Crystallization and preliminary X-ray crystallographic analysis of the TM1442 gene product from *Thermotoga maritima*, a homologue of *Bacillus subtilis* anti-anti-sigma factors

A 110-residue protein encoded by the TM1442 gene of *Thermotoga maritima* shows amino-acid sequence similarity to *Bacillus subtilis* anti-anti-sigma factors RsbV and SpoIIAA. It has been over-expressed in *Escherichia coli* and the recombinant protein exists primarily as both a monomer and a dimer in solution. The dimeric form has been crystallized using polyethylene glycol (PEG) 8000 as a precipitant. Native X-ray diffraction data have been collected at 100 K to 2.0 Å resolution. The crystals are monoclinic, belonging to the space group *P*2₁, with unit-cell parameters *a* = 31.54 (13), *b* = 116.83 (37), *c* = 31.39 (7) Å, *α* = 90, *β* = 119.84 (9), *γ* = 90°. The asymmetric unit contains two monomers of the recombinant polypeptide, with a corresponding *V*_M of 2.24 Å³ Da⁻¹ and a solvent content of 45.0%.

1. Introduction

The most significant gene regulation in eubacteria occurs at the level of transcription initiation. A layer of transcriptional regulation utilizes the anti-sigma (anti-σ) factors, antagonists of the sigma factors. A wide range of cellular processes are regulated by anti-σ factors, including bacteriophage growth, sporulation, stress response, flagellar biosynthesis, pigment production, ion transport and virulence expression. The anti-σ factors act by binding to the cognate σ factors, thus preventing the interaction of the latter with RNA polymerases. However, the anti-σ factors themselves are heterogeneous in size and the mechanism of their regulation is also quite varied (Hughes & Mathee, 1998).

In *B. subtilis*, the activities of at least two σ factors, σF and σB, are modulated by anti-σ factor proteins (Dufour & Haldenwang, 1994; Yang *et al.*, 1996). Sigma factor *F* (σF) is a key transcription factor that initiates prespore development in *B. subtilis*. Its activity is controlled by an anti-σ factor, SpoIIAB, which inhibits σF activity by direct interaction (Magnin *et al.*, 1996; Min *et al.*, 1993). SpoIIAB is regulated in turn by its antagonist, the anti-anti-σ factor SpoIIAA, which binds the SpoIIAB-σF complex to release σF in the forespore (Alper *et al.*, 1994; Diederich *et al.*, 1994; Duncan *et al.*, 1996). Subsequent work proved that purified SpoIIAB not only inhibits σF in vitro (Duncan & Losick, 1993; Min *et al.*, 1993) but also acts as a protein kinase that catalyses the phosphorylation of SpoIIAA (Min *et al.*, 1993). Phosphorylation of SpoIIAA leads to a major change in conformation and its ability to interact with SpoIIAB is lost (Magnin *et al.*, 1996). In the presence of ADP, the non-phosphorylated form of SpoIIAB binds to SpoIIAB. In the presence of ATP, SpoIIAA is phosphorylated and the SpoIIAB released from the SpoIIAA-SpoIIAB complex can bind to and inhibit σF (Alper *et al.*, 1994; Diederich *et al.*, 1994). Sigma factor B (σB), a secondary σ factor of *B. subtilis*, mediates the general stress responses (Haldenwang & Losick, 1980). The transcription factors σF and σB are closely related to each other and the regulatory mechanisms affecting their activities are strikingly similar. Just as the σF activity is regulated by the anti-σ factor SpoIIAB and its antagonist SpoIIAA, σB is regulated by the anti-σ factor RsbW and its antagonist RsbV. RsbW also possesses a kinase activity directed toward its RsbV antagonist (Kang *et al.*, 1996; Yang *et al.*, 1996; Dufour & Haldenwang, 1994). Furthermore, the anti-σ factors RsbW and SpoIIAB are themselves similar to each other, with a 27% amino-acid identity, suggesting evolution from a common ancestor. The same is true for the anti-anti-σ factors RsbV and SpoIIAA, which have a 32% amino-acid identity (Kalman *et al.*, 1990).

