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Purification, crystallization and preliminary X-ray diffraction studies of the soluble domain of the oligosaccharyltransferase STT3 subunit from the thermophilic archaeon *Pyrococcus furiosus*

Oligosaccharyltransferase catalyzes the transfer of preassembled oligosaccharides onto asparagine residues in nascent polypeptide chains. The STT3 subunit is thought to bear the catalytic site. The C-terminal domain of the STT3 protein of *Pyrococcus furiosus* was expressed in *Escherichia coli* cells. STT3 protein prepared from two different sources, the soluble fraction and the inclusion bodies, produced crystals that diffracted to 2.7 Å. During crystallization screening, cocrystals of *P. furiosus* STT3 with an *E. coli* 50S ribosomal protein, L7/L12, were accidentally obtained. This cross-species interaction is not biologically relevant, but may be used to design a built-in polypeptide substrate for the STT3 crystals.

1. Introduction

Asparagine-linked glycosylation (N-glycosylation) is widespread in eukaryotic, archaeal and some eubacterial organisms (Szymanski *et al.*, 2003; Upreti *et al.*, 2003; Szymanski & Wren, 2005; Kelleher & Gilmore, 2006) and is essential for the structural and functional integrity of many secretory and membrane proteins. Oligosaccharyltransferase (OST) catalyzes the transfer of preassembled oligosaccharides on lipid carriers onto asparagine residues in the sequon Asn-X-Thr/Ser, where X can be any residue except Pro, in nascent polypeptide chains (Bause & Hettkamp, 1979; Gavel & von Heijne, 1990).

The OST enzyme is a multisubunit protein complex residing in the endoplasmic reticulum membrane in eukaryotic cells (Knauer & Lehle, 1999). In contrast, the OST of the Gram-negative eubacterium *Campylobacter jejuni* is composed of one subunit (Szymanski *et al.*, 2003). Several lines of biochemical and genetic evidence have indicated that the STT3 protein is the catalytic subunit of the OST enzyme in eukaryotes and *C. jejuni* (Yan & Lennarz, 2002; Nilsson *et al.*, 2003; Karamyshev *et al.*, 2005; Feldman *et al.*, 2005; Glover *et al.*, 2005). The inactivation of the *stt3* gene in the archaeon *Methanococcus voltae* resulted in the underglycosylation of flagellin and S-layer proteins (Chaban *et al.*, 2006). This experiment suggested that the STT3 protein also functions as the catalytic subunit of the OST enzyme in archaea. The STT3 protein in all three domains of life consists of an N-terminal half segment, containing 10–13 predicted transmembrane helices (TMs), and a C-terminal globular domain (Zufferey *et al.*, 1995; Knauer & Lehle, 1999). The C-terminal domain contains a highly conserved five-residue motif, Trp-Trp-Asp-Tyr-Gly, and is thought to bear the catalytic site.

Here, we report the expression, crystallization and preliminary crystallographic analysis of the C-terminal domain of the STT3 protein from the thermophilic archaeon *Pyrococcus furiosus*.

2. Materials and methods

2.1. Expression of the soluble domain of *P. furiosus* STT3 protein

The soluble domain (residues 471–967) of the STT3 protein (sSTT3) from *P. furiosus* (accession No. PF0156, Q8U4D2_PYRFU) was produced in *Escherichia coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene) using the *Nco*I and *Bam*HI restriction sites of the



pET21d vector (Invitrogen). The transformed *E. coli* cells were cultured at 310 K in LB media containing 50 mg l⁻¹ ampicillin. After the OD₆₀₀ had reached 0.6, the culture was supplemented with 0.5 mM IPTG for induction. The cells were cultured at 310 K for 4 h or at 289 K overnight for the production of the sSTT3 protein in inclusion bodies or as soluble protein, respectively. After the cells had been harvested by centrifugation, the cell pellet was resuspended in buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl and 2 mM EDTA) supplemented with 4 mM MgCl₂ and 10 U ml⁻¹ Benzonase (Merck). The cells were then disrupted by sonication. The resultant solution was separated into supernatant and pellet fractions by centrifugation at 10 000g for 30 min.

