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Crystallization and preliminary crystallographic study of a component of the *Escherichia coli* Tol system: TolB

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Abstract

TolB from *Escherichia coli* is part of the Tol system used by the group A colicins to penetrate and kill cells. A TolB derivative tagged with six histidines was overexpressed, purified by chelation on a nickel affinity column and crystallized using the *SAmBA* software to define the optimal crystallization protocol. The crystals belong to the monoclinic system, space group $P2_1$ with unit-cell parameters a = 64.48, b = 41.06, c = 78.41 Å, β = 110.78°. Frozen crystals diffract to 1.9 Å resolution. Screening for heavy-atom derivatives both on the native TolB and various cysteine-substituted mutants is in progress. In addition, a selenomethionine-substituted protein is being produced in order to use the MAD method for structure determination.

1. Introduction

TolB is a periplasmic protein of *Escherichia coli*. It is synthesized in a precursor form in the cytoplasm and the 409-residue mature protein results from the cleavage of the 21-residue signal sequence upon export across the cytoplasmic membrane (Isnard, Rigal, Lazzaroni, Lazdunski & Lloubès, 1994). TolB is a component of the Tol system (Vianney, Muller, Clavel, Lazzaroni, Portalier & Webster, 1996; Webster 1991; Bénédetti & Géli, 1996) which comprises six other proteins: TolA, TolQ, TolR, PAL, Orf2 and Orf1. TolA and TolR are single transmembrane domain proteins anchored in the inner membrane by their N-terminal regions, TolQ crosses the inner membrane three times, PAL is a lipoprotein anchored to the outer membrane *via* the fatty acid which acylates its first residue. Orf2, like TolB, is a periplasmic protein, and Orf1 is a cytoplasmic protein that may play a role in the expression of the proteins of the system. All these proteins are encoded by clustered genes and expressed in two operons (Vianney, Muller, Clavel, Lazzaroni, Portalier & Webster, 1996; Webster, 1991; Bénédetti & Géli, 1996). Furthermore, they are believed to form a complex of definite stoichiometry preferentially localized in the contact sites between the inner and outer membranes of bacteria (Guihard, Boulanger, Bénédetti, Lloubès, Besnard & Letellier 1994). Recently, genetic and biochemical approaches have provided information on the organization of this complex; TolA, TolQ and TolR interact with each other inside the inner membrane (Derouiche, Bénédetti, Lazzaroni, Lazdunski & Lloubès, 1995; Lazzaroni, Vianney, Popot, Bénédetti, Samatey, Lazdunski, Portalier & Géli, 1995) and TolB interacts with PAL (Bouveret, Derouiche, Rigal, Lloubès, Lazdunski & Bénédetti, 1995).

The physiological role of this system is unknown. However, its function might be important since it has been described in several other Gram-negative bacteria (*Haemophilus influenzae, Brucella abortus, Pseudomonas putida* and *Pseudomonas aeruginosa*) (Deich, Metcalf, Finn, Farley & Green, 1988; Tibor, Weynants, Denoel, Lichtfouse, de Bolle, Saman, Limet & Letesson, 1994; Rodriguez-Herva, Ramos-Gonzalez & Ramos, 1996; Cornelis, Sierra, Lim, Malur, Tungpradabkul, Tazka, Leitao, Martins, Di Perna, Brys, De Baetselier & Harners, 1996). It might play a role in the cell integrity of the envelope since *tol* and *pal* mutants are hypersensitive to drugs (vancomycin and bacitracin), EDTA and detergents, and tend to release periplasmic proteins into the extracellular medium (Nagel de Zwaig & Luria, 1967; Fognini-Lefebvre, Lazzaroni & Portalier, 1987). Recently, TolA has been shown to interact with trimeric porins (Derouiche, Gavioli, Bénédetti, Prilipov, Lazdunski & Lloubès, 1996). The level of assembled porins in the outer membrane has been shown to be affected in *tolA* mutants (Lazzaroni, Fognini-Lefevre & Portalier, 1986); thus the Tol system might be involved in the assembly of outer membrane LPS-associated porins.

