The periplasmic sensing domain of *Pseudomonas fluorescens* chemotactic transducer of amino acids type B (CtaB): Cloning, refolding, purification, crystallization, and X-ray crystallographic analysis

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Summary

*Pseudomonas fluorescens* is a plant growth promoting rhizobacterium that provides nutrients for growth and induces systemic resistance against plant diseases. It has been linked with a number of human diseases including nosocomial infections and bacterial cystitis. Chemotactic motility of *P. fluorescens* towards root exudates plays a crucial role in establishing a symbiotic relationship with host plants. The *P. fluorescens* chemotactic transducer of amino acids type B (CtaB) mediates chemotaxis towards amino acids. As a step towards elucidation of the structural basis of ligand recognition by CtaB, we have produced crystals of its recombinant sensory domain and performed their X-ray diffraction analysis. The periplasmic sensory domain of CtaB has been expressed, purified, and crystallized by the hanging-drop vapor diffusion method using ammonium sulfate as a precipitating agent. A complete data set was collected to 2.2 Å resolution using cryocooling conditions and synchrotron radiation. The crystals belong to space group *P*

1. Introduction

*Pseudomonas fluorescens* and other fluorescent pseudomonads belong to the group of plant growth promoting rhizobacteria (PGPR) that form a symbiotic relationship with host plants (¹,²). PGPR strains exhibit beneficial effects on plants by fixing nitrogen, producing siderophores and solubilizing essential elements in soil (¹,²). In addition, they exert indirect beneficial effects by preventing growth or activity of phytopathogens and inducing systemic resistance against plant diseases (²). They produce different types of secondary metabolites including fungicides and hydrogen cyanide, which protect roots against pathogens (³). Furthermore, some strains of *P. fluorescens* participate in biodegradation of xenobiotic compounds and bioremediation of heavy metals (³,⁴).

*P. fluorescens* is considered to be an opportunistic pathogen for humans (⁵). It has been linked with a number of human diseases including nosocomial infections and bacterial cystitis. In addition, *P. fluorescens* has been isolated from a large number of respiratory specimens taken from hospital patients,
although its association with pulmonary infections is not well understood (5). Furthermore, Sutton CL et al. (5) reported that more than half of the patients with Crohn’s disease develop antibodies against P. fluorescens. P. fluorescens can also cause blood transfusion-related bacteremia and catheter-associated bacteremia amongst the immunosuppressed patients (6). Nosocomial outbreaks of bacteremia due to P. fluorescens have been reported (5). However, the information on pathogenesis mechanism of P. fluorescens is very limited.

Flagella-mediated motility and chemotaxis of P. fluorescens towards different nutrients present in root exudates or the rhizosphere play a crucial role in establishing a symbiotic relationship with plants (2,7-9). Previous mutagenesis studies demonstrated that chemotaxis is important for the root-tip colonization by P. fluorescens (9-11). Furthermore, motility and chemotaxis are important virulence factors of many pathogenic bacteria, and it is likely that they play an important role in P. fluorescens pathogenesis in humans. The environmental chemical signals are sensed by bacterial membrane-embedded methyl-accepting chemotaxis protein (MCP) receptors (12). Upon binding of the signal molecule, MCPs trigger a chemotactic signaling cascade and control bacterial movement towards or away from chemoeffectors and repellents, respectively (12).

Of the 37 putative MCPs identified in the genome of P. fluorescens Pf0-1 to date, ligands are known for only seven. The MCPs termed chemotactic transducers of amino acids (CtaB, CtaB, and CtaC) sense amino acids as attractants (13). MCPs Pf01_3768 and Pf01_0728 were identified as receptors for L-malate, succinate, and fumarate (14). Finally, the chemoreceptor for 2-nitrobenzoate NbaY was shown to be involved in the metabolism of aromatic compounds (15).

The periplasmic sensing domain of CtaB has been shown to recognize a broad range of amino acids (16 in total) (13). The structural basis of how CtaB recognizes its ligands and transmits the signal across the membrane in response to ligand binding is yet to be determined. Data on bacterial receptors that are structurally and functionally homologous to CtaB is limited. The periplasmic sensing domain of CtaB (CtaB PTPSD, residues 107-185) (Figure 1). The sequence encoding PTPSD of Tlp3 and Mcp37, respectively. This protein provides an example of a PTPSD-type receptor with an extremely broad substrate specificity. To elucidate the structural basis of the CtaB’s ligand promiscuity, we have initiated X-ray crystallographic studies on recombinant CtaB PTPSD. Here, we report its cloning, refolding, purification and crystallization together with the analysis of the diffraction data.

