

# Anti-TRAP protein from *Bacillus subtilis*: crystallization and internal symmetry

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Anti-TRAP protein regulates the expression of tryptophan biosynthetic genes by binding to TRAP and preventing formation of the TRAP–RNA complex. Anti-TRAP from *Bacillus subtilis* has been crystallized by vapour diffusion. The crystals belong to space group *P*1, with unit-cell parameters  $a = 51.6$ ,  $b = 60.1$ ,  $c = 60.4$  Å,  $\alpha = 114.0$ ,  $\beta = 101.4$ ,  $\gamma = 100.5^\circ$ . X-ray data have been collected to 2.8 Å resolution. Peaks in the self-rotation function correspond to four trimers in the unit cell related by twofold and threefold rotational axes. The symmetry and gel-filtration data suggest that the protein exists as a trimer or a dodecamer in solution.

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## 1. Introduction

*Bacillus subtilis* anti-TRAP regulates the expression of tryptophan biosynthetic genes by binding to the *trp* RNA-binding attenuation protein (TRAP; Valbuzzi & Yanofsky, 2002) and preventing interaction of TRAP with RNA by masking its binding site (Valbuzzi *et al.*, 2002). In the absence of anti-TRAP, TRAP, when activated by bound L-tryptophan, binds to the mRNA region within the 5' leader region of the *trpECDFBA* operon, resulting in the formation of a transcription-termination hairpin (Gollnick, 1994; Babitzke & Gollnick, 2001, 2002). The RNA-binding segment is organized as 11 (G/U)AG repeats separated by two or three variable 'spacer' nucleotides (Babitzke *et al.*, 1994, 1995). TRAP also regulates the translation of several genes by binding to specific regions of mRNAs (Merino *et al.*, 1995). Expression of anti-TRAP protein is induced by the accumulation of uncharged tRNA<sup>Trp</sup> in the cell (Valbuzzi & Yanofsky, 2001). Therefore, regulation of tryptophan synthesis in *B. subtilis* occurs by sensing both the level of charged tRNA<sup>Trp</sup> and the level of free cellular tryptophan.

Anti-TRAP is a small oligomeric protein (5.6 kDa) composed of several identical 53-amino-acid polypeptides (Valbuzzi & Yanofsky, 2001). Each monomer binds one Zn atom, which is essential for its activity; removal of zinc converts anti-TRAP to inactive monomers (Valbuzzi & Yanofsky, 2002). The amino-acid sequence of anti-TRAP shows some homology to the cysteine-rich domain of the chaperone protein DnaJ (Valbuzzi & Yanofsky, 2001), the sequence identity for residues segment 9–39 containing two cysteine-rich motifs being 32%. The cysteine-rich motif CXXCXGXG (where X represents a variable amino acid) is repeated four times in DnaJ and

appears twice in anti-TRAP. The NMR structure of DnaJ (Martinez-Yamout *et al.*, 2000) showed that two such motifs are involved in binding one Zn atom, with four cysteine side chains coordinated to the metal. Substitution of any anti-TRAP cysteine residue by alanine results in rapid degradation of the mutant proteins *in vivo* (Valbuzzi & Yanofsky, 2002). Chemical cross-linking experiments have suggested that anti-TRAP exists as a hexamer composed of two identical trimers. Within the trimer, subunits appear to be bound *via* Zn atoms, with the metal atom coordinated by four cysteine residue side chains: two from one subunit and two from an adjacent subunit (Valbuzzi & Yanofsky, 2002).

It has recently been shown by analytical ultracentrifugation that anti-TRAP exhibits concentration-dependent and reversible self-association. Experiments, which were performed over the concentration range 12.5–214 µM, indicated that most of the protein exists as a trimer at low concentration and as a dodecamer at high concentration (Snyder *et al.*, 2004).

The three-dimensional structures of tryptophan-activated TRAP (Antson *et al.*, 1995; Chen *et al.*, 1999) and of several TRAP–RNA complexes have been determined (Antson *et al.*, 1999; Hopcroft *et al.*, 2002). TRAP is a toroid-shaped molecule composed of 11 identical subunits with 11 tryptophan-binding sites located at subunit–subunit interfaces. Tryptophan binding stabilizes the active conformation of TRAP, enabling it to interact with RNA (McElroy *et al.*, 2002). In the TRAP–RNA complex, the RNA chain binds to 11 binding sites, forming a belt around the protein molecule.

To date, there is no structural information available for anti-TRAP. Here, we describe the crystallization of anti-TRAP from *B. subtilis*.

We also describe preliminary X-ray data analysis, which suggests the possible internal symmetry of anti-TRAP.

