Crystallization and preliminary X-ray analysis of the 9 kDa protein of the mouse signal recognition particle and the selenomethionyl-SRP9

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Abstract Two different crystal forms of the 9 kDa protein of the signal recognition particle (SRP9) have been prepared by the hanging drop vapor diffusion technique using 28% (w/v) PEG8000 or 28% saturated ammonium sulphate as precipitant. The crystals are hexagonal bipyramids with average dimensions of $0.2 \times 0.1 \times 0.1$ mm^3 and they diffract to a resolution of 2.3 Å. They belong to the space groups $P6_122/P6_422$ or $P3_121/P3_221$ with cell dimensions $a = b = 63.0$ Å, and $c = 111.5$ Å. Crystals have also been grown from the selenomethionyl protein and multiwavelength data sets have been collected.

Key words: Signal recognition particle; Incomplete factorial design; Selenomethionyl protein; Crystallization; X-ray diffraction

1. Introduction

The signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein that plays an essential role in sorting proteins to the rough endoplasmic reticulum (RER) [1–3]. SRP recognizes and binds to the signal sequence of the nascent chain as it emerges from the ribosome. This interaction triggers a pause in the synthesis of the polypeptide chain and the ribosome-nascent chain-SRP complex is then targeted to RER via the interaction of SRP with its receptor. Protein synthesis is resumed and the nascent chain engages in the actual translocation process.

Canine SRP, the first example to be discovered and the most studied, is composed of an RNA molecule (SRP RNA), two heterodimeric protein subunits (SRP68/72 and SRP9/14) and two monomeric polypeptides (SRP54 and SRP19). The two polypeptides SRP9/14 bind to the sequences at the 5′ and 3′ end of SRP RNA that are homologous to the Alu family of repetitive sequences [4]. SRP9/14 is absolutely required to mediate the pause in the translation of secretory proteins.

It is of particular interest that all SRP proteins lack apparent structural similarities to already characterized RNA binding motifs. Moreover, no structural data have been obtained so far for any of the SRP proteins. In this paper we describe the crystallization and characterization of mouse SRP9. In the accompanying paper [5] we describe the crystallization of SRPΦ14-9, a single polypeptide fusion protein that can functionally replace the SRP9/14 heterodimeric subunit in the SRP [6].

2. Material and methods

2.1. Cloning, expression and purification

The coding region of the SRP9 cDNA (Accession number X78304) was cloned into the expression vector pET3a [7] linearized with *Nde*I and *Bam*HI.

E. coli BL21/lysS were grown in 1 liter of LB medium containing 50 mg/l ampicillin at 37°C. At $OD_{600} = 0.6$ the expression of SRP9 was induced for 4 h by adding 0.8 mM IPTG. The cells were harvested by centrifugation, suspended in 10 ml of lysis buffer: 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10% glycerol, 0.5 mg DNAse, 0.5 mM Pefabloc and 200 mM NaCl. Cells were lysed in a French press (1000 psi) and 10 mM EDTA was added.

After centrifugation (15 min at $15000 \times g$) the supernatant was precipitated with 0.2% polymin P for 15 min on ice and centrifuged for 15 min at $15000 \times g$. The supernatant was then loaded on a 5-ml HiTrap heparin column (Pharmacia Biotech) equilibrated with 200 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM β-mercaptoethanol, and 1 mM EDTA. After washing the column, a 60 ml gradient was applied (0.2 to 1.5 M NaCl in the same buffer). Pure SRP9 eluted at approximately 1.2 M NaCl.

The protein was precipitated with 70% saturated ammonium sulphate and centrifuged. Prior to crystallization, the protein was resuspended in 200 mM ammonium sulphate, 10 mM Tris pH 8.0, dialyzed against the same buffer and concentrated to 4–8 mg/ml using Centricon-10 concentrators.

2.2. Production of selenomethionyl SRP9

Selenomethionine containing protein was produced according to VanDyne et al. [8]. Cells from 1 ml of an overnight culture in LB medium were spun down, resuspended in 1 ml of minimum medium M9 with carbon source (glucose) at 4 g/liter and then added to 1 liter of the same, pre-warmed medium. Cells were grown to mid-log phase before addition of the amino acids: lysine, phenylalanine, and threonine at 100 mg/liter, isoleucine, leucine, and valine at 50 mg/liter, L-selenomethionine at 60 mg/liter. Induction was done 15 min after addition of the amino acids. Purification of selenomethionyl-SRP9 was essentially similar to that of the native protein, except that 10 mM beta mercaptoethanol and 1 mM EDTA were added during purification to prevent the oxidation of the selenomethionyl protein.

2.3. Crystallization

Crystallization conditions were found using an incomplete factorial

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Abbreviations: EDTA, *N,N′*-1,2-ethanediylbis[*N*-(carboxymethyl)glycine; ESRF, European Synchrotron Radiation Facility; EMBL, European Molecular Biology Laboratory; IPTG, isopropyl-β-D-thiogalactopyranoside; MES, 2-[*N*-Morpholino]ethanesulphonic acid; PEG, polyethylene glycol; RER, rough endoplasmic reticulum; SRP, signal recognition particle; SRP9/14, signal recognition particle proteins SRP9 and SRP14 heterodimer; SRPΦ14-9, SRP9/14 fusion protein; Tris-HCl, Tris[hydroxymethyl]aminomethane hydrochloride.

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design [9]. Crystals were grown using the hanging drop vapor diffusion technique in Linbro plates. A drop of 2–10 μl of protein solution was mixed with an equal volume of the reservoir solution, sealed against 1 ml of reservoir solution, and set at room temperature.