The TM1442 gene of *T. maritima* encodes a polypeptide chain comprising 110 amino-acid residues with a molecular mass of 12 300 Da (Nelson *et al.*, 1999). It shows overall sequence identities of 30 and 25% with *B. subtilis* anti-anti-σ factors RsbV and SpoIIAA, respectively. The solution structure of SpoIIAA has been determined (Kovacs *et al.*, 1998). It is a monomeric protein with a fold consisting of a four-stranded β-sheet and four α-helices. No experimental data are available regarding the function of the TM1442 gene product. However, on the basis of sequence similarity, it
Table 1
Synchrotron data-collection statistics.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray wavelength</td>
<td>0.9794 Å (Photon Factory, BL-18B)</td>
</tr>
<tr>
<td>Space group</td>
<td>P2_1</td>
</tr>
<tr>
<td>Unit-cell parameters (Å)</td>
<td>a = 31.54, b = 116.83, c = 31.39, α = 90°, β = 119.84, γ = 90°</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>19.9–2.0</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>12868</td>
</tr>
<tr>
<td>Data completeness (%)</td>
<td>96.8 (96.8)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>13.9 (5.2)</td>
</tr>
</tbody>
</table>

\[ R_{merge} = \sum_i |\langle h \rangle_i - \langle h \rangle| / \sum_i \langle h \rangle_i \], where \( \langle h \rangle_i \) is the intensity of reflection \( h \), \( \sum_i \langle h \rangle_i \) is the sum over all reflections and \( \sum_i \) is the sum over \( i \) measurements of reflection \( h \).

\[ \text{merge}_i = |\langle h \rangle_i - \langle h \rangle| / \langle h \rangle_i \], where \( \langle h \rangle_i \) is the sum over all reflections and \( \sum_i \) is the sum over \( i \) measurements of reflection \( h \).

is believed to be a homologue of \( B. subtilis \) anti-anti-\( \sigma \) factors RsbV and SpoIIA. In order to obtain clues for a possible biological role of the TM1442 gene product, we have initiated its structure determination. Here, we report its overexpression, crystallization and preliminary X-ray crystallographic data.

2. Experimental

2.1. Protein expression and purification

The TM1442 gene was amplified by the polymerase chain reaction using the \( T. maritima \) genomic DNA as template. The amplified DNA was inserted into the \( NdeI/BamHI \)-digested expression vector pET-21a. The protein was overexpressed in soluble form in \( E. coli \) strain C41(DE3) upon induction by 0.5 mM isopropyl-\( \beta \)-D-thiogalactopyranoside (IPTG) at 303 K. Cells were grown in Luria–Bertani medium for 13 h at 293 K after IPTG induction and were harvested by centrifugation at 4200g (6000 rev min\(^{-1}\); Sorvall GSA rotor) for 10 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer (50 mM Tris–HCl pH 8.5, 15 mM NaCl) and was then disrupted by ultrasonication. The crude cell extract was centrifuged at 36 000g (18 000 rev min\(^{-1}\); Hanil Supra 21K rotor) for 40 min at 277 K. The supernatant fraction was heated and maintained between 340 and 358 K for 10 min and then placed in an ice bath for 10 min. The cell extract was centrifuged at 36 000g (18 000 rev min\(^{-1}\); Hanil Supra 21K rotor) for 50 min at 277 K. The supernatant was loaded onto a Q-Sepharose anion-exchange chromatography column (Amersham-Pharmacia) which was previously equilibrated with buffer A (50 mM Tris–HCl pH 8.5). Most of the TM1442 protein was present in the flowthrough. This fraction was heated and maintained between 340 and 358 K for 20 min. Final purification of the enzyme was performed by gel filtration on a HiLoad XK16 Superdex 200 prep-grade column (Amersham-Pharmacia) which was previously equilibrated with buffer A containing 200 mM sodium chloride. The protein solution of the dimeric form (see § for more details) was concentrated using a YM1 membrane (Amicon) to ~25 mg ml\(^{-1}\). The protein concentration was estimated by measuring the absorbance at 280 nm employing the calculated molar extinction coefficient of 2560 M\(^{-1}\) cm\(^{-1}\).