## 2. Material and methods

### 2.1. Overexpression and purification of anti-TRAP

Anti-TRAP was purified by a modification of the procedure described by Valbuzzi & Yanofsky (2001). Anti-TRAP was expressed using the T7 expression system and pET17b (Novagen). The resulting plasmid was transformed into *Escherichia coli* Rosetta (DE3) competent cells (Novagen). Cells were grown in LB medium until early log phase ( $A_{600} = 0.4$ ), at which point anti-TRAP expression was induced by addition of 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside and the cells were grown for an additional 3 h. Cells were collected by centrifugation and broken in a French Pressure cell (Aminco) in buffer A (100 mM Tris-HCl pH 7.8, 1 mM dithiothreitol). Cell debris was removed by centrifugation at 30 000g for 20 min. Streptomycin sulfate was added to the supernatant to 3% (w/v), with stirring on ice for 20 min, and the precipitate was removed by centrifugation as above. The supernatant was heated at 363 K for 8 min and the insoluble proteins were removed by centrifugation. Anti-TRAP is a thermostable protein and on heating the extract most (>90%) of the *E. coli* proteins are denatured and precipitate but anti-TRAP does not. The supernatant was dialysed overnight against buffer B (50 mM Tris-HCl pH 7.8, 1 mM dithiothreitol) and then loaded onto a MonoQ Sepharose column (Pharmacia) equilibrated with buffer B. Anti-TRAP was eluted with a 0–1.0 M NaCl gradient and the fractions containing anti-TRAP were collected and concentrated using an Amicon Ultra-15 (Millipore) device. After dialysis overnight against buffer A, the sample was passed over a Sephadex G-75 column equilibrated with buffer A. Anti-TRAP fractions were pooled and concentrated as above. The purity of the anti-TRAP was monitored by SDS gel electrophoresis and its concentration was determined by BCA protein assay (Pierce).

### 2.2. Gel filtration

Gel-filtration experiments were performed using a Superdex-200 column with a total elution volume of 120 ml. The column was equilibrated with 5 mM triethanolamine pH 8.0, 20 mM NaCl, 1 mM dithiothreitol and calibrated using standard protein molecular-weight markers. The

protein concentration used was 1 mg ml<sup>-1</sup>, with an overall sample volume of 1 ml. The flow rate during the column run was 1 ml min<sup>-1</sup>. Because of the absence of tryptophan residues and the presence of only a single tyrosine residue (one per monomer), the elution profile of the protein was monitored at 215 nm.

### 2.3. Crystallization

Anti-TRAP protein was dialysed against a solution containing 1 mM dithiothreitol, 20 mM NaCl and 5 mM triethanolamine pH 8.0 and concentrated to 15 mg ml<sup>-1</sup> using an ultrafiltration membrane (Filtron). The protein concentration was estimated using the Bradford reagent (Pierce) with bovine serum albumin as standard (Bradford, 1976).

Crystals of anti-TRAP were grown using hanging-drop vapour diffusion (McPherson, 1982). Initial screening was performed using sparse-matrix kits from Hampton Research (Jancarik & Kim, 1991). The protein solution was allowed to equilibrate against 0.5 ml of reservoir solution at 293 K. Each droplet was formed by mixing equal volumes (1  $\mu$ l) of protein solution and reservoir solution. After the initial crystallization trials, the best conditions were optimized.

### 2.4. X-ray data collection

For the diffraction experiment, crystals were flash-frozen in a cryosolution composed of crystallization ingredients with a slightly higher concentration of precipitating agent (PEG) and with the addition of 16% (v/v) glycerol. X-ray data were collected at 110 K and a wavelength of 0.87 Å using synchrotron radiation at beamline 9.6 (Daresbury) and an ADSC Quantum 4 CCD detector. During the experiment, the oscillation angle was 1.0°, the crystal-to-detector distance was 300 mm and the exposure time per oscillation was 80 s with three oscillations per image. A total of 183 images were collected. Diffraction data were integrated using *MOSFLM* (Leslie, 1993) and scaled and merged with *SCALA* (Evans, 1997). All crystallographic calculations were performed using the *CCP4* suite of programs (Collaborative Computational Project, Number 4, 1994). The self-rotation function was calculated using *MOLREP* (Vagin & Teplyakov, 1997).

