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Crystallization and preliminary crystallographic study of the peptidoglycan-associated lipoprotein from *Escherichia coli*

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The peptidoglycan-associated lipoprotein (Pal) from *Escherichia coli* is part of the Tol–Pal multiprotein complex used by group A colicins to penetrate and kill cells. Pal homologues are found in many Gram-negative bacteria and the Tol–Pal system is thought to play a role in bacterial envelope integrity. The Pal protein comprises 152 amino acids. Crystals of the C-terminal 109-amino-acid fragment of the Pal protein have been produced. The crystals belong to the tetragonal space group $I4_1$, with unit-cell parameters $a = b = 89.3$, $c = 67.2$ Å. There are two molecules in the asymmetric unit. Frozen crystals diffract to at least 2.8 Å resolution using synchrotron radiation. Selenomethionine-substituted truncated Pal protein is currently being produced in order to use multiwavelength anomalous dispersion (MAD) for phasing.

1. Introduction

The Pal protein of *E. coli* is a peptidoglycan-associated lipoprotein (Lazzaroni & Portalier, 1992; Mizuno, 1979). It is synthesized as a precursor of 173 amino acids. After cleavage by signal peptidase II at the lipoprotein consensus sequence Ala-Cys-Ser, a lipoprotein of 152 residues is obtained which carries an *N*-acyl diglyceride moiety on the N-terminal cysteine (Mizuno, 1979). The serine residue at position +2 is a sorting signal for localization of this protein in the outer membrane (Gennity & Inouye, 1991). The Pal protein is bound to the outer membrane by its amino-terminus lipid moiety and strongly interacts with the peptidoglycan *via* its C-terminal region (Lazzaroni & Portalier, 1992). Pal maintains membrane stability and integrity and *E. coli pal* mutants exhibit increased sensibility to detergents and drugs, permeability to periplasmic proteins (Lazzaroni & Portalier, 1992) and the formation of outer membrane vesicles (Bernadac *et al.*, 1998). Pal is part of the Tol–Pal system. This system is composed of seven proteins encoded by a cluster of genes organized into two operons: *orf1-tolQ-tolR-tolA* and *tolB-pal-orf2* (Vianney *et al.*, 1996; Webster, 1991). It is organized into two subcellular complexes: TolA, TolR and TolQ form an inner membrane complex (Dérouiche *et al.*, 1995) and TolB and Pal form an outer membrane-associated complex (Bouveret *et al.*, 1995). These two subcellular complexes interact with each other through the interaction of TolA with TolB (Walburger, personal communication) and with Pal (Cascales *et al.*, 2001). The function of this system is unknown. Mutations in any of the *tol* genes exhibit the same phenotype as obtained with a *pal* mutant (Bernadac *et al.*, 1998). In regard to its multiple interactions with other proteins of the envelope involved in outer membrane stability such as OmpA, lpp (Clavel *et al.*, 1998), TolB (Bouveret *et al.*, 1995) and recently TolA (Cascales *et al.*, 2001), it is proposed that Pal may anchor the peptidoglycan to the inner and the outer membranes.

Recently, it has been shown that Pal interacts with TolA and that this interaction requires the proton motive force (Cascales *et al.*, 2001). Thus, Pal may have another role in addition to its suspected structural function. It may participate in an active mechanism of transport of molecules through the membranes. Finally, Pal, in common with the Tol system, is highly conserved among Gram-negative bacteria (Mizuno, 1979, 1981; Zlotnick *et al.*, 1988; Engleberg *et al.*, 1991; Spinola *et al.*, 1992; Frey *et al.*, 1996; Rodriguez-Herva *et al.*, 1996). It has also been shown that antibodies directed against the *Haemophilus influenzae* Pal protein are protective and bactericidal (Munson & Granoff, 1985; Murphy *et al.*, 1986). Therefore, Pal could be a valuable diagnostic tool for sero detections and could be used as a protective antigen for vaccines.

A Pal derivative (PalC1A) deleted of its signal sequence, substituted in its first residue

(Cys→Ala) and tagged with six histidines at the N-terminus has been produced in the cytoplasm and purified by chelation on a cobalt affinity column. Here, we report crystallization conditions and preliminary data of the crystals.

2. Results and discussion

2.1. Construction of pet14PalC1A

The DNA sequence encoding Pal was amplified from pBP (Bouveret *et al.*, 1995) by the polymerase chain reaction (PCR).

The primers PAL3 5′-GCCATATGG-CGTCTTCCAACAAGAACGCC-3′ and PAL2 5′-GCGGATCCTTAGTAAACCAG-TACCGC-3′ introduce *Nde*I and *Bam*HI, respectively, at each end of the PCR product. PAL3 also brings about three base substitutions (TGT→GCG), resulting in a change in the first amino acid (Cys→Ala) of the mature sequence of Pal. The resulting fragment was ligated into the pet14 plasmid (Novagen) and the plasmid obtained was named pet14PalC1A.

