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Crystallization and preliminary crystallographic analysis of RSB-66, a novel round spermatid-specific protein

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Crystals of the RSB-66 protein have been grown at 291 K using NaCl as precipitant. In the refinement of the crystallization this protein, the crystallographic PCR method was used and was found to help in obtaining the best crystals more quickly and easily. The diffraction pattern of the crystal extends to 2.7 Å resolution in-house. A full set of X-ray diffraction data were collected to 2.7 Å from a single crystal. The crystals belong to space group $P4212$, with unit-cell parameters $a = 90.4$, $b = 90.4$, $c = 122.2$ Å, $\alpha = \beta = \gamma = 90^\circ$. The presence of two or three molecules per asymmetric unit gives a crystal volume per protein mass (V_M) of 3.22 or 2.14 Å^3 Da^{-1}, respectively.

1. Introduction

Spermatogenesis in mammals is a process of cell differentiation in which stem cells undergoing mitotic proliferation proceed into meiosis, followed by a remodelling of the haploid spermatids to form mature spermatozoa (Hecht, 1998). The progression of spermatogenesis occurs over a specified period of time, duration and spatial orientation (Kerr, 1995) associated with the expression of genes and the production of specific proteins under stringent temporal and spatial regulation. It has been estimated that about 5% of the proteins are produced during each stage of germ-cell differentiation (Boitani *et al.*, 1980). To better understand the molecular mechanisms and to identify the factors involved in the regulation of spermatogenesis, genes expressed during the specific stages of germ-cell differentiation have been studied (Yu *et al.*, 2003). In these studies, however, genes unique to specific spermatogenic cells were not identified.

In rat testis, germ cells are organized in well defined groups at stages of differentiation (Leblond & Clermont, 1952). Although delineation of the cycle into 14 stages is considered to be a milestone achievement, there are other issues concerned with the differentiation of germ cells that need to be resolved, *e.g.* determination of the specific genes expressed in germ cells at various stages of differentiation, which changes with time and in spatial association. To proceed with these studies, a method to isolate specific germ cells from the testis in sufficient quantities needs to be developed that will allow investigators to perform biochemical and biological experiments. We have captured the primary spermatocytes and round spermatids from rat testis sections by the application of the laser capture microdissection (LCM) technique (Simone *et al.*, 1998; Bonner *et al.*, 1997; Emmert-Buck *et al.*, 1996; Liang *et al.*, 2003). Primary spermatocytes are diploid cells undergoing meiosis I to form two secondary spermatocytes, while round spermatids are haploid cell derived from secondary spermatocytes undergoing meiosis II. They subsequently differentiate into elongated spermatozoa. The mRNAs of the two populations of primary spermatocytes and round spermatids were extracted. Moreover, the prepared mRNAs are of good quality and, having an integrated 5′ end, they can be amplified using the SMART kit. SSH is a PCR-base technique that is widely utilized to identify differentially expressed genes in cells involved in various biological phenomena (Diatchenko *et al.*, 1996, 1999; Jin *et al.*, 1997). Using the cDNAs from primary spermatocytes and round spermatids, the cDNA subtractive libraries of these cells were constructed by application of the suppression subtractive hybridization technique (SSH; Dey *et al.*, 2001; Xiong *et al.*, 2001). A novel cDNA was identified in round spermatids and designated RSB-66 (AY121839). RSB-66 was obtained from the SSH library of round spermatid-specific cDNAs against those of primary spermatocyte. Evidence is presented showing that the expression of RSB-66 gene is unique to spermatids and may play a role in spermiogenesis. Northern blotting showed that the RSB-66 gene is transcribed only in the testis. Immunohistochemical analysis showed that the RSB-66 protein appears in round spermatids (Liang *et al.*, 2003). In the present study, the crystallization and preliminary crystallographic analysis of RSB-66 are reported.

2. Methods

2.1. Protein expression and purification

The RSB-66 gene, which codes 168 amino acids including six Cys residues, was cloned

into the prokaryotic expression vector pGEX-6p-1 with *Bam*HI and *Xho*I restriction enzyme sites. The expected protein was expressed in *Escherichia coli* BL21 (DE3) in high quantity. The bacterial cell pellet was resuspended in lysis buffer (1× PBS, 1 m*M* PMSF, 1 m*M* DTT) and was homogenized by sonication. The lysate was centrifugated at 20 000*g* for 25 min to remove cell debris. The supernatant was applied onto a GST-affinity column (1 ml glutathione Sepharose 4B) and the contaminant protein was washed away with wash buffer (lysis buffer plus 400 m*M* NaCl). The protein was then cut by PreScission protease at 277 K. After 36 h, the protein with an additional five-amino-acid head (GPLGS) was eluted with lysis buffer. The eluant was changed to buffer *A* (25 m*M* MES pH 6.15, 1 m*M* DTT) using a G-25 resin column using the gel-filtration chromatography method and applied onto a Resource S column (Pharmacia) (buffer *A*: 25 m*M* MES pH 6.15, 1 m*M* DTT; buffer *B*: 25 m*M* MES pH 6.15, 500 m*M* NaCl, 1 m*M* DTT). The protein was concentrated and purified using an Ultrafree 5000 molecular-weight cutoff filter unit (Millipore) and a Superdex-75 (Pharmacia) (using buffer *C*: 10 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 1 m*M* DTT, 1 m*M* EDTA, 1 m*M* NaN_3). The purified protein was analyzed on SDS–PAGE (Fig. 1) and native PAGE, which showed that the protein was >95% pure. The dynamic light-scattering data showed the protein had 70–80% homogeneity as a monomer.