2. Materials and Methods

2.1. Cloning and overexpression of CtaB PTPSD as inclusion bodies (IBs)

The membrane topology and the boundaries the periplasmic sensing domain of CtaB (CtaB PTPSD, residues 32-272) from P. fluorescens Pf0-1 (UniProt ID Q3KK38) were predicted by TOPCONS server (http://topcons.net/) (19) (Figure 1). The sequence encoding CtaB PTPSD was codon optimized for expression in Escherichia coli, synthesized and ligated into the pET151/D-TOPO vector (Invitrogen) by Genscript to generate an expression vector that harbors a N-terminal His6 tag followed by a TEV protease cleavage site. The expression vector was introduced into E. coli BL21 (DE3) (Novagen) and cells were grown in Luria-Bertani medium supplemented with 50 µg/mL ampicillin to an OD600 of 0.6 at 310 K. Overexpression of CtaB PTPSD was induced with 0.5 mM isopropyl-b-D-1-thiogalactopyranoside (Thermo Scientific) and growth was continued for 3.5 h at 210 K. The cells were harvested by centrifugation at 6,000 g for 15 min at 277 K. The cells were resuspended in buffer A (10 mM Tris-HCl buffer pH 8.0 and 200 mM NaCl), lysed by sonication and centrifuged at 10,000 g for 30 min at 277 K. SDS-PAGE gel electrophoresis of clarified supernatant and pellet confirmed that CtaB PTPSD expressed in inclusion bodies (IBs).

2.2. Solubilization of IBs, protein refolding and purification

Purification of CtaB PTPSD from IBs was performed following the procedure described earlier with some modifications (20). Briefly, IBs were washed twice with buffer B (10 mM Tris-HCl pH 8.0, 0.2 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich), 1% Triton X-100 (Sigma-Aldrich)) and once in buffer C (10 mM Tris-HCl pH 8.0, 0.2 mM PMSF), and centrifuged at 10,000 g for 30 min to purify IBs. The IBs were then solubilized in buffer D (10 mM Tris/
Prior to crystallization, the protein sample was concentrated to 10 mg/mL, centrifuged for 20 min at 13,000 g and transferred into a clean tube. The preliminary crystallization screening was carried out by the hanging-drop vapour-diffusion method using an automated Phoenix crystallization robot (Art Robbins Instruments). Commercial crystallization screens (Crystal Screen, Index Screen HT, The JCSG Screen and PEG/Ion Screen HT (Hampton Research, Laguna Niguel, CA) were used. Crystals appeared after one day in the condition No. 54 of The JCSG Screen consisting of 0.2 M zinc acetate, 20% (w/v) polyethylene glycol (PEG) 3000 and 0.1 M imidazole pH 8.0. Refinement to improve the quality of the crystals (Figure 3) resulted in the final optimized condition that contained 14% (w/v) PEG 3000, 0.15 M zinc acetate sulfate and 0.1 M imidazole pH 7.5.

2.4. Data collection and processing

Prior to data collection, crystals were briefly soaked in a cryoprotectant solution consisting of 0.18 M zinc acetate, 20% (w/v) polyethylene glycol (PEG) 3000 and 0.1 M imidazole pH 7.5. Refinement to improve the quality of the crystals (Figure 3) resulted in the final optimized condition that contained 14% (w/v) PEG 3000, 0.15 M zinc acetate sulfate and 0.1 M imidazole pH 7.5.

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with a cleavable N-terminal His6-tag from the pET151/D-TOPO plasmid in E. coli BL21 (DE3) upon induction of T7 polymerase. The protein was found in inclusion bodies (IBs). It was refolded from IBs and purified to >95% electrophoretic homogeneity based on Coomassie Blue staining of SDS-PAGE gels (Figure 2). The protein migrated as a single band on SDS-PAGE with a molecular weight of ~25 kDa. This value was close to molecular weight (26.7 kDa) calculated from the amino acid sequence. When subjected to size-exclusion chromatography, the protein eluted as a single peak with a retention volume of 225 mL corresponding to an approximate molecular weight of 24.5 kDa, which suggested that *P. fluorescens* CtaB PTPSD is a monomer in solution under the tested buffer conditions.