## 3. Results and discussion

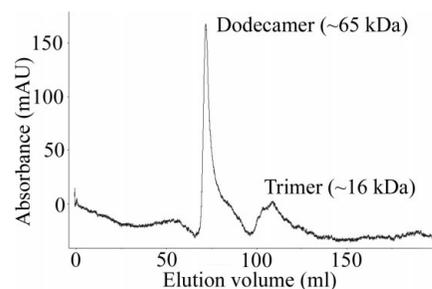
### 3.1. Gel filtration

Two peaks were observed on the gel-filtration profile: one major peak, which is

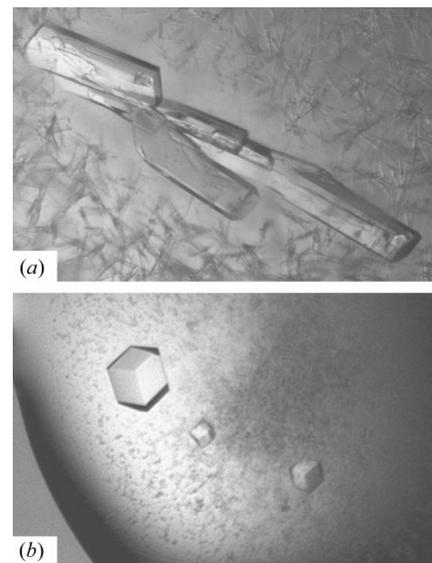
consistent with a weight of about 65 kDa, and a second less abundant peak with a weight corresponding to 16 kDa. The weight of one anti-TRAP monomer is ~5.6 kDa. Therefore, the first peak in the gel-filtration profile probably corresponds to a dodecamer (67.2 kDa) and the second peak corresponds to a trimer (16.8 kDa) (Fig. 1).

### 3.2. Crystals of anti-TRAP

The best crystals of anti-TRAP were obtained with a reservoir containing 21–23% (w/v) poly(ethylene) glycol 5000 monomethyl ether, 0.1 M sodium cacodylate pH 6.5 and 50 mM magnesium acetate. Plate-shaped crystals with approximate dimensions of 0.4 × 0.1 × 0.05 mm grew in 3–4 d (Fig. 2a). These crystals proved to be anisotropic and highly mosaic (1–2°), with several plates often grown into each other. These crystals belonged to space group *P1*, with unit-cell parameters  $a = 51.6$ ,  $b = 60.1$ ,  $c = 60.4$  Å,  $\alpha = 114.0$ ,  $\beta = 101.4$ ,  $\gamma = 100.5^\circ$ . A total of more than 50 crystals were tested before one single crystal diffracting to 3 Å



**Figure 1**  
Gel-filtration elution profile of anti-TRAP.



**Figure 2**  
Crystals of anti-TRAP. (a) Plate-like crystals; (b) cubic shaped crystals.

resolution was identified. Interestingly, when the pH of the reservoir was increased to 8.0 (0.1 M triethanolamine), small cube-shaped crystals with dimensions of  $0.2 \times 0.2 \times 0.2$  mm appeared (Fig. 2*b*). These crystals produced limited diffraction to  $\sim 8$  Å resolution.

### 3.3. Data-collection statistics and self-rotation function analysis

X-ray data were collected to a resolution of 2.8 Å using a single crystal belonging to space group *P1*. Although the mosaicity was relatively high ( $1.6^\circ$ ), the data were processed and merged reasonably well. The main statistics of the data set are shown in Table 1.

To deduce the internal symmetry of anti-TRAP, we calculated the solvent content and analysed the self-rotation function  $R(\Phi, \Psi, K)$  (Crowther, 1972). 12 monomers (four trimers), with a molecular weight of

$12 \times 5648$  Da per asymmetric unit, result in a Matthews coefficient of  $2.4 \text{ \AA}^3 \text{ Da}^{-1}$  and a solvent content of 48% (Matthews, 1968). Three or five trimers of anti-TRAP per asymmetric unit would correspond to solvent contents of 61 and 35%, with Matthews coefficients of 3.2 and  $1.9 \text{ \AA}^3 \text{ Da}^{-1}$ , respectively.

The self-rotation function was calculated with a radius of integration of 30 Å using diffraction data within the 10.0–3.0 Å resolution shell. Peaks corresponding to four threefold axes (Fig. 3*a*) and three twofold axes (Fig. 3*b*) were observed. These peaks remained present when the radius of integration was varied. The constellation of peaks could be explained by four trimers arranged at the corners of a tetrahedron, as shown in Fig. 4. In that model, each trimer sits on a threefold axis, with twofold axes perpendicular to the faces of a cube.

The symmetry and gel-filtration data suggest that the anti-TRAP protein exists as

**Table 1**

Data-collection statistics.