2.2. Crystallization

The purified protein was concentrated to 20–25 mg ml^{-1} using an Ultrafree 5000 molecular-weight cutoff filter (Millipore). Protein concentrations were estimated spectroscopically by absorbance at 280 nm (Wetlaufer, 1962), assuming an A_{280} of 0.630 for a 1.0 mg ml^{-1} solution. Crystallization was performed by the hanging-drop vapour-diffusion method at 291 K in 16-well plates. Because the protein would have positive net charge in solutions of pH lower than 7.50, we used Riès-Kautt's method (Riès-Kautt *et al.*, 1997) to screen the initial parameters. Sets of screening reagents were supplied by the Positive Net Charge Screening Kit, which is based on the effect of the Hofmeister series and was used for initial screening. NaCl, sodium acetate, ammonium citrate and ammonium sulfate were initially screened as precipitants and 2-propanol was used as an additive. We screened the pH range 4.0–7.0 using 100 m*M* sodium acetate and MES as buffer, with a pH interval of 0.5. Typically, 3 µl droplets were prepared on siliconized cover slips by mixing 1.5 µl of protein solution and 1.5 µl of the reservoir solution and the mixture was vapour-equilibrated against 150 µl reservoir solution. Small needle-like crystals were obtained using 2.5 *M* NaCl pH 5.0 after 3 d (Fig. 2*a*). Further optimization was performed by refinement of the protein concentration, pH value, precipitants and additives. Using the crystallographic PCR method (Prompt Crystallization Reaction, a systematic micro/macroseeding method) (Yang & Rao, 2003), pyramid-shaped crystals (Fig. 2*b*) were obtained that were reproducible and suitable for X-ray diffraction. The crystals were obtained using 1.76 *M* NaCl in 100 m*M* sodium acetate buffer pH 4.75 containing 10% 2-propanol as an additive and the mixture was vapour-equilibrated against 150 µl reservoir solution.

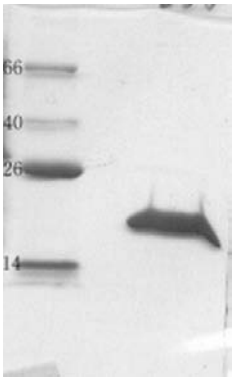

Figure 1
15% SDS–PAGE of the purified RSB-66 protein.

2.3. X-ray crystallographic studies

Preliminary diffraction data sets were collected at 100 K in-house using a Rigaku RU2000 rotating-anode Cu $K\alpha$ X-ray generator at 48 kV and 98 mA (λ = 1.5418 Å) with a MAR 345 mm image-plate detector. The beam was focused using an Osmic mirror. For more detailed analysis, flash-cooled crystals were used. Crystals were immersed in a freezing solution for 5–10 s, picked up in a loop and then flash-cooled in a stream of nitrogen gas at 100 K. 5 m*M* RSB-66 protein was mixed with 1.76 *M* NaCl, 100 m*M* sodium acetate pH 4.75 and 25% glycerol and used as the cryosolution for cryoprotection. All intensity data were indexed, integrated and scaled with the *HKL* programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

Table 1
Intensities of systematic absences.

h	*k*	*l*	Intensity	σ	I/σ
5	0	0	−14.0	25.1	−0.6
7	0	0	60.1	39.7	1.5
9	0	0	32.6	49.6	0.7
11	0	0	91.6	59.8	1.5
13	0	0	−165.5	68.4	−2.4
15	0	0	129.2	79.8	1.6
17	0	0	54.9	90.1	0.6
19	0	0	24.1	97.9	0.2
21	0	0	141.4	111.4	1.3
23	0	0	0.0	122.0	0.0
25	0	0	−87.0	130.1	−0.7
27	0	0	−238.9	132.3	−1.8
29	0	0	−94.3	131.9	−0.7
31	0	0	37.5	127.1	0.3
33	0	0	12.1	127.2	0.1

