not accept sacculi as a substrate, neither native nor D-amino acid modified. In fact, the relative proportions of muropeptides with tripeptidic and tetrapeptidic side chains were virtually identical in digested and control samples in both cases. In this particular respect, LD-CPase would resemble the *E. coli* amidase, another periplasmic enzyme which only accepts solubilized muropeptides as substrates [1,22].

Assuming that the in vivo properties of LD-CPase were accurately reflected in the described in vitro experiments, the inability of the enzyme to accept sacculi as a substrate conditions its possible role in murein metabolism. In fact, it would rule out a direct involvement of the enzyme in the elimination of D-amino acids from modified murein and in the generation of MUR-3 muropeptides in the sacculus. The specificity of the enzyme, however, fits well with a degradative function in murein turn-over, as previously proposed [23]. If no other LD-CPase were present in *E. coli*, the origin of MUR-3 muropeptides would be difficult to understand. The fact that newly synthesized murein has a very low porportion of MUR-3 muropeptides [24] questions the possibility of LD-CPase generating MUR-3 in nascent linear peptidoglycan chains or at the lipid-linked precursor level. Studies performed in ether-treated cells lead to proposal of the existence of two LD-CPase activities in *E. coli* [25]. However, to our knowledge LD-CPase purification work performed up to now has not confirmed the second activity. The implications which the origin of MUR-3 muropeptides may have on murein biosynthesis and cell division models should stimulate further investigations on LD-CPase activities.

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