3. Results and discussion

An incomplete factorial design was set up using 3 values of pH (5.5, 6.5, 7.5,), 3 concentrations of PEG 8000 (22, 25, and 28% (w/v)), 2 concentrations of ammonium sulphate (50 and 200 mM), and the presence or absence of Nikkol, a detergent that had previously been used during purification (K. Strub, personal communication). This 4-variable incomplete factorial design resulted in a 12 experiment matrix. Two conditions gave rise to crystals: condition 7 (0.1 M MES pH 6.5, PEG 8000 28%, 50 mM ammonium sulphate) and condition 11 (0.1 M sodium citrate pH 5.5, PEG 8000 28%, 50 mM ammonium sulphate). The more acidic pH increased nucleation and resulted in a large number of smaller crystals, so condition 7 was chosen to be improved on. Ammonium sulphate concentration was varied from 50 to 200 mM in the reservoir solution, and the best results were obtained with the lowest concentration. Addition of dithiothreitol was found to prevent crystallization.

The protein, dialysed against 0.2 M ammonium sulphate in 10 mM Tris-HCl pH 8.0, was concentrated to 4–8 mg/ml and centrifuged at 13 000 rpm for 5 min prior to crystallization. The protein was then equilibrated against 28% polyethylene glycol (PEG) MW = 8000, 50 mM ammonium sulphate in 100 mM MES pH 6.5. Hexagonal bipyramids appear after 5–7 days with typical dimensions $0.2 \times 0.1 \times 0.1$ mm^3. These crystals belong to the hexagonal space group $P6_222/P6_422$ with cell dimensions $a = b = 63.0$ Å, $c = 111.5$ Å. The unit cell volume is consistent with the presence of one molecule per asymmetric unit, corresponding to a specific volume of 3.14 Å^3/Da and a solvent content of 61%, which lie within the range found for other protein crystals [10].

Due to the small crystal size, it was necessary to use synchrotron radiation for diffraction experiments. The crystals also showed signs of radiation damage after being irradiated for a few hours using a rotating anode as X-ray source, so it was also essential to find a cryoprotectant solution. Cryoprotectant solution was found by adding glycerol to the reservoir solution. Sucrose was also tried but was found to be detrimental to the crystals. A 2.8 Å native data set has been collected on a single flash-frozen crystal at 100 K. The crystal was first harvested in 28% PEG 8000, 50 mM ammonium sulphate, 100 mM MES pH 6.5 and 10% glycerol. Glycerol concentration was increased by steps of 5%, to reach a final concentration of 25%. A fine loop made of eye surgery thread was used to fish the crystal out of the cryoprotectant solution. Data were collected at $\lambda = 0.93$ Å, on a Mar Research area detector at the Hamburg EMBL outstation, on the X11 beamline and processed using the CCP4 program package [11]. The overall merging R-factor of intensities is 8.2%, and the completeness of the data is 93% to 2.8 Å. The redundancy is 6.2 and 73.3% of the reflections are stronger then $3\sigma(I)$, with an average $I/\sigma(I)$ of 2.6 for data in the 2.9 to 2.8 Å resolution shell.

Further screening for crystallization conditions resulted in a second crystal form. These crystals were obtained in 28% of ammonium sulphate, 100 mM Na-Citrate buffer at pH 5.5 using a protein concentration of 7 mg/ml. In this condition crystals appear within three days and grow to a maximum size of 0.5 mm. These crystals diffract to higher resolution than form 1 crystals and belong to the space group $P3_221/P3_121$ with cell dimensions $a = b = 63.0$ Å, $c = 111.5$ Å. The unit cell volume is consistent with the presence of two molecules per asymmetric unit. A native data set has been collected on the ESRF-beamline BL19 on a single flash-frozen crystal using a Mar Research imaging plate detector at $\lambda = 0.98$ Å. The data are 95% complete to 2.3 Å with an R_{sym} on intensities of 4.4%. The redundancy is 5.0 and 73% of the reflections are stronger then $3\sigma(I)$, with an average $I/\sigma(I)$ of 3.6 for data in the 2.4 to 2.3 Å resolution shell.

In parallel, we have produced the selenomethionyl-SRP9. SRP9 has 4 internal methionines for a total number of 85 residues, the N-terminal methionine being cleaved off in native protein and when expressed in *E. coli*. The anomalous and isomorphous signals coming from the replacement of sulphur for selenium should be high enough for phasing. We chose a recently developed technique [8] based on the inhibition of methionine biosynthesis that does not require the use of a methionine auxotroph strain, and thus does not necessitate either transformation or transduction. High concentrations of isoleucine, lysine, and threonine are used to block methionine biosynthesis in *E. coli* by inhibiting by negative feed-back their aspartokinases (see section 2. Selenomethione incorporation was checked using mass spectroscopy. A difference in molecular weight of 188.5 between the native and selenomethionyl proteins was measured corresponding to full incorporation of 4 selenium atoms (data not shown).

Selenomethionyl-SRP9 crystals have been grown using the native crystallization protocol, with the addition of 5% glycerol in the mother liquor. They belong to the same space groups as native crystals $P6_222/P6_422$ or $P3_121/P3_221$, respectively. Complete data to 2.5 Å have been collected on BL19 (ESRF, Grenoble), at four different wavelengths (0.9797 Å, 0.9799 Å, 0.9463 Å, and 1.1000 Å) from a single flash-frozen crystal which was grown with ammonium sulphate as precipitant. An energy scan clearly showed an absorption edge corresponding to the selenium K-edge (data not shown). Data are being processed and used for phase determination by multiwavelength anomalous dispersion (MAD) methods in order to determine the three-dimensional structure of the protein.

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