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Crystallization and preliminary X-ray diffraction studies of the quorum-sensing regulator TraM from *Agrobacterium tumefaciens*

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TraM is a 11.4 kDa protein involved in the control of the conjugal transfer of *Agrobacterium tumefaciens* Ti plasmids by quorum-sensing. TraM was overexpressed and purified from *Escherichia coli*. This protein binds to the transcriptional regulator TraR, abolishing its function. Size-exclusion chromatography and dynamic light scattering show that the recombinant protein has an apparent molecular weight of 30 kDa in solution. Crystals have been obtained of both native and selenomethionine-substituted TraM by the vapour-diffusion method. Crystals diffract to 1.67 Å and belong to the space group $P2_12_12$, with unit-cell parameters $a = 76.43$, $b = 47.09$, $c = 47.46$ Å and two molecules in the asymmetric unit. A two-wavelength MAD data set for the selenomethionine-substituted form has been collected to a resolution of 2.0 Å. The selenium substructure (five out of six possible sites) has been solved using direct methods.

1. Introduction

Quorum-sensing is a form of bacterial cell–cell communication used by both Gram-positive and Gram-negative bacteria to regulate a variety of physiological functions. This mechanism is based on the production, the release and the response to small signal molecules, called autoinducers or pheromones, produced by bacteria themselves (reviewed in Fuqua & Winans, 1994). Conjugal transfer of Ti plasmids in *Agrobacterium tumefaciens*, the bacterium responsible for crown gall disease in plants, is regulated by quorum-sensing *via* the LuxR-type transcriptional regulator TraR and oxooctanoyl-L-homoserine lactone, the *Agrobacterium* autoinducer (Piper *et al.*, 1993; Piper & Farrand, 2000). Recently, the three-dimensional structure of the quorum transcriptional regulator TraR bound to its autoinducer and target DNA has been reported (Vannini *et al.*, 2002; Zang *et al.*, 2002). The structure shows that the autoinducer molecule plays a key role in the correct folding of the nascent protein instead of acting as an allosteric effector.

In contrast to most other LuxR-type transcriptional regulators, the activity of TraR is under the influence of the Ti plasmid-encoded TraM regulator, a protein thus far only identified in *A. tumefaciens* and other members of the family *Rhizobiaceae* (Freiberg *et al.*, 1997; Fuqua *et al.*, 1995; Hwang *et al.*, 1995). TraM acts as an inhibitor, forming an extremely stable complex with the transcriptional regulator TraR, preventing the latter from recognizing its target DNA operators and, as a consequence, preventing the activation of specific gene expression. Directed mutational analysis of TraM identified a number of amino acids that play important roles in the inhibition of TraR clustering in two regions of the protein (Luo *et al.*, 2000; Swiderska *et al.*, 2001). This inhibition is absolutely required for normal operation of the entire quorum-sensing pathway and TraM plays a key role in determining the threshold level of the bacterial population, called a quorum, required for initiating the Ti plasmid conjugal transfer (Fuqua *et al.*, 1995; Swiderska *et al.*, 2001).

In this scenario, structural studies of TraM regulator and, eventually, its complex with TraR could greatly enhance our understanding of the molecular basis of quorum-sensing in *A. tumefaciens*.

We have overexpressed, purified and characterized the recombinant regulator TraM in an *Escherichia coli* expression system. Furthermore, we have crystallized both the native and the selenomethionine-substituted protein. Because at present no relevant sequence homology has been detected with other proteins of known structure, we applied the multiwavelength anomalous diffraction method (Hendrickson, 1991) using Se atoms as anomalous scatterers to obtain initial phases.

2. Experimental and results

2.1. Protein production

The *A. tumefaciens* TraM (SWISS-PROT Q57471) synthetic gene was purchased from Entelechon GmbH (Germany). The gene, encoding a total of 102 residues, was subcloned into PT7.7 vector as an *Nde*I/*Eco*RI fragment. The plasmid harbouring the TraM sequence was transformed into *E. coli* strain BL21(DE3)pLysS for protein expression. A

total of 3 l of cells were grown at 310 K in LB medium until the OD_{600nm} reached 0.8. Expression was induced for 3 h by the addition of 0.4 m*M* isopropyl-β-D-thiogalactoside. Cells were resuspended in 150 ml lysis buffer [50 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 1 m*M* dithiothreitol (DTT)] and the soluble fraction was loaded onto a weak cation-exchange column (Heparin) pre-equilibrated with lysis buffer. The flow-through, which contained the protein, was then loaded onto an anion-exchange column (Resource Q) pre-equilibrated with the same buffer. Surprisingly, the protein was found, virtually pure, in the flowthrough, despite its theoretical pI of 5.9. This flow-through was concentrated to an appropriate volume and loaded onto a gel-filtration column (Superdex G-200) pre-equilibrated with the same buffer containing 150 m*M* NaCl. The purified protein was concentrated to 2.2 m*M* (25 mg ml^{-1}) by ultrafiltration. TraM concentration was determined by UV spectroscopy (ε_{280nm} = 3840 M^{-1} cm^{-1}). The protein was then flash-frozen in liquid nitrogen and stored at 193 K.

