

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

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Received 26 November 1993; revised version received 7 January 1994

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)-*mA*₂pm-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 *mA*₂pm 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 *mA*₂pm at position 3 by growing *mA*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 μ g/ml) for ten generations. Ornithine-containing muropeptides were purified from murein of *Thermus thermophilus* ([18]; manuscript in preparation). Muramidase (Cellosyl, Hoechst, Germany) digests of normal and modified mureins were fractionated by HPLC on an Hypersil RP18 column (3 μ m particle size, 250 \times 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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Table 2

Apparent kinetic parameters of LD-carboxypeptidase for modified mucopeptides

| Muropeptide ^a | K_m (mM) | V_{max}^b (nmol · min ⁻¹ · μg ⁻¹) | r^c |
|-------------------------------|---------------|---|-------|
| <i>R</i> -mA2pm-D-Ala | 0.20 | 0.279 | 0.995 |
| <i>R</i> -mA2pm-D-Phe | 0.34 | 0.084 | 0.960 |
| <i>R</i> -mA2pm-D-Met | 0.42 | 0.038 | 0.941 |
| <i>R</i> -mA2pm-Gly | 1.02 | 3.191 | 0.940 |
| <i>R</i> -m-Lanthionine-D-Ala | 0.25 | 0.370 | 0.992 |
| <i>R</i> -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-mA2pm-D-Ala (0.20 mM) was moderately higher than published values for the related compound UDP-*N*-acetyl-muramyl-L-Ala-D-Glu-mA2pm-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 mucopeptides 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 particular 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 mucopeptides 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 mucopeptides 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 mucopeptides 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 mucopeptides would be difficult to understand. The fact that newly synthesized murein has a very low porportion of MUR-3 mucopeptides [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 mucopeptides may have on murein biosynthesis and cell division models should stimulate further investigations on LD-CPase activities.

Acknowledgements The technical assistance of J. de la Rosa is greatly appreciated. This work was supported by Grant BIO91-0523 from the CICYT and an institutional grant from Fundación Ramón Areces. J.I.L. was supported by a grant from the Basque Country Government.

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