2.2. Protein preparation by refolding from inclusion bodies

The pellet of the sonicated lysate contained sSTT3 in inclusion bodies. The inclusion bodies were washed three times by resuspension and centrifugation in buffer A containing 1% Triton X-100 and then three times in buffer A without Triton X-100. The washed inclusion bodies were dissolved in buffer A containing 6 M urea and the insoluble materials were removed by centrifugation. The supernatant was loaded onto an anion-exchange Q-Sepharose FF column (GE Healthcare). The sSTT3 protein was eluted as a single peak with a linear gradient of NaCl in the presence of 6 M urea and the fractions containing sSTT3 were collected. Refolding was carried out by direct injection into a gel-filtration Superdex 200 column (GE Healthcare) pre-equilibrated with buffer A without urea. The fractions containing the refolded sSTT3 protein were combined and concentrated to 10 mg ml⁻¹ by ultrafiltration (10 kDa cutoff, Amicon Ultra, Millipore) in 10 mM Tris-HCl pH 8.0.

2.3. Protein preparation by heat treatment of the supernatant

The supernatant of the sonicated lysate contained soluble sSTT3. After the supernatant had been heated to 353 K for 15 min, the denatured *E. coli* proteins were removed by centrifugation at 16 000g for 15 min. The resultant supernatant was diluted with buffer (50 mM Tris-HCl pH 8.0, 2 mM EDTA) and loaded onto a Resource Q anion-exchange column (GE Healthcare). The sSTT3 protein was eluted with a linear gradient of NaCl. The fractions containing sSTT3 were combined and concentrated by ultrafiltration. The protein was further purified by gel-filtration chromatography on a Superdex 200 column (GE Healthcare) pre-equilibrated with buffer A. The fractions at the top of the peak (peak-top) containing sSTT3 were combined and concentrated to 10 mg ml⁻¹ by ultrafiltration in 10 mM Tris-HCl pH 8.0. The fractions at the tailing edge of the peak were also collected and processed in the same manner.

A selenomethionyl (SeMet) derivative of sSTT3 was expressed in minimal medium containing seleno-L-methionine at a concentration of 25 mg l⁻¹. SeMet sSTT3 was purified by the same procedure as described for the peak-top fraction of the supernatant sSTT3 and the incorporation of selenium was confirmed by MALDI-TOF mass spectrometry.

2.4. Crystallization

Initial screening was carried out using the sitting-drop vapour-diffusion method in 96-well Intelli-plates (Art Robbins Instruments) and optimization was performed using the hanging-drop vapour-diffusion method in 24-well VDX greased plates (Hampton Research). Sitting drops were set up by mixing equal volumes (0.2 µl each) of the protein solution and the reservoir solution using an automated dispenser (Hydra II Plus One system; Apogent Discov-

eries), whereas hanging drops were prepared manually by mixing 0.7 µl protein solution and 0.7 µl reservoir solution. Each sitting drop was placed over 0.1 ml reservoir solution and each hanging drop was placed over 0.4 ml reservoir solution.

3. Results and discussion

3.1. Preparation

The C-terminal 497-residue fragment of the STT3 protein (sSTT3) from *P. furiosus* was expressed in *E. coli* cells. The majority of the expressed sSTT3 resided in the pellet, but the supernatant also contained a small but significant amount of sSTT3. The insoluble sSTT3 contained in the pellet fraction was purified by anion-exchange chromatography in the presence of urea and was refolded by direct injection into a gel-filtration column pre-equilibrated without urea. The majority of the sSTT3 protein eluted as a single peak (upper chromatogram in Fig. 1). By assuming a monomeric form (55.9 kDa), the point corresponding to the peak-top elution volume is on the calibration curve (inset in Fig. 1), suggesting successful refolding as the monomeric form. The typical yield of sSTT3 from the pellet was 30 mg per litre of culture. On the other hand, the supernatant fraction was heated at 353 K. The thermostable sSTT3 remained in the solution, but the majority of the *E. coli* proteins precipitated. After centrifugation, the sSTT3 was recovered in the supernatant and was further purified by anion-exchange and gel-filtration chromatography. The sSTT3 protein eluted as a single peak in the gel-filtration chromatography at the same elution volume as the refolded sSTT3, but with a tailing peak (lower chromatogram in Fig. 1). The peak-top fractions contained pure sSTT3. The typical yield of sSTT3 from the supernatant was 2 mg per litre of culture. The fractions at the tailing edge were also collected. All protein stock