The pure protein at 1 m*M* elutes as a single species of apparent molecular weight 32 kDa from an analytical gel-filtration column (Superdex G-200). This result was supported by dynamic light scattering (DLS) performed at 297 K with globular mode settings using a DynaPro-801 molecular-sizing instrument with temperature control. The TraM protein is monodisperse with a hydrodynamic radius of 2.61 nm, corresponding to a molecular weight of 30 kDa. This would account for a trimer in solution in the case of a globular protein. Another possibility is that TraM is a dimer, assuming an elongated α-helical macromolecule, which would explain the larger hydrodynamic radius. This assumption is supported by circular-dichroism analysis of TraM (data not shown), which reveals that 70% of the protein is α-helical, in line with secondary-structure prediction (Fig. 1). The purified recombinant protein binds TraR tightly and completely abolishes its DNA-binding activity, as determined by direct binding assays (data not shown).

Selenomethionine-labelled TraM was overexpressed in the methionine-auxotrophic *E. coli* strain B834(DE3) (Novagen) in M9 medium supplemented with 40 mg ml^{-1} of all amino acids except methionine and 60 mg ml^{-1} seleno-L-methionine and was grown at 310 K until the OD_{600nm} reached 0.8. Expression was induced for 18 h at 296 K by addition of 0.4 m*M* isopropyl-β-D-thiogalactoside. The labelled protein was purified using the same procedure as for the native protein, except that the DTT concentration was increased to 5 m*M* in order to prevent selenomethionine oxidation. ESI mass spectrometry confirmed the full incorporation of three selenomethionines per monomer (data not shown). The yield of the recombinant TraM is 80 mg per litre of cells for the native protein and 30 mg per litre of cells for the selenomethionine-substituted protein.

Table 1
Summary of X-ray diffraction data.

Values in parentheses are for the highest resolution shell.

	SeMet		Native
Wavelength (Å)	0.9791 (peak)	0.9611 (remote)	0.9611
Resolution (Å)	50–2.0 (2.12–2.00)	50–1.8 (1.91–1.80)	50–1.67 (1.78–1.67)
R_{sym} (%)	4.6 (18.5)	3.6 (22.4)	5.2 (22)
$I/\sigma(I)$	9.8 (3.4)	13.9 (3.3)	6.9 (3.4)
Observations	130145	161543	247930
Unique observations	11970	15965	19766
Completeness	99.3 (99.6)	96.9 (83.5)	93.5 (71)
Redundancy	3.6 (3.1)	3.3 (2.1)	3.4 (1.9)
Mosaicity (°)	1.5	1.5	0.9

2.2. Crystallization

TraM concentrated to 2.2 m*M* (25 mg ml^{-1}) in 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl and 1 m*M* DTT was used for crystallization trials. Trials were conducted at 297 K in 24-well plates using the hanging-drop and sitting-drop vapour-diffusion methods (McPherson, 1999). In all experiments, 1–4 µl protein solution was mixed with an equal volume of reservoir solution and equilibrated against 0.6 ml of reservoir solution. Initial screenings were carried out using the crystallization reagent kits Crystal Screen I and Crystal Screen II (Hampton Research) and also non-commercial screens. Crystals of TraM emerged from several conditions. After optimization, the best crystallization conditions were 100 m*M* Tris pH 8.5, 30–35%(*w*/*v*) PEG 4000, 200 m*M* ammonium sulfate, 1 m*M* DTT. Large single crystals appeared in 2 d and grew at 297 K to maximum dimensions of 0.5 × 0.5 × 0.2 mm in a week (Fig. 2).

Crystals were flash-cooled for data collection at 100 K in a nitrogen stream after stepwise transfer from the drop to the final cryosolution, which had the same composition of the mother liquor but with an increase in PEG 4000 to 40%, by gradually equilibrating them with increasing concentrations of PEG.