The Tol system is also used by a group of bacterial toxins (group A colicins) to penetrate into the cells and kill them. The mode of action of colicins involves three steps: (i) binding to a specific receptor at the cell surface; (ii) translocating across the cell envelope to reach the cellular target; and (iii) cell killing. A particular domain of the toxin is involved at each step. The colicin N-terminal domain is involved in the translocation and it has been demonstrated for some colicins that this domain is able to interact with TolA and TolB (Bénédetti, Lazdunski & Lloubès, 1991; Bouveret, Rigal, Lazdunski Bénédetti, 1997). Therefore, TolB appears to play a key role in the translocation of some colicins like E3 (Davies & Reeves, 1975) and A (E. Bouveret, unpublished results).

A TolB derivative (tolBHis), deleted of its signal sequence and tagged with six histidines in the N terminus, has been produced in the cytoplasm and purified by chelation on a HiTrap affinity column (Pharmacia). Here we report crystallization conditions and preliminary data on the crystals.

2. Results and discussion

2.1. *Purification of TolBHis*

The cleared lysate obtained as described by Bouveret, Rigal, Lazdunski & Bénédetti (1997) was applied to a 5 ml column of chelating Sepharose–agarose beads charged with Ni^{2+} and equilibrated with buffer *A* (sodium phosphate 50 m*M* pH 8, 100 m*M* NaCl and 5 m*M* imidazole). The column was washed with buffer *A*. Elution was performed with a linear gradient [start buffer: buffer *A*, final buffer: buffer *B* (sodium phosphate 50 m*M* pH 8, 100 m*M* NaCl and 300 m*M* imidazole)]. The eluates

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were collected and the presence and purity of TolBHis in the different fractions were assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting with polyclonal TolB/PAL antiserum (Bouveret, Derouiche, Rigal, Lloubès, Lazdunski & Bénédetti, 1995). The fractions corresponding to the elution with 180–250 m*M* imidazole contained at least 98% pure TolBHis and were, therefore, pooled. The resulting preparation was concentrated and dialyzed against 50 m*M* potassium phosphate buffer pH 6.8, 50 m*M* NaCl, 10 m*M* β-octylglucoside. About half of TolBHis was lost during these manipulations because of a tendency to aggregate and adsorb on plastic tubes. The concentration of the final TolBHis preparation was approximately 4 mg ml^{-1}. Iso-electrofocalization revealed a single band around 8.6.

2.2. *Crystallization*

The TolBHis protein was concentrated to 4 mg ml^{-1} in 10 m*M* Tris buffer pH 7.5 using a centrifugal filter device (Ultrafree Biomax 30K, Millipore, Bedford MA, USA). Precipitation experiments were carried out on the TolBHis protein using different precipitating agents [*i.e.* $(NH_4)_2SO_4$, PEG, NaCl, 2 methyl-2,4-pentane diol (MPD), ethanol] at five pH levels (*i.e.* 4.5, 5.5, 6.5, 7.5, 8.5). We obtained promising precipitates when using polyethylene glycol (PEG) 8000 as a precipitating agent. The experimental protocol to search for TolB crystallization conditions was designed using the *SAmBA* software (Table 1) (Audic, Lopez, Claverie, Poirot & Abergel, 1997; http://igs-server.cnrs-mrs.fr/samba/). We tested two kinds of PEG at three different concentrations, five different pH's and two different salts. Crystallization trials were performed at 293 K by hanging-drop vapor-diffusion using 24-well culture plates. Each hanging drop was prepared by mixing 0.5 µl of the 4 mg ml^{-1} TolB with an equal volume of the reservoir solution. The hanging drop on the cover glass was vapor equilibrated against 1 ml of the reservoir solution in each well of the tissue-culture plate. Crystals appeared after 4 weeks using the optimized design provided by *SAmBA* (Audic, Lopez, Claverie, Poirot & Abergel, 1997) and the best crystals were obtained at pH 6.5 2-morpholinoethanesulfonic acid (MES) 0.1 *M*, PEG 8000 15%(*w/v*) and NaCl 0.1 *M*.

2.3. *Data collection and processing*

The crystals were sealed in thin-walled glass capillaries and subjected to X-ray diffraction. A complete data set was collected on a 18 cm MAR Research imaging-plate detector, placed on a Rigaku RU-200 rotating anode running at 40 kV and 80 mA, using a copper target. The data collection was carried out with oscillation angles of 2.0° and with a crystal-to-detector distance ranging from 110 to 120 mm. The total oscillation range collected was 120°. The native data set was processed using the *DENZO* package (Otwinowski, 1993), and programs from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) were used for scaling of the native and the potential heavy-atom derivatives.

