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Crystallization and preliminary X-ray analysis of the helicase domains of Vasa complexed with RNA and an ATP analogue

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The helicase fragment of Vasa was purified and its RNA-binding activity was examined by a UV cross-linking assay. The fragment was crystallized in complex with poly(U) RNA (U_{10}) and a non-hydrolyzable analogue of ATP. The crystal belonged to space group $P2_1$, with unit-cell parameters a = 71.06, b = 142.35, c = 130.47 Å, β = 90.86°. The cryocooled crystal diffracted to about 2.2 Å using synchrotron radiation from station BL41XU at SPring-8.

1. Introduction

Spatiotemporal regulation of mRNA translation is one of the major strategies for the establishment of cellular diversity, thus contributing to the development of multicellular organisms (Johnstone & Lasko, 2001). The *Drosophila melanogaster* Vasa protein (Hay *et al.*, 1988; Lasko & Ashburner, 1988) functions in this process. In the developing embryo, Vasa is localized to the posterior pole and positively regulates the translation of specific mRNAs to produce protein factors required for embryonic patterning and germline differentiation (Breitwieser *et al.*, 1996; Carrera *et al.*, 2000; Markussen *et al.*, 1997; Styhler *et al.*, 1998; Tinker *et al.*, 1998). Homologues of Vasa are expressed specifically in the germlines of higher eukaryotes, including humans, implying a widely conserved mechanism for germline differentiation (Raz, 2000).

Vasa is a member of the DEAD-box RNA helicases, which regulate RNA functions by catalyzing the rearrangement of RNA tertiary structures (*e.g.* the separation of complementary double strands) accompanied by ATP hydrolysis (Tanner & Linder, 2001). Indeed, RNA helicases participate in essentially all processes involving RNA molecules: transcription, editing, splicing, ribosome biogenesis, RNA export, translation and RNA degradation (de la Cruz *et al.*, 1999). In the catalytic cycle, RNA helicases alter their own conformations in conjunction with ATP binding and hydrolysis, causing structural rearrangement of the substrate (Caruthers & McKay, 2002). Therefore, determination of the functional structures in the catalytic cycle is a prerequisite for the elucidation of the helicase mechanism. So far, two structures of DEAD-box RNA helicases have been solved (those of eIF4A from yeast and MjDEAD from *Methanococcus jannaschii*; Caruthers *et al.*, 2000; Story *et al.*, 2001), revealing the common architectures of their two RecA-like domains. The ATP- and ADP-bound structures of the eIF4A N-terminal RecA-like domain have also been solved, offering insight into the ATP-binding mechanism (Benz *et al.*, 1999). However, no structure of the entire helicase region complexed with a substrate RNA or cofactor nucleotide has been solved. Here, we report the crystallization and preliminary diffraction analysis of the Vasa helicase fragment complexed with RNA and AMP-PNP, a non-hydrolyzable analogue of ATP.

2. Experimental procedures

2.1. Purification

Full-length and N-terminally truncated (residues 200–661; Vasa-51) forms of Vasa were produced in *Escherichia coli* as GST-fusion proteins and were purified by glutathione-Sepharose chromatography followed by GST removal and anion-exchange chromatography (Uno-Q, Bio-Rad). An amino- and carboxyl-terminally truncated form (residues 200–623; Vasa-47) was produced and purified similarly, but heparin-Sepharose chromatography (HiTrap Heparin, Amersham Biosciences) was used instead of anion-exchange chromatography. Single-stranded RNA was chemically synthesized and purified by urea–PAGE.

2.2. Partial proteolysis

Full-length Vasa was incubated with trypsin (0.1 µg per 120 µg of Vasa) in 100 m*M* Na HEPES buffer pH 7.0 containing 300 m*M* NaCl and 1 m*M* DTT for 30 min at 310 K and was analyzed by SDS–PAGE.