2.3. Data collection

All data were collected at 100 K using synchrotron radiation at the European Synchrotron Radiation Facility (ESRF), Grenoble. MAD data were collected on beamline ID29 using an ADSC Quantum 210 charge-coupled device (CCD) detector. A crystal scan was performed to determine the wavelengths for data collection. Data

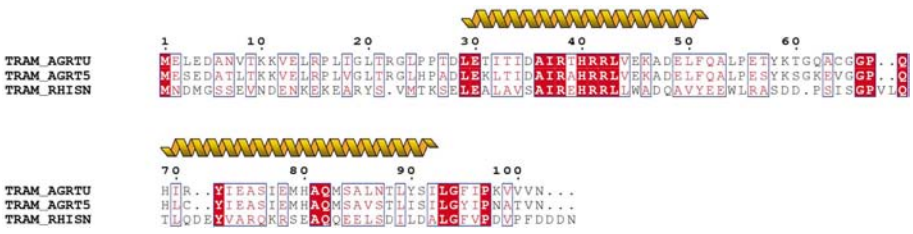

Figure 1
Multiple sequence alignment and secondary-structure prediction of TraM from octopine-type Ti plasmid pTiA6NC (tram_agrtu), TraM from nopaline-type Ti plasmid pTiC58 (tram_agrt5), both from *A. tumefaciens*, and TraM from *Rhizobium* sp. strain NGR234 (tram_rhisn). Identical and conserved residues are denoted by a red vertical bar and a blue vertical box, respectively. TraM secondary structure and residue numbering are shown above the alignment. The program *ESPript* (Gouet *et al.*, 1999) was used to generate the figure.

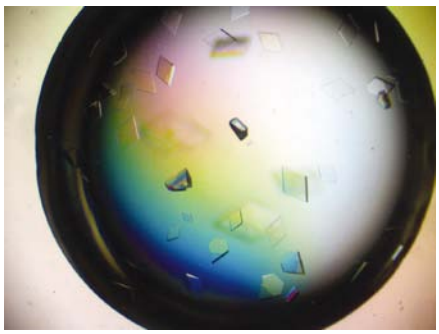

Figure 2
Typical crystals of TraM.

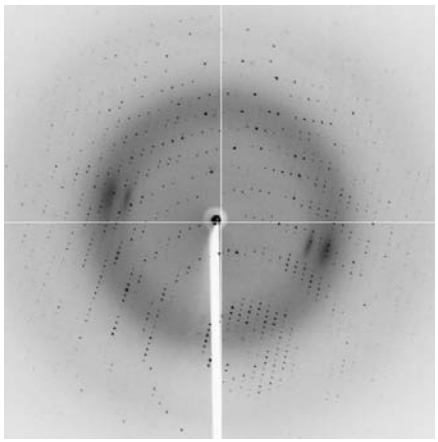

Figure 3
Diffraction image from the SeMet-derivatized TraM crystal.

were collected from a randomly oriented crystal at the Se absorption peak (12.663 keV) and at a remote reference energy (12.900 keV) (Fig. 3). Higher resolution data were collected at the same beamline at 1.67 Å from a native crystal. Data were processed with *MOSFLM* (Leslie *et al.*, 1986) and scaled with *SCALA* (Evans, 1993). Crystals belong to space group $P2_12_12$, with unit-cell parameters a = 76.43, b = 47.09, c = 47.46 Å. Despite the high degree of crystal mosaicity, data show very good statistics after processing and scaling (Table 1). Assuming the presence of two TraM molecules in the asymmetric unit gave a reasonable Matthews coefficient (Matthews, 1968) of 1.87 $Å^3\,Da^{-1}$ and a solvent content of 34.3%. Therefore, six crystallographically independent selenium sites had to be located (three selenomethionines per protein monomer, including the starting methionine).

2.4. Substructure solution and initial phases

The selenium substructure was determined with the *SnB* v.2.2 package (Weeks & Miller, 1999). The peak-wavelength anomalous differences were processed with the *DREAR* suite (Blessing *et al.*, 1996) to generate difference normalized structure factors (diff*E* values). The largest 300 diff*E* values were used in *SnB* v.2.2 to generate 3000 triplet invariants. Each trial was processed for 20 cycles of dual-space refinement. All these trials were carried out using an electron-density grid size of 0.67 Å and a minimum inter-peak distance of 3.0 Å. After 1000 trials had been processed, one trial was identified that had a significantly lower minimal function value ($R_{\rm min}$ = 0.170) than the others ($R_{\rm min}$ = 0.443–0.752). In all, five of the six independent selenium positions were located. These were confirmed in *CNX* (Accelrys, Pharmacopeia Inc.) by calculating and checking the anomalous difference Patterson maps (15–2.5 Å) from the experimental data and from the known locations of the selenium sites. Heavy-atom refinement, phase calculations and solvent flattening were carried out at 2.0 Å resolution using the remote wavelength as a reference in *CNX*. The figure of merit before and after solvent flattening was 0.532 and 0.912, respectively. The electron-density map is of excellent quality and clearly shows a boundary for two molecules of TraM arranged as a dimer. Model building and refinement are in progress.

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