Biochemical and Biophysical Research Communications xxx (2018) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The crystal structure and oligomeric form of *Escherichia coli* L,D-carboxypeptidase A

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ARTICLE INFO

Article history:
Received 5 February 2018
Accepted 26 March 2018
Available online xxx

Keywords:
Antibiotic
Crystal structure
Recycle pathway
Analytical ultracentrifugation

ABSTRACT

Bacterial peptidoglycan is constructed by cross-linking sugar chains carrying pentapeptide building blocks with two D-alanine residues at the C-terminus. Incorporation into the polymer and subsequent breakdown of peptidoglycan releases a tetrapeptide with a single D-alanine residue. Removal of this residue is necessary for the tripeptide to receive a new D-Ala-D-Ala dipeptide in the synthetic pathway, but proteases are generally unable to work with substrates having residues of unusual chirality close to the scissile bond. Processing of the tetrapeptide is carried out by a dedicated LD-carboxypeptidase, which is of interest as a novel drug target. We describe the high resolution crystal structure of the enzyme from *E. coli*, and demonstrate the dimeric structure is highly conserved.

1. Introduction

Both Gram positive and Gram negative bacteria possess a cell wall that includes a protective layer of peptidoglycan (PG), built from sugar chains cross-linked via short peptides.

Synthesis involves adding fresh units consisting of a disaccharide with an attached pentapeptide that generally terminates with two D-alanine residues. In Gram negative species the peptide has the sequence L-Ala-γ-D-Glu-m-A2pm-D-Ala-D-Ala, where m-A2pm stands for meso-diamopimelic acid [1,2]. Penicillin-binding proteins (PBPs) [3] are a large group of proteins responsible for the transpeptidation reaction that cross-links the peptides, and also include non-essential enzymes that trim the peptides, and so help to shape the cell and also recycle PG components [4,5]. The importance of PG to bacteria is underlined by the fact that numerous antibiotics, including the penicillins, function by blocking key enzymes involved in its synthesis. Penicillin and its analogues mimic the D-alanine residues to make suicide substrates [6].

PG is continually reworked as the cell grows and divides [7–9], so that during exponential growth bacterial cells release a considerable quantity of PG break-down products into the growth medium. Some of these cell-wall fragments function as messengers, for example as triggers of immune responses; the mammalian protein NOD1 detects specific PG fragments [10,11], initiating inflammatory responses. Hydrolases are found that can break each of the glycosidic or amide bonds in PG, and many appear to have overlapping roles [3,12]. This creates a functionally redundant system that is robust to mutation or chemical inhibition, and allows the cell to re-use much of the material in fresh PG synthesis. Lytic transglycosylases and endopeptidases release anhydromuropeptides that are transported into the cell by the AmpG permease before further degradation through the action of N-acetylglucosaminidase (NagZ), N-acetylmuramyl-L-alanine amidase (AmpD) and L,D-carboxypeptidase (LdcA) [12]. LdcA uniquely cleaves the peptide bond between the third and fourth residues of the tetrapeptide, releasing the C-terminal D-alanine [13].

It is estimated that about half of the PG breakdown products are recaptured in this way, to offset the very considerable metabolic cost of PG synthesis [14]. Growth in rich media affords bacteria the luxury of building PG entirely *de novo*, so that loss of recycling pathways does not impact the speed of cell multiplication. Both Gram positive and negative cells however are found to lyse in stationary phase if recycling is blocked [15,16]. This discovery has prompted efforts to develop specific inhibitors of LdcA for use as anti-bacterial agents with an entirely novel mechanism of action, and high-throughput screening conducted some years ago produced a lead candidate [17]. Since then it has become clear that PG recycling plays an important role in triggering the expression of beta-lactamase in pathogenic *Pseudomonas*, and further that strong impairment of PG recycling may severely weaken both fitness and

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https://doi.org/10.1016/j.bbrc.2018.03.195
0006-291X/

virulence [18,19]. Given the interest in LdcA as a possible novel drug target, different groups have determined crystal structures of the protein from *Pseudomonas aeruginosa* [20] and *Novosphingobium aromaticivorans* [21]. These models show the protein is a member of the Peptidase_S66 family (Pfam family PF02016). This family includes the self-immunity protein MccF, whose structure has also been solved by X-ray crystallography [22]. The oligomeric state of *Na*LdcA appears to be dimeric from the crystal structure, but size-exclusion chromotography indicated a monomer [21]. *E. coli* LdcA shows significant sequence differences from the known structures, and includes sequence insertions that might be involved in formation of homo- or hetero-oligomer formation (Supplementary Fig. 1). To clarify these issues we have solved the crystal structure of *Ec*LdcA and a mutant at the active site.

2. Materials and methods

2.1. Cloning

The wild-type *E.coli ldcA* gene was amplified from genomic DNA by PCR, and cloned into pET-28b expression vector between the restriction sites NdeI and BamH1 to allow expression of recombinant protein with a hexa-histidine tag at the N-terminus, cleavable with thrombin. The PCR product before and after restriction enzyme treatment was purified using Nippon Genetics Purification Kit. The insert DNA was ligated into the cut vector using T4 DNA ligase at 16 °C for 3 h before transformation for plasmid preparation. To introduce the site-directed mutation S106A using PCR the following primers were used: GCTCATTTGCGGACATGCGGATTTTACCGCCATTC and GAATGGCGGTAAAATCCGCATGTCCGCAAATGAGC. The expression vector for the mutant protein was produced in the same way as the wild-type.