An X-ray diffraction data set was collected from a cryo-cooled crystal of CtaB PTPSD to 2.2 Å using the AS facility (Figure 4). Processing of the diffraction data using the autoindexing routine in *iMosflm* and the analysis of systematic absences implemented in *AIMLESS* suggested that the crystals have the *P*2₁2₁2₁ symmetry, with unit cell parameters a = 34.5, b = 108.9, c = 134.6 Å. The average *I*/σ(*I*) value was 16.2 for all reflections (resolution range 35.0-2.2 Å) and 4.9 in the highest resolution shell (2.3-2.2 Å). A total of 216,640 measurements were made of 26,718 independent reflections. Data processing gave a *R*<sub>min</sub> of 0.027 for intensities (0.157 in the resolution shell 2.3-2.2 Å), and these data were 99.9% complete (100% completeness in the highest resolution shell).

Under the assumption that there are two molecules of CtaB PTPSD in the asymmetric unit, the calculated Matthews coefficient was 2.64 Å³ Da⁻¹ and the corresponding solvent content was approximately 53%. Analysis of the self-rotation function computed using POLARRFN (23) with diffraction data in the resolution range 30-6 Å and an integration radius of 16 Å revealed the presence of a twofold symmetry axis (κ = 180°) represented by a peak at (φ = 44.7°, ω = 0°) with a height of 4σ (Figure 5). Together, this analysis suggests that the CtaB PTPSD crystals contain two molecules per unit cell. Phasing by molecular replacement has not been possible due to low sequence similarity with the known structures deposited in the RCSB PDB database. A search for heavy-atom derivatives with the aim to solve the structure using multiple isomorphous replacement and/or multi-wavelength anomalous dispersion methods is in progress.

We have previously observed that expression of periplasmic sensory domains of bacterial MCP receptors in *E. coli* often results in their deposition predominantly in inclusion bodies (16,20,25-27). The recombinant ligand sensing domain of *P. fluorescens* CtaB is another example of a molecule of this type that required extraction from IBs and refolding. We succeeded in producing folded protein and high-quality crystals by following the refolding procedure that we have recently developed (25,26). The purified protein was monomeric in solution, in line with previous studies that showed PTPSDs from other receptors to be also monomeric in solution (16,20,25-27).

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**Table 1. Data collection and processing**

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<th>Parameter</th>
<th>Value</th>
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<td>Space group</td>
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<tr>
<td>a, b, c (Å)</td>
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<tr>
<td>α, β, γ (°)</td>
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<tr>
<td>Resolution range (Å)</td>
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<tr>
<td>Total No. of reflections</td>
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<tr>
<td>No. of unique reflections</td>
<td>26,718 (3,862)</td>
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<tr>
<td>Completeness (%)</td>
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<td><em>I</em>/σ(<em>I</em>)</td>
<td>16.2 (4.9)</td>
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<tr>
<td><em>R</em>&lt;sub&gt;min&lt;/sub&gt;</td>
<td>0.027 (0.157)</td>
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<tr>
<td>Overall B factor from Wilson plot (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
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Values for the outer shell are given in parentheses.
X-ray crystallographic analysis of CtaB PTPSD in complex with various amino acid ligands is expected to provide an explanation of the structural basis behind the broad ligand specificity of this receptor. We note that the crystal structure of PTPSD of another ‘promiscuous’ amino acid MCP receptor, *V. cholerae* Mcp37, has been recently reported (18). However, only the crystal complexes of that protein with alanine and serine have been characterised, which makes it difficult to predict how larger amino acids can fit into its relatively small ligand-binding pocket.

The results presented here are important because they lay the foundation for future systematic structural studies that will be able to address the fundamental biological question of how this receptor, and similar receptors in other important bacteria, sense environmental cues, how they transduce the signal across the membrane and thus control bacterial movement.

Acknowledgements

Part of this research was undertaken on the MX1 beamline of the AS, Victoria, Australia. We thank the AS staff for their assistance with data collection. We are also grateful to Dr. Danuta Maksel and Dr. Robyn Gray at the Monash University Protein Crystallography Unit for assistance with the robotic crystallization trials.

References


(Received November 22, 2016; Revised December 20, 2016; Accepted January 16, 2017)


(Received November 22, 2016; Revised December 20, 2016; Accepted January 16, 2017)