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Cloning, purification, crystallization and preliminary X-ray analysis of a bacterial GABA receptor with a Venus flytrap fold

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In response to infection by the pathogen *Agrobacterium tumefaciens*, plants synthesize several stress amino acids, including γ-aminobutyric acid (GABA), which modulates the expression of bacterial virulence factors. GABA penetrates into the bacterial cytoplasm *via* an ABC transporter that is associated with the periplasmic receptor Atu2422. Mature receptor Atu2422 (without its signal peptide) was overexpressed in *Escherichia coli*, purified and crystallized. A complete data set was collected to 1.35 Å resolution at 100 K. The crystals belonged to the monoclinic space group *C*2 and contained one molecule in the asymmetric unit. Molecular replacement was performed and the initial electron-density maps revealed a closed form of this Venus flytrap (VFT) receptor, suggesting the presence of an endogenous *E. coli* ligand.

1. Introduction

The pathogen *Agrobacterium tumefaciens* is responsible for crown gall disease in numerous plants of agronomical interest (reviewed by Escobar & Dandekar, 2003). Several plant signals are required to control the expression of virulence. γ-Aminobutyric acid (GABA) acts as a plant signal that attenuates virulence functions by inducing the expression of the lactonase AttM in *A. tumefaciens* (Chevrot *et al.*, 2006; Yuan *et al.*, 2008). This lactonase can cleave the bacterial signal 3-oxooctanoyl homoserine lactone (OC8HSL) when *A. tumefaciens* colonizes the plant tissues. Therefore, it may affect the expression of OC8HSL-dependent functions, such as the dissemination of virulence genes by horizontal transfer of the tumour-inducing (Ti) plasmid (Zhang *et al.*, 2002) and the number of emerging tumours at the infection site (Chevrot *et al.*, 2006).

The import of GABA into several bacteria requires a periplasmic bacterial receptor that traps the GABA molecule and transfers it to the ABC transporter Bra. The GABA receptor is called Atu2422 in *A. tumefaciens* strain C58 (Chevrot *et al.*, 2006) and BraC (Rl3745) in *Rhizobium leguminosarum* bv. *viciae* strain 3841 (Hosie *et al.*, 2002). The peptide sequence of Atu2422 shares around 40% sequence identity with two *Escherichia coli* periplasmic amino-acid receptors of known structure: Leu-Ile-Val-binding protein (LIVBP, LivJ; Sack, Saper *et al.*, 1989; Trakhanov *et al.*, 2005) and leucine-binding protein (LBP, LivK; Sack, Trakhanov *et al.*, 1989; Magnusson *et al.*, 2004). These amino-acid receptors, which consist of two domains separated by a deep cleft, exhibit the Venus flytrap (VFT) architecture, which is conserved among a large number of ligand-binding proteins in bacteria and eukaryotes, including the $GABA_B$ receptors in mammals (Galvez *et al.*, 1999), as shown by modelling based on sequence alignment (Acher & Bertrand, 2005). The unliganded receptor adopts an open form, whereas it adopts a closed form upon ligand binding.

Here, we report the successful purification, crystallization and preliminary X-ray analysis of the bacterial GABA receptor Atu2422.

2. Cloning, expression and purification

Genomic DNA purified from *A. tumefaciens* strain C58 was used as a template to amplify the DNA encoding the mature protein Atu2422

(consisting of 349 residues without its signal peptide). Suitable restriction sites (*Nde*I and *Not*I; shown in bold) were added to the primers (5′-GGAATTC**CATATG**GATGTCGTCATCGCTGTC and 5′-TTT**GCGGCCGC**TTAATGGTGATGGTGATGGTGGCTGCC-CTGCTGG) for cloning into a pET-9aSN1 expression vector (a gift from S. Chéruel, IBBMC, University Paris Sud, Orsay, France). An oligonucleotide coding for a six-histidine tag was added to the 3′ end of the gene to facilitate protein purification.

The PCR product was purified with a QIAquick PCR purification kit (Qiagen), digested with *Nde*I and *Not*I enzymes and cloned into the expression vector pET-9aSN1, which was digested with the same enzymes. The construct was then introduced by heat shock into XL1-Blue chemically competent cells. The nucleotide sequence of one selected clone was confirmed by DNA-sequence analysis.