2.2. Expression and purification

The pET-28 plasmid with *ldcA* was transformed into *E. coli* BL-21(DE21) cells and the cells were grown at 37 °C with shaking in 1 L LB medium containing kanamycin (20μg ml^{-1}). When the OD_{600} of the culture reached 0.6, protein expression was induced by adding IPTG to a final concentration of 0.5 mM and growth was continued overnight at 20 °C. The cells were collected by centrifugation at 4600 *g* at 4 °C for 20 min. The pellet was suspended in 10 ml 50 mM Tris-HCl pH 8.5 and 50 mM NaCl per 1 L culture and then lysed by sonication on ice. The lysate was centrifuged at 48000 g at 4 °C for 30 min. The supernatant was micro-filtered before loading onto a 15 ml nickel Sepharose column equilibrated with 50 mM Tris-HCl pH 8.5, 50 mM NaCl and 10 mM imidazole. After washing, the protein was eluted with 50 mM Tris-HCl pH 8.5, 50 mM NaCl and 250 mM imidazole. After dialysis the protein was loaded onto a HiTrap Q HP column equilibrated with 50 mM Tris-HCl pH 8.5, and eluted with a salt gradient to 1 M NaCl. The pooled fractions containing LdcA were concentrated and loaded onto a gel filtration column equilibrated with 50 mM Tris-HCl pH 8.5 and 50 mM NaCl. The eluted protein was concentrated to 12 mg ml^{-1} using Amicon centrifugal filter units. The same protocol was used to express and purify the mutant LdcA.

2.3. Crystallisation and data collection

Purified protein, both native and mutant, was crystallised by vapor diffusion using the hanging-drop method. The reservoir solution (12% (w/v) PEG 3350, 0.2 M sodium thiocyanate, 20 mM TrisHCl pH 8.5) was mixed in a 1:1 ratio with the protein, and the drop then equilibrated against 0.5 mL of reservoir solution at 20 °C. A separate crystal form (used for heavy atom soaks) was grown using 85 mM HEPES pH 7.5, 3.66 M sodium chloride and 15% glycerol. Diffraction data used for the final refinements were collected from crystals held at 100K at BL17A of the Photon Factory, Tsukuba using a PILATUS-2M detector. Data processed with XDS [23]. Initial structure solution was performed using PHENIX [24], and data manipulation was performed with the CCP4 suite [25]. Refinement was carried out with REFMAC [26] and COOT [27]. All data collection and refinement statistics are shown in Supplementary Table 1. The structures and X-ray data were deposited in the Protein Data-Bank and assigned the identifiers PDB: 5Z01 (native) and PDB: 5Z03 (mutant).

3. Results

3.1. Native crystal structure

The cloning, expression and purification of native LdcA were carried out as described in the Materials and Methods section. The protein crystallised in space group *I*222 and $P3_12$. The former crystals, grown in PEG 3350, diffracted to 1.75 Å at beam-line BL17A of the Photon Factory, but the trigonal crystals (grown in sodium chloride) gave weaker data to a maximum resolution of only 2.54 Å. The trigonal crystals however proved robust to soaking with ethylmercury-*p*-toluenesulphanilide, allowing SAD phasing with a single dataset collected at BL5A using a wavelength of 1.00659 Å. Five mercury sites could be identified and reliably refined using the Autosol [28] procedure of PHENIX [24]. Automatic model building produced a largely connected structure of 248 residues with R-factor/Rfree of 33.26% and 38.67%. PHASER [29] was used to place this search model in the space group and cell of the native data, giving rotation and translation scores (RFZ and TFZ) of 12.5 and 15.7 respectively. Manual improvements were carried out using COOT [27], giving a model with a single continuous chain of 305 residues and 212 water molecules. The data collection and final refinement statistics are given in Supplementary Table 1. The N-terminal histidine tag proved difficult to remove with thrombin and so this step was dropped from the purification protocol, but only a few residues of the tag are visible in the electron density. The Ramachandran plot shows Ser 10 and Ser 106 have unusual backbone angles; the latter residue is the catalytic serine, but even this is not considered an outlier by Molprobity [30]. The ϕ/ψ angles of 68° and 129° are energetically unfavourable, but typical for serine proteases. Some side-chains show unusual rotamers, which can mostly be explained by the fact these are surface residues with no strong side-chain interactions. One exception is Arg 41, which makes strong hydrogen bonds with Asp 56, both residues being very well defined in the electron density.

*Ec*LdcA has a mixed α/β structure with two β sheets, one highly twisted and surface exposed, and the other being sandwiched between α helices (Fig. 1). A search for similar models using the monomer model and DALI [31] gave a list of related structures, with the most strongly related (PDB: 3TLC) having a Z-score of 35.9 and 26% sequence identity. This model is of the self-immunity protein MccF, a serine carboxypeptidase that hydrolyzes a natural antimicrobial compound called microcin C7 [22]. The catalytic triads of both enzymes overlay closely, although the active site serine of the MccF model (PDB: 3TLC) has been replaced with alanine so that a substrate peptide can bind without undergoing hydrolysis. Overlaying *Ec*LdcA with MccF using the automated procedure SSM [32] shows that 256 core residues of the two models may be aligned with an rmsd of 1.76 Å. The similarity of the two models is highlighted in Supplementary Fig. 2. The principal difference between them is an insertion from residue 174 to 202 in MccF (equivalent to residues 158 and 160 in *Ec*LcdA), which creates a long loop stretching towards the active site. Trp 186 of MccF provides a

Please cite this article in press as: K. Meyer, et al., The crystal structure and oligomeric form of *Escherichia coli* L,D-carboxypeptidase A, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.03.195