3. Results

When the crystals were exposed to X-rays, diffraction spots were observed to a Bragg spacing of 2.7 Å (Fig. 3). A set of data was collected from this crystal. The crystals belong to space group *P*4212 (the intensities of systematic absences are shown in Table 1), with unit-cell parameters a = 90.4, b = 90.4, c = 122.2 Å, $\alpha = \beta = \gamma = 90^\circ$. Scaling and

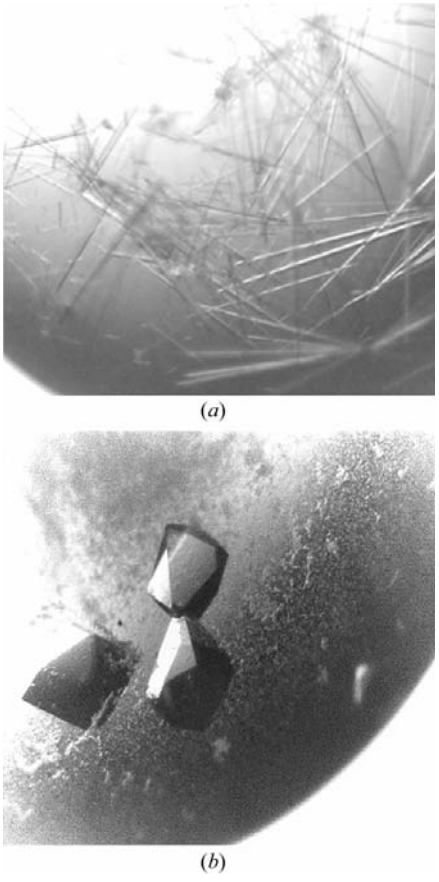

Figure 2
Crystals of RSB-66. The dimensions of the largest crystal are 0.4 × 0.4 × 0.4 mm.

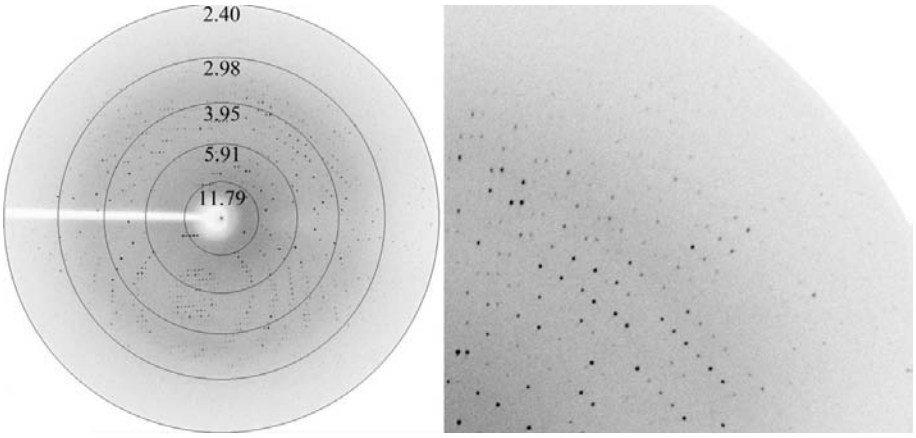

Figure 3
Typical diffraction pattern of RSB-66 crystals. The detector edge corresponds to 2.7 Å resolution. An enlarged image is shown on the right. The exposure time was 300 s, the crystal-to-detector distance was 225 mm and the oscillation range per frame was 1°.

Table 2
Data-collection and processing statistics.

Space group	*P*4212
Unit-cell parameters (Å, °)	$a = 90.4$, $b = 90.4$, $c = 122.2$, $\alpha = \beta = \gamma = 90.0$
Matthews coefficient ($Å^3$ Da^{-1})	3.22 or 2.14
Resolution (Å)	50–2.7
Total observations	67037
Unique reflections	14231 (1556)
Redundancy	4.71
Average $I/\sigma(I)$	15.69 (4.01)
R_{merge}† (%)	9.6 (37.4)
Data completeness (%)	97.8 (97.6)

† $R_{merge} = 100|I_i - \langle I\rangle|/\sum I_i$, where I_i is the intensity of the ith observation.

merging of the crystallographic data resulted in an overall R_{merge} of 9.6% and an R_{merge} in the highest resolution shell (2.80–2.70 Å) of 37.4%. Based on the Matthews coefficient (Matthews, 1968), we estimated there to be two or three molecules in the asymmetric unit. If there are two molecules in the asymmetric unit, the Matthews coefficient is 3.22 $Å^3$ Da^{-1} and the estimated solvent content is 61.4%. The value of the Matthews coefficient is 2.14 $Å^3$ Da^{-1} for three molecules in the asymmetric unit and the estimated solvent content is 42.2%. Complete data-collection statistics are given in Table 2.

We have obtained selenomethionine-derivative crystals and are waiting for free synchrotron time. We are also attempting to use the MIR method to solve the structure of RSB-66.

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