2.3. UV cross-linking assay

Vasa-51 (10 µg) was incubated for 30 min at room temperature with 90 ng synthetic 5′-^{32}P-labelled 10-mer poly(U) RNA in 20 m*M* Tris–

HCl buffer pH 8.0 containing 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol and cofactors. The reaction mixtures were then irradiated with a 254 nm UV lamp (Stratalinker 1800, Stratagene) for 30 min. After irradiation, samples were subjected to SDS–PAGE on an 11% polyacrylamide gel, which was then analyzed by an FLA-2000 imager (Fujifilm).

2.4. Crystallization

Purified Vasa-51 was incubated with a 10-mer poly(U) RNA at a protein to RNA ratio of 1:1.2 in 50 m*M* Tris–HCl buffer pH 8.0 containing 200 m*M* $NaCl_2$, 1 m*M* DTT, 1 m*M* $MgCl_2$ and 1 m*M* AMP-PNP. The mixture was then concentrated using a Centricon-10 filter (Amicon) and was buffer-exchanged to 20 m*M* Tris–HCl buffer pH 8.0 containing 1 m*M* DTT, 1 m*M* $MgCl_2$ and 1 m*M* AMP-PNP. Finally, an equimolar amount of the 10-mer poly(U) RNA was again added to the sample, which was then used for crystallization. The Vasa-47 crystallization sample was prepared similarly, but 100 m*M* NaCl was added to the solution. Initial screening was carried out at 277 K by the hanging-drop vapour-diffusion method using Crystal Screen I, II, Natrix and Membfac kits (Hampton Research).

2.5. Crystallographic analysis

Diffraction experiments were carried out on BL41XU at SPring-8 at 100 K using a MAR CCD165 detector (MAR). The crystallization solutions containing 20%(*v*/*v*) ethylene glycol were used as cryoprotectant buffers. Data processing and scaling were performed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

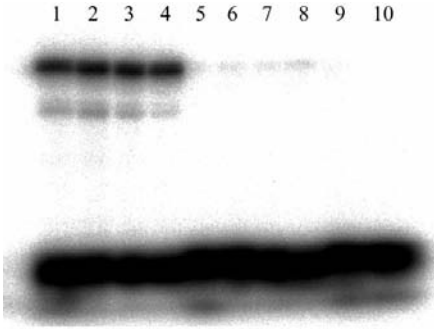


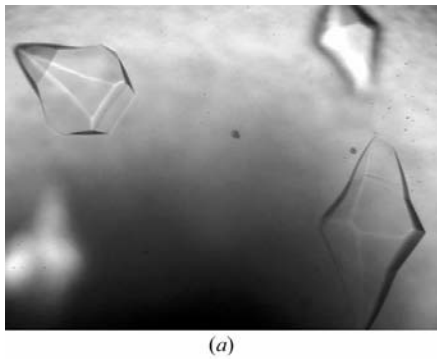
Figure 1
UV cross-linking assay. Cofactors for each lane are 1, 1 m*M* AMP-PNP and 1 m*M* $ZnCl_2$; 2, 1 m*M* AMP-PNP; 3, 2 m*M* AMP-PNP; 4, 4 m*M* AMP-PNP; 5, 1 m*M* ADP and 1 m*M* $ZnCl_2$; 6, 1 m*M* ADP; 7, 2 m*M* ADP; 8, 4 m*M* ADP; 9, no cofactors; 10, no protein (control).

3. Results and discussion

Initial attempts to crystallize the complex of full-length Vasa, RNA and AMPPNP failed, probably because of the structural heterogeneity of the N-terminal region, which contains arginine-glycine-glycine (RGG) repeats. Trypsin treatment of the full-length Vasa protein produced a stable fragment of approximately 50 kDa, as estimated by SDS–PAGE (data not shown). Amino-acid sequence analysis of this fragment revealed the removal of the N-terminal 199 residues; however, we did not determine whether the C-terminal region of the protein was affected. Thus, the N-terminally truncated Vasa (Vasa-51) was cloned.

To assess the RNA-binding ability of Vasa-51 and its dependence on nucleotide cofactors, we performed a UV cross-linking assay (Fig. 1). The cross-linking of RNA to Vasa-51 was strong with AMP-PNP, but was weak with ADP and without the nucleotide.