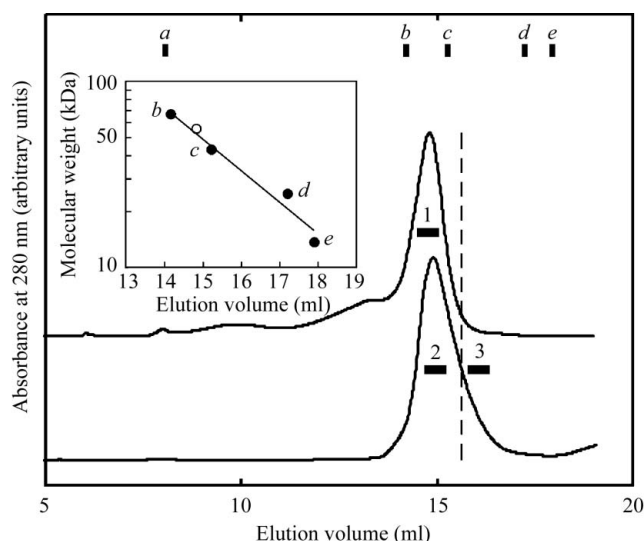


Figure 1 Comparison of gel-filtration chromatograms of the two different preparations of the C-terminal soluble domain of the STT3 protein from *P. furiosus*. The upper chromatogram shows the refolded sSTT3 recovered from the pellet fraction and the lower chromatogram shows the sSTT3 recovered from the heat-treated cell lysate. The pooled fractions for crystallization are indicated by horizontal bars. The inset shows the calibration curve for the estimation of apparent molecular weight. The open circle represents the peak-top position of the chromatograms, assuming the molecular weight of the monomeric form (55.9 kDa). a, Void volume; b, albumin, 67 kDa; c, ovalbumin, 43 kDa; d, chymotrypsinogen, 25 kDa; e, ribonuclease A, 13.7 kDa.

solutions used for crystallization contained 10 mg ml⁻¹ protein in 10 mM Tris-HCl pH 8.0.

3.2. Crystallization

Screenings were carried out at 293 K using Crystal Screens 1 and 2 (Hampton Research). Crystals of sSTT3 purified as the peak-top fractions in the gel filtration from the supernatant fraction (Fig. 1, sample 2) were obtained with Crystal Screen 1 solution No. 22 (0.1 M Tris-HCl pH 8.5, 30% PEG 4000, 0.2 M sodium acetate) and Crystal Screen 2 solution No. 30 (0.1 M HEPES pH 7.5, 10% PEG 6000, 5% MPD). After optimization, the crystals grew from a hanging drop with a 1:1 volume ratio of 10 mg ml⁻¹ protein stock solution and reservoir solution (0.1 M Tris-HCl pH 9.0, 22% PEG 4000, 0.3 M sodium acetate) at 303 K in 1–3 weeks (Fig. 2a). Crystallization at 303 K was essential to obtain large rod-like crystals. The SDS-PAGE of the crystals revealed their purity (Fig. 2a, lane C). Crystals of sSTT3 prepared by refolding from the pellet fraction (Fig. 1, sample 1) grew under the same conditions.

Small crystals were obtained when the peak-tailing edge fractions in the gel filtration from the supernatant fraction were used (Fig. 1, sample 3), but they grew much faster and with a different shape.

Optimization resulted in a lower PEG concentration and the addition of Y³⁺ ion as an additive. Crystals grew from a hanging drop with a 1:1 volume ratio of 10 mg ml⁻¹ protein stock solution and reservoir solution (0.1 M Tris-HCl pH 9.0, 15% PEG 4000, 0.3 M sodium acetate, 5 mM YCl₃) at 293 K in 2 d (Fig. 2b). EXAFS measurements showed that the Y³⁺ ion was not incorporated into the crystals. Surprisingly, SDS-PAGE analysis revealed that the washed crystals contained a stoichiometric amount of an unknown protein band with a molecular weight of about 12 kDa in addition to the sSTT3 protein band (Fig. 2b, lane C). The protein solution used for crystallization contained some extra protein bands (Fig. 2b, lane S), but apart from the 12 kDa protein these contaminating proteins were not incorporated into the crystals. In-gel trypsin-digestion and LC-ESI-MS/MS analysis revealed that the 12 kDa protein was an *E. coli* 50S ribosomal protein, L7/L12 (entry name RL7_ECOLI). L7/L12 is a ribosomal subunit protein (for recent reviews, see Wahl & Moller, 2002; Gonzalo & Reboud, 2003). L7 is the N-terminally acetylated form of L12. L7/L12 is present in each 50S ribosomal subunit in four copies, organized as two dimers, and is necessary for the proper function of the elongation factors EF-G and EF-Tu. Functional counterparts, termed P1/P2 proteins, exist in archaeal and eukaryotic ribosomes but have no sequence homology.