2.2. Purification of PalC1A

A 1 l culture of BL21(DE3) (Novagen) pet14PalC1A (OD_{600} of 0.5 ml^{-1}) was induced overnight with IPTG (100 m*M*). The cells were harvested and resuspended in 10 ml 50 m*M* sodium buffer pH 6.8 containing 50 m*M* NaCl. Protease inhibitors [1 m*M* PMSF, 10 ml Cocktail inhibitor (Sigma–Aldrich)] were added and the cells lysed by two passages through a French press. After a 30 min centrifugation at 20 000 rev min^{-1} and an ultracentrifugation of 30 min at 45 000 rev min^{-1}, the cleared lysate obtained was passed through an 8 ml column of cobalt metal-affinity resin (Clontech). The column was then extensively washed with 50 m*M* sodium buffer pH 6.8 containing 50 m*M* NaCl. PalC1A was eluted from the column with an 0–1 *M* imidazole gradient in 50 m*M* sodium buffer pH 6.8 containing 50 m*M* NaCl. The purity of the different fractions was assessed by SDS–PAGE and Western blotting with a polyclonal Pal antiserum (Bouveret *et al.*, 1999). The fractions corresponding to elution with 450–600 m*M* imidazole contained at least 98% pure PalC1A and were therefore pooled. The resulting preparation was dialyzed against 10 m*M* Tris at pH 7.

2.3. Crystallization

The PalC1A protein was concentrated to 26 mg ml^{-1} in 20 m*M* Tris pH 8.0 using a centrifugal filter device (Ultrafree Biomax 5K, Millipore, Bedford, MA, USA). Precipitation experiments were carried out on the PalC1A protein using various precipitating agents [*i.e.* $(NH_4)_2SO_4$, PEG, NaCl, MPD, ethanol] at various pHs (*i.e.* 5, 6, 7, 8, 9). Interesting precipitates were obtained from both the MPD and PEG precipitation experiments at pH 6 and 7. The *SamBA* software (Audic *et al.*, 1997) was then used in order to prospect around these conditions and to optimize the crystallization conditions once the first crystals were obtained. Crystallization trials were performed at 293 K by hanging-drop vapour diffusion using 24-well culture plates. Each hanging drop was prepared by mixing 0.5 µl of the 26 mg ml^{-1} PalC1A with an equal volume of reservoir solution. The hanging drop on the cover glass was vapour-equilibrated against 500 µl of the reservoir solution in each well of the tissue-culture plate. Crystals were obtained after three months from solution containing 40%(*v*/*v*) MPD, 10%(*w*/*v*) PEG 4000, 0.2 *M* ammonium sulfate, 10 m*M* imidazole and 0.1 *M* MES buffer pH 6.0. Once the first crystals were obtained, they were easily reproduced and required only a few days to appear. Owing to the time discrepancy between the first and last experiments it was decided to verify the exact nature of the crystallized protein using N-terminal sequencing. It was then established that the crystals consist of the final 109 C-terminal amino acids of the mature Pal protein. The N-terminal Pal sequence is probably a flexible tail only serving to anchor the protein to the outer membrane through an *N*-acyl diglyceride moiety. It is likely that its spontaneous excision over the long crystallization process allowed the truncated protein to crystallize.

2.4. Data collection and processing

A single crystal was collected in a 0.5 mm^3 Hampton Research loop, flash-frozen to 105 K in a cold nitrogen-gas stream and subjected to X-ray diffraction. This data set was collected on a MAR Research imaging-plate detector at the ESRF synchrotron-radiation facility (BM30) at a wavelength of 0.9794 Å. Data collection was carried out with oscillation angles of 1.0° and with a crystal-to-detector distance of 250 mm. The total oscillation range collected was 90°. Space-group determination was performed using the autoindexing option in *DENZO* (Otwinowski, 1993). The crystals belong to the tetragonal space group $I4_1$, with unit-cell parameters a = 89.35, c = 67.22 Å. The packing density for two monomers of the truncated PalC1A (12.3 kDa) in the asymmetric unit of these crystals (volume 536 646 $Å^3$) is 2.83 $Å^3$ Da^{-1}, a reasonable value for globular proteins and indicating an approximate solvent content of 56.55% (Matthews, 1968).

The data set was processed using the *MOSFLM* package (Leslie, 1990; Kabsch, 1993; Campbell, 1995; Steller *et al.*, 1998); the *SCALA* program from the *CCP*4 package (Collaborative Computational Project, 1994) was used for the scaling and data reduction of the native data set. The crystal diffracted to 2.84 Å and 60 098 reflections were measured in the resolution range 34.35–2.84 Å. This was reduced to a data set of 5974 unique reflections with an R_{sym} value of 7.3. This represents a completeness of 96.2% with a multiplicity of 3.6 and an average $I/\sigma(I)$ of 5.6. For the highest resolution shell, 1418 reflections were measured in the resolution range 2.94–2.84 Å, corresponding to 456 unique *hkl*, an R_{sym} value of 17.3 and an average $I/\sigma(I)$ of 4.4, a completeness of 96.2% and a multiplicity of 2.8. We are currently producing selenomethionine-substituted PalC1A in order to solve the Pal structure using the MAD method (Hendrickson *et al.*, 1990).

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