A dynamic light-scattering experiment (DynaPro, Protein Solutions) revealed that the concentrated Vasa-51 was severely aggregated (Ferré-d'Amare & Burley, 1997), with an apparent molecular weight of over 1 MDa. This aggregation was dramatically reduced when ssRNA and AMP-PNP were added to the protein solution before concentration, yielding an apparent molecular weight of about 70 kDa, which is roughly consistent with that of the monomeric Vasa-51–ssRNA complex (54 kDa). Very small needle-like polycrystals of Vasa-51 were observed with a buffer containing 0.1 *M* ADA pH 6.5, 12%(*w*/*v*) polyethylene glycol (PEG) 4000, 0.1 *M* Li_2SO_4 and 2%(*v*/*v*) isopropyl alcohol within a few days. However, trials to improve the crystallization conditions by varying the pH, PEG concentration and temperature and by including additives did not yield better crystals.


(*a*)

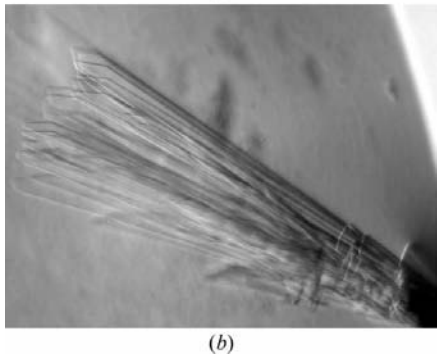

(*b*)

Figure 2
Crystals of Vasa-47 complexed with RNA and AMP-PNP. (*a*) Form *A*, (*b*) form *B*.

Table 1
Data-collection statistics.

Values in parentheses are for data in the highest resolution shell.

Space group	$P2_1$
Wavelength (Å)	0.9120
Resolution range (Å)	50–2.20 (2.24–2.20)
Measured reflections	316996
Unique reflections	116785
Completeness (%)	89.0 (73.7)
Mean $I/\sigma(I)$	15.9 (2.2)
$R_{\rm merge}$† (%)	7.5 (29.8)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_{hkli} - \langle I_{hkl}\rangle|/\sum_{hkl}\sum_i \langle I_{hkl}\rangle$.

To improve the crystallization conditions, we re-examined the protein-expression construct. Based on the amino-acid conservation, we cloned a C- and N-terminally truncated protein (Vasa-47), which further eliminated the 38 C-terminal residues. Under the same crystallization conditions used for Vasa-51, Vasa-47 grew a few rod-like single crystals, indicating a slight improvement of the crystal quality. Further improvement by the addition of the detergent Cymal-3 produced two different forms of crystals, depending on the PEG 4000 concentration. Large crystals (form *A*; 0.3 × 0.3 × 0.2 mm) and thin plate-shaped crystals (form *B*; 0.5 × 0.1 × 0.03 mm) appeared within a few days with 4–6%(*w*/*v*) and 9–12%(*w*/*v*) PEG 4000, respectively (Fig. 2). The final crystallization conditions were 0.1 *M* ADA pH 6.5, 0.1 *M* Li_2SO_4, 1–2%(*v*/*v*) isopropyl alcohol, 69 m*M* Cymal-3 and PEG 4000 at the concentrations described above. SDS–PAGE and urea–PAGE analyses of the thin plate-shaped crystals indicated the presence of the intact Vasa-47 protein and RNA (data not shown).

A diffraction experiment on BL41XU at SPring-8 showed that the form *A* crystals diffracted (to about 20 Å resolution) and seemed to be hexagonal. On the other hand, the form *B* crystals diffracted to about 2.2 Å resolution and a data set was collected from this form (Table 1). The form *B* crystal belongs to space group $P2_1$, with unit-cell parameters a = 71.06 Å, b = 142.35, c = 130.47 Å, β = 90.86°. Therefore, if the asymmetric unit of the form *B* crystal

contains three or four complexes, the V_M value should be 4.4 or 3.3 Å^3 Da^{-1}, respectively.

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