For the expression of Atu2422 (residues 24–372)-6×His, transformed BL21 (DE3) STAR *E. coli* cells were grown at 310 K to an OD_{600} of about 0.8 in 2×TY medium containing 50 µg ml^{-1} kanamycin. Expression of the His-tagged receptor was induced with 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside. The cells were grown for a further 3 h at 310 K before centrifugation (4000*g*, 10 min). The pellet was resuspended in 25 m*M* Tris–HCl pH 8.5, 150 m*M* NaCl. Cell lysis was achieved by sonication after the addition of an anti-protease cocktail (Sigma). Cell debris was spun out by centrifugation at 40 000*g* for 30 min at 277 K. Recombinant mature protein with a C-terminal His tag (Atu2422-His) was purified by IMAC chromatography using Ni–NTA resin (Qiagen) equilibrated in 25 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 10 m*M* imidazole. Low-affinity binding contaminants were washed from the column with 35 m*M* imidazole prior to the elution of Atu2422 with 300 m*M* imidazole. The eluted sample was further purified by size-exclusion chromatography using a Superdex 200 HiLoad column (Amersham Biosciences) equilibrated in 20 m*M* HEPES pH 7.5, 150 m*M* NaCl. Both chromatographic steps were performed on an ÄKTA Purifier 10 (Amersham Biosciences) at 277 K. The purity of the sample was determined by SDS–PAGE. Atu2422-His was concentrated to 11 mg ml^{-1} using Vivaspin-10 centrifugal concentrators (Vivasciences) and stored frozen.

3. Crystallization

In crystallization trials, mature Atu2422-His protein was used at a concentration of 11 mg ml^{-1} in a buffer consisting of 20 m*M* HEPES pH 7.5 and 150 m*M* NaCl. Commercial crystallization kits (the PEG II and Classics Suites from Qiagen) were screened in sitting-drop vapour-diffusion experiments using a nanodrop robot (Cartesian Proteomic Solutions) at 293 K. Small crystals appeared within 3 d in only one condition (PEG II condition D4). Crystallization conditions were then manually optimized with home-made solutions. 2 µl protein solution was mixed with 2 µl reservoir solution consisting of 0.1 *M* Tris–HCl pH 8.5, 35%(*w*/*v*) PEG 4000, 100 m*M* $CaCl_2$ and equilibrated over 1 ml reservoir solution, leading to large thin plate-shaped crystals (Fig. 1).

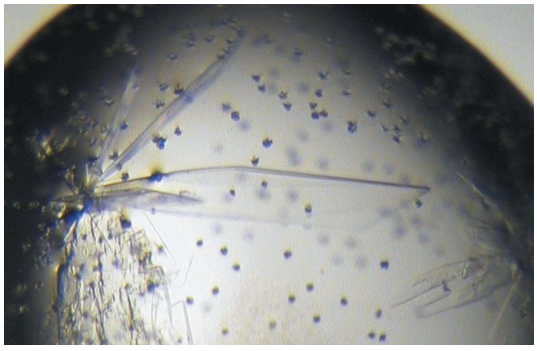

Figure 1
Large thin plate-shaped crystals of Atu2422 (dimensions 0.6 × 0.1 × 0.04 mm).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell (of nine).

Space group	*C*2
Unit-cell parameters (Å, °)	*a* = 116.2, *b* = 38.9, *c* = 71.6, β = 95.7
Resolution (Å)	30.0–1.35 (1.42–1.35)
No. of observed reflections	283328
No. of unique reflections	69760
Completeness (%)	99 (98.2)
$R_{\rm merge}$† (%)	7.3 (68)
$I/\sigma(I)$	13 (2.1)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observed amplitude of reflection hkl and $\langle I(hkl)\rangle$ is the mean amplitude for all observations i of reflection hkl.

4. Data collection and processing

Crystals were flash-frozen in a cryoprotectant solution containing 0.1 *M* Tris–HCl pH 8.5, 37% PEG 4000, 100 m*M* $CaCl_2$ and 20% glycerol. Data-collection experiments were carried out at 100 K on the PROXIMA I beamline at SOLEIL (Saint Aubin, France). 360° of data were collected in 1° frames, with 1 s exposure per frame. Diffraction intensities were evaluated with the program *XDS* (Kabsch, 1993) and further processed using the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The crystal diffracted to 1.35 Å resolution and belonged to the monoclinic space group *C*2, with unit-cell parameters *a* = 116.2, *b* = 38.9, *c* = 71.6 Å, β = 95.7°. Data-collection and processing statistics are given in Table 1.

5. Molecular replacement

The asymmetric unit can contain only one molecule, corresponding to a Matthews coefficient (Matthews, 1968) of 2.15 Å^3 Da^{-1} and a solvent content of 42.9%. Molecular replacement using the program *Phaser* (Storoni *et al.*, 2004) gave an incorrect solution (RFZ = 5.2, TFZ = 2.8, LLG = −70 and an initial *R* factor of 51%) using the apo structure of *E. coli* LBP (PDB code 1usg) as a search model. Taking into account the fact that periplasmic amino-acid receptors are known to bind endogenous amino acids during the production/purification procedure (Trakhanov *et al.*, 2005; Huvent *et al.*, 2006), a liganded form of LBP (PDB code 1usk; leucine-bound) was used as a model and led to a solution with good scores (RFZ = 9.5, TFZ = 10.2, LLG = 158) and an initial *R* factor of 44%. Examination of the resulting model and density maps clearly showed that Atu2422 adopts a closed conformation, meaning that the receptor was crystallized with a bound fortuitous ligand that needs to be identified. Refinement of the mature receptor Atu2422 structure and determination of its amino-acid selectivity are under way.

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