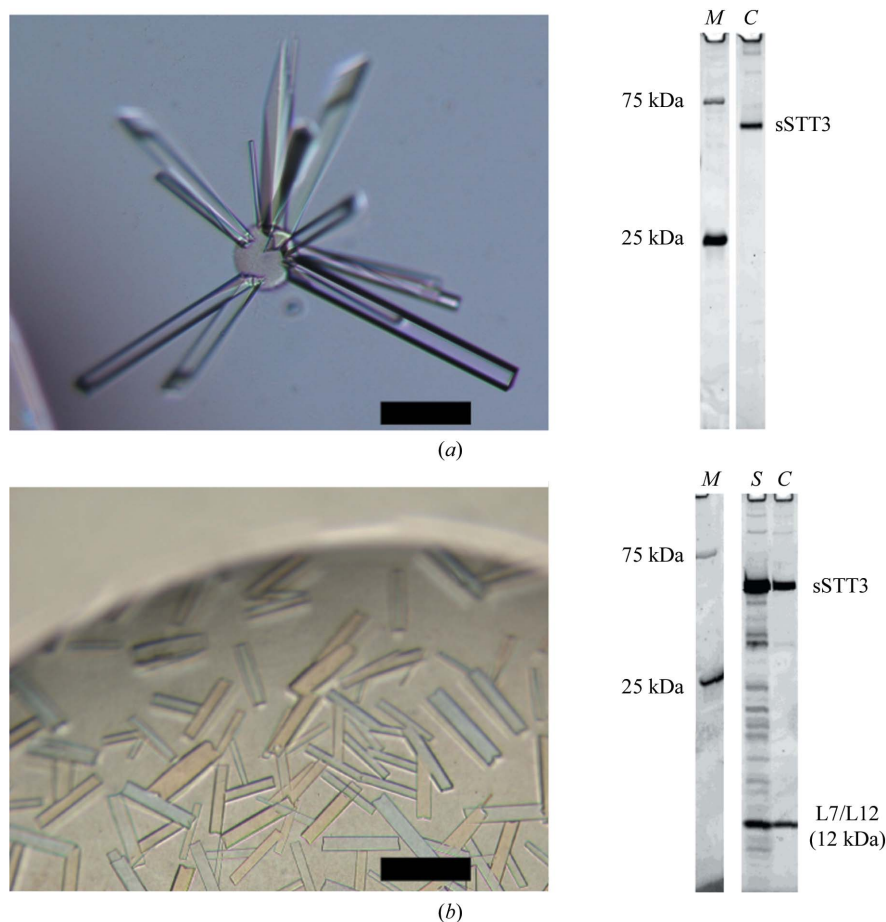


Figure 2

Crystals of *P. furiosus* sSTT3 protein prepared from the heat-treated cell lysate and SDS-PAGE analysis of the crystals. (a) Crystals obtained from the peak-top fractions (Fig. 1, sample 2) after two weeks at 303 K; (b) crystals obtained from the tailing-edge fractions (Fig. 1, sample 3) after 2 d at 293 K. The scale bars represent 0.1 mm. Crystals were transferred to the corresponding mother liquors for washing, mixed with 5× SDS sample buffer, heated for 5 min at 368 K and then subjected to SDS-PAGE (10–20% gradient gel, Daiichi). The gels were stained with SYPRO Orange protein gel stain (Invitrogen) and were photographed with an LAS-3000 multicolour image analyzer (Fuji Film) using a Blue LED (460 nm) illuminator and a Y515-Di filter. Lane M, prestained markers (Precision Plus protein standards, dual colour, Bio-Rad); lane S, protein solution used for crystallization; lane C, washed crystals. The amount of the markers used was extremely small, so that only the two bands prestained with a fluorescent dye are visible. The 12 kDa band was excised from the CBB-stained gel and subjected to in-gel digestion with trypsin and the eluted peptides were loaded onto LC-ESI-MS/MS (LC Q DECA, Finnigan). The protein was identified by a peptide-fingerprint search using the Mascot software (Matrix Science).