Values in parentheses are for the highest resolution shell.	
Wavelength (Å)	0.87
Space group	<i>P1</i>
Unit-cell parameters (Å, °)	$a = 51.6, b = 60.1,$ $c = 60.4, \alpha = 114.0,$ $\beta = 101.4, \gamma = 100.5$
Resolution (Å)	35–2.8 (2.95–2.8)
No. of measurements	26628 (458)
No. of unique reflections	13148 (229)
Completeness (%)	93.4 (82.2)
$R_{\text{merge}}^\dagger$ (%)	11.0 (18.6)
Average $I/\sigma(I)$	4.3 (3.4)
Multiplicity	1.9 (1.8)

$^\dagger R_{\text{merge}} = \sum_h \sum_i |I(h)_i - \langle I(h) \rangle| / \sum_h \sum_i I(h)_i$ , where  $I(h)_i$  is the intensity of reflection  $h$ ,  $\sum_h$  is the sum over all measured reflections and  $\sum_i$  is the sum over  $i$  measurements of a reflection.

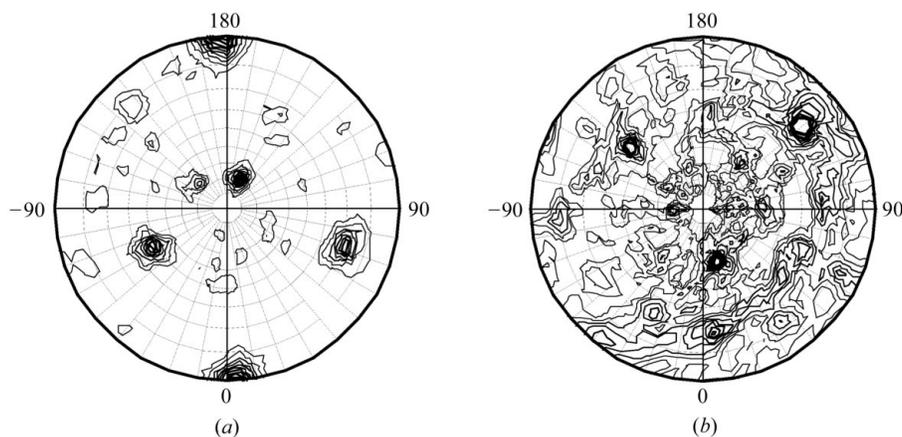
a trimer or dodecamer in solution. Our data do not support the association of two trimers into a hexamer, as proposed previously on the basis of chemical cross-linking experiments (Valbuzzi & Yanofsky, 2002). In agreement with our results, data obtained recently by analytical ultracentrifugation (Snyder *et al.*, 2004) indicate that anti-TRAP exists as a dodecamer at high concentrations and as a trimer at low concentrations. It is reasonable to assume that physiological concentrations of anti-TRAP in the cell are lower than those required for association of trimers into dodecamers (subunit concentration of  $12.5 \mu\text{M}$  or higher). We therefore predict that anti-TRAP functions as a trimer.

The results reported here lay the groundwork for structure determination. The presence of one Zn atom per subunit should permit structure determination by the MAD technique.

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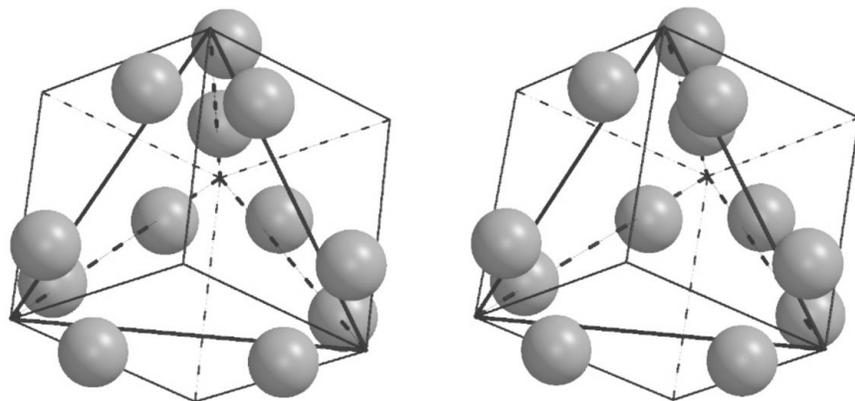
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**Figure 3**

Stereographic projection of the self-rotation function, with the crystallographic  $a$  axis along  $\varphi = 0^\circ$ ,  $\psi = 90^\circ$  and the  $c^*$  axis perpendicular to the plane of the figure. The  $\varphi$  angle varies from  $0$  to  $\pm 180^\circ$  around the plot, while the  $\psi$  angle varies from  $0^\circ$  at the centre to  $90^\circ$  at the edge of the ring. (a) Section with  $\kappa = 120^\circ$ ; (b) section with  $\kappa = 180^\circ$ . The resolution of data used was 10.0–3.0 Å and the radius of integration was 30 Å. Contours start at the zero level and increase in  $0.6\sigma$  intervals.



**Figure 4**

Stereo figure showing the arrangement of four trimers of anti-TRAP that constitute a crystallographically independent unit. Each monomer is represented by a sphere.

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