Space-group determination was carried out using the autoindexing option in *DENZO*. The crystals belong to the monoclinic system, space group $P2_1$ with unit-cell parameters a = 64.48, b = 41.06, c = 78.41 Å, β = 110.78° and diffract to 2.6 Å resolution at room temperature. Frozen crystals diffract to 1.9 Å resolution with unit-cell parameters a = 64.13, b = 40.97, c = 76.84 Å, β = 110.29°. The packing density of a monomer of TolB (45 507 Da) in the asymmetric unit of these crystals (volume 194 090 $Å^3$) is 2.13 $Å^3$ Da^{-1}, a reasonable value for globular proteins indicating an approximate solvent content of 42% (Matthews, 1968). These crystals are suitable for a complete three-dimensional structure determination by the method of isomorphous replacement and this work is in progress. 54 754 reflections have been measured in the resolution range from 20.72 to 2.12 Å and reduced to a final data set of 19 149 unique reflections with an R_{sym} value of 0.107 representing a completeness of 95.2% and average $I\sigma$ = 1.1; for the highest resolution range, 2.27–2.12 Å, R_{sym} = 0.28 with a completeness of 97.4% and an average I/σ (I) = 1.6. In an attempt to collect a better native data set from frozen crystals, we are currently searching for a cryoprotectant inducing no loss of diffraction. Since there is no cysteine in the TolB sequence, heavy-atom binding may not occur, and we are currently preparing a selenomethionine-substituted protein to be able to use the MAD method to circumvent the problem (Hendrickson, Horton & LeMaster, 1990). In addition, we are producing cysteine-substituted mutants and crystals of a $Ala_{12} \rightarrow Cys_{12}$ TolB mutant isomorphous to the native crystals are currently under test for heavy-atom binding.

Table 1. *TolB crystallization protocol*

Variable 1 corresponds to the polyethylene glycol type [8000 or 5000 monomethyl ether (MME)] and its concentration. Variable 2 corresponds to the pH (0.1 *M* MES buffer was used for pH 6.0 and 6.5, 0.1 *M* HEPES buffer for pH 7.0–8.0). Variable 3 corresponds to the two salts tested: ammonium sulfate ($AmSO_4$) and sodium chloride (NaCl) at two concentrations. The last column corresponds to the scoring of the experiment (0 = soluble, 1 = precipitate, 2 = crystals).

Experiment number	PEG %(*w/v*)	pH	Salts (*M*)	Score
1	8000 15	6.5	NaCl 0.1	2
2	MME 5	7.5	$AmSO_4$ 0.1	0
3	MME 10	8.0	NaCl 0.1	2
4	8000 10	6.5	NaCl 0.1	2
5	MME 15	7.0	NaCl 1	0
6	8000 10	7.0	$AmSO_4$ 0.1	0
7	8000 15	7.0	$AmSO_4$ 0.1	0
8	MME 15	6.5	0	2
9	MME 15	6.0	$AmSO_4$ 0.1	1
10	8000 5	8.0	0	2
11	MME 5	7.0	0	0
12	MME 10	7.5	NaCl 1	1
13	MME 10	7.0	NaCl 0.1	2
14	MME 5	6.5	NaCl 1	0
15	8000 10	6.0	NaCl 1	1
16	MME 10	6.5	$AmSO_4$ 0.1	1
17	MME 10	6.0	0	2
18	8000 15	7.5	0	2
19	8000 5	7.0	0	0
20	8000 5	7.5	$AmSO_4$ 0.1	0
21	8000 5	6.5	NaCl 1	1
22	8000 5	6.0	NaCl 0.1	1
23	8000 15	8.0	$AmSO_4$ 0.1	1
24	8000 15	6.0	NaCl 1	1
25	MME 15	7.5	NaCl 0.1	2
26	8000 10	8.0	$AmSO_4$ 0.1	1
27	MME 5	6.0	NaCl 0.1	1
28	8000 10	7.5	0	2
29	MME 5	8.0	0	1
30	MME 15	8.0	NaCl 1	1

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