2.2. Dynamic light-scattering studies

The dynamic light-scattering experiment was performed on a Model DynaPro-801 instrument from Protein Solutions (Charlottesville, Virginia). The data were measured at room temperature with 3 mg ml\(^{-1}\) of protein in buffer A containing 200 mM sodium chloride.

2.3. Crystallization

Crystallization was achieved by the hanging-drop vapour-diffusion method at 296 K using 24-well tissue-culture plates (Hampton Research). A hanging drop was prepared by mixing equal volumes (2 μl each) of the protein solution and the reservoir solution. The protein concentration was 25 mg ml\(^{-1}\) before mixing with the reservoir solution. Each hanging drop was placed over a 1.0 ml reservoir solution. Initial crystallization conditions were established using sparse-matrix sampling (Jancarik & Kim, 1991).

2.4. X-ray diffraction experiment

A crystal of the anti-anti-\( \sigma \) factor was transferred to a solution consisting of 27.5% (w/v) PEG 8000, 200 mM magnesium acetate and 100 mM sodium cacodylate pH 6.5 within a minute in one step before being flash-frozen. A set of native X-ray diffraction data was collected at 100 K at the BL-18B experimental station of the Photon Factory, Tsukuba, Japan (Sakabe, 1991) with an ADSC Quantum 4R CCD detector. The wavelength of the synchrotron X-rays was 0.9794 Å. The crystal was rotated through a total of 120°, with a 1° oscillation range per frame. The raw data were processed and scaled using the program MOSFLM (Leslie, 1992).

3. Results

The TM1442 gene of \( T. maritima \) has been overexpressed in \( E. coli \) as a soluble form. The recombinant protein was separated into two peaks upon gel filtration. The native molecular masses of the two peaks were estimated to be about 14.8 and 31.7 kDa by dynamic light-scattering measurements. This suggests that the protein exists in solution primarily as a monomer and a dimer. The yield was approximately 250 mg for the dimer fraction and 350 mg for the monomer fraction from a 3.0 l culture. The dimeric form of the recombinant protein gave well diffracting crystals using a reservoir solution containing 200 mM magnesium acetate, 100 mM sodium cacodylate pH 6.5 and 20% (w/v) PEG 8000. The crystals grew to maximum dimensions of 0.30 × 0.15 × 0.05 mm within 2 d (Fig. 1). Under similar conditions, the monomeric form gave much smaller crystals of poor morphology, which were too small to be characterized crystallographically. This observation seems to suggest that conversion between a monomer and a dimer is possible under the crystallization conditions. However, it is difficult to assess how rapidly this process may take place.

The native diffraction data were collected to 2.0 Å resolution at 100 K using synchrotron X-rays. A total of 57 327 measured reflections were merged into 12 868 unique reflections with an \( R_{merge} \) (on intensity) of 3.2%. The merged data set is 96.8% complete to 2.0 Å resolution. The crystals belong to the monoclinic space group \( P2_1 \), with unit-cell parameters \( a = 31.54 (13) \), \( b = 116.83 (37) \), \( c = 31.39 (7) \) Å, \( α = 90° \), \( β = 119.84 (9) \), \( γ = 90° \), where the estimated standard deviations are given in parentheses. The presence of two monomers of TM1442 polypeptide in the asymmetric unit gives a crystal volume per protein mass (\( V_M \)) of...
2.24 Å³ Da⁻¹, with a corresponding solvent content of 45.0% (Matthews, 1968). Table 1 summarizes the statistics for the synchrotron data collection. Molecular-replacement trials using the NMR structure of *B. subtilis* spoIIAA (25% sequence identity) as a search model did not give any reasonable solution. Therefore, the structure will be solved by multiwavelength anomalous diffraction or the multiple isomorphous replacement method.

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**References**