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	SeMet sSTT3	Native sSTT3	Native sSTT3 + L7/L12
Data collection			
Space group	$P2_12_12$	$P2_12_12$	$C222_1$
Unit-cell parameters (Å)			
<i>a</i>	137.34	136.93	102.23
<i>b</i>	266.60	265.29	106.04
<i>c</i>	73.95	74.13	289.38
Wavelength (Å)	0.97915 (peak)	0.96419	1.00000
Resolution range (Å)	30–3.0 (3.1–3.0)	40–2.7 (2.8–2.7)	50–3.35 (3.5–3.35)
No. of measured reflections	610027	458643	168317
No. of unique reflections	55387 (5441)	75226 (7371)	23023 (2238)
Completeness (%)	100.0 (100.0)	99.9 (99.4)	100.0 (100.0)
Multiplicity	11.0 (11.1)	6.1 (5.8)	7.3 (7.5)
Average $I/\sigma(I)$	7.4 (3.8)	12.4 (3.5)	7.8 (4.5)
$R_{\text{merge}}^{\dagger}$	0.136 (0.598)	0.081 (0.443)	0.127 (0.465)
Mosaicity (°)	0.254	0.261	0.458
Molecules per AU	4	4	2 (sSTT3) + 2 (L7/L12)
Phasing statistics			
Se sites (found/all)	16/20		
Phasing power (centric/acentric)	0.00/0.608		
(FOM) (after SOLVE/ after SHARP)	0.298/0.737		

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \bar{I}(hkl)| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\bar{I}(hkl)$ is the average intensity obtained from multiple observations of symmetry-related reflections.

3.3. Data collection and phase determination

The sSTT3 crystals (Fig. 2a) were cryoprotected by increasing the concentration of PEG 400 to 24% and were cryocooled in a nitrogen-gas stream (100 K). The crystals diffracted to 2.7 Å at BL41XU, SPring-8 (Harima, Japan). Crystals of SeMet sSTT3 grew under the same conditions and were processed in the same manner and diffracted to 3.0 Å. The diffraction data were processed with *HKL-2000* (Otwinowski & Minor, 1997). The crystals belonged to space group $P2_12_12$, with unit-cell parameters $a = 137$, $b = 266$, $c = 74$ Å. A search for Se-atom sites was carried out with *SOLVE* (Terwilliger & Berendzen, 1999), resulting in 16 out of 20 Se sites, assuming four SeMet residues in each molecule. Phases were further improved by density-modification methods using *autoSHARP* (Vonrhein *et al.*, 2006), with a mean figure-of-merit value of 0.737. The statistics of data collection and phasing are summarized in Table 1. The resulting electron-density map allowed tracing of the main chains of the polypeptides. Model building and structure refinement are now in progress.

The crystals containing sSTT3 and *E. coli* L7/L12 (Fig. 2b) were cryoprotected by transferring them to a mixture of mother liquor and ethylene glycol (8:2 volume ratio) and were cryocooled in a nitrogen-gas stream. The crystals diffracted to 3.4 Å at BL41XU and optimization of the crystallization conditions is under way. The space group was $C222_1$, with unit-cell parameters $a = 102$, $b = 106$, $c = 289$ Å (Table 1). As the preliminary electron densities indicated extra short α -helices in addition to the sSTT3 molecules, the interactions

between sSTT3 and L7/L12 seem to be stoichiometric and specific in the crystals. The interaction probably also occurred weakly in solution, as the *E. coli* L7/L12 protein coeluted with sSTT3 at an elution volume earlier than that expected for the 24 kDa L7/L12 dimer (Fig. 1). We do not think that this cross-species interaction is biologically relevant, but L7/L12 might mimic the interaction with the partner protein(s) *in situ*. Finally, such ‘xenogeneic cocrystals’ may provide chances for unexpected research and applications. For example, L7/L12 will serve as the template for a polypeptide substrate specifically designed for cocrystallization by engrafting an Asn-X-Thr/Ser sequon.

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