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Crystallization of a ZRANB2–RNA complex

ZRANB2 is a zinc-finger protein that has been shown to influence alternative splice-site selection. The protein comprises a C-terminal arginine/serine-rich domain that interacts with spliceosomal proteins and two N-terminal RanBP2-type zinc fingers that have been implicated in RNA recognition. The second zinc finger bound to a six-nucleotide single-stranded RNA target sequence crystallized in the hexagonal space group \(P_{6_5}2_2\) or \(P_{6_1}2_2\), with unit-cell parameters \(a = 54.52\), \(b = 54.52\), \(c = 48.07\) Å; the crystal contains one monomeric complex per asymmetric unit. This crystal form has a solvent content of 39% and diffracted to 1.4 Å resolution using synchrotron radiation.

1. Introduction

Alternative splicing is an essential process in higher eukaryotes that provides an important level of gene regulation and contributes to the diversity of the proteome. A number of proteins that play roles in alternative splicing contain regions that are rich in arginine–serine repeats (RS domains); the best characterized of these are a small family known as SR (serine/arginine-rich) proteins (Graveley, 2003), which additionally contain one or two RNA-recognition motifs (RRMs). ZRANB2 is a widely expressed protein that is conserved from nematodes through to humans and displays many of the properties of SR proteins. It is localized to the nucleus and interacts with the spliceosomal proteins U1-70K and U2AF35 (Adams et al., 2001); in minigene reporter assays it can alter the distribution of splice variants of GluR-B, SMN2 and Tra2β reporter genes (Adams et al., 2001; Li et al., 2008). Unlike many SR and SR-related proteins, ZRANB2 contains two RanBP2-type zinc fingers (ZnFs) in place of the RRMs.

RanBP2-type zinc fingers (also called NZF-type ZnFs) contain the consensus sequence \(W-X-C-X_2-4-C-X_3-N-X_6-C-X_2-C-X_2-K/R\) and consist of two \(β\)-hairpins sandwiching a zinc ion. They occur in one to eight copies within a functionally diverse range of proteins and appear to act as interaction motifs. Specifically, the RanBP2-type ZnF of Npl4 recognizes ubiquitin (Alam et al., 2004) and the RanBP2-type ZnFs in RanBP/Nup358 and Nup153 bind RanBTP/GDP (Higa et al., 2007). In contrast, the two RanBP2-type ZnFs of ZRANB2 can recognize single-stranded RNA (ssRNA) with micromolar affinities (Plambeck et al., 2003 and unpublished results). We have previously determined the solution structure of both the first ZnF of ZRANB2 (ZRANB2-F1; PDB code 1n0z; Plambeck et al., 2003) and the second finger (F. E. Loughlin and J. P. Mackay, unpublished work; PDB code 2klp); the two domains share 45% sequence identity. In order to understand the molecular basis of RNA recognition by RanBP2-type ZnFs, we have crystallized ZRANB2-F2 bound to ssRNA.

2. Materials and methods

2.1. Cloning, expression and purification

A construct encoding amino acids 65–95 of human ZRANB2 (ZRANB2-F2, from Swiss-Prot entry O95218) was subcloned into pGEX-2T (GE Healthcare) and expressed overnight at 298 K in
BL21 Rosetta2 *Escherichia coli* following induction with 0.4 mM IPTG (and the addition of 0.1 mM ZnSO₄). Cells were harvested and treated with lysozyme followed by DNaseI. ZRNAB2-F2 was affinity-purified on glutathione Sepharose-4B beads (GE Healthcare), cleaved from the immobilized GST tag with thrombin and further purified by gel filtration (Superdex 30, GE Healthcare) in an RNase-free buffer containing 50 mM MOPS pH 7.2, 150 mM NaCl and 1 mM DTT. Fractions containing ZRNAB2-F2 were pooled and dialyzed against a buffer containing 20 mM MOPS pH 7.3 and 1 mM DTT.

ZRANB2–RNA complex

**2.2. Crystallization**

Initial crystallization trials were set up at 298 K as vapour-diffusion hanging drops using a Mosquito robot (Molecular Dimensions) by mixing 150 nl sample solution (ZRANB2–RNA, 1:1 complex at 6.8 mg ml⁻¹ in 20 mM MOPS, 1 mM DTT pH 7.3, 0.5 U ml⁻¹ RNasin) and 150 nl reservoir solution and placing the resultant drop over 80 ml reservoir solution in flat-bottom 96-well PS microplates (Greiner Bio-One). SaltRX, Crystal Screen and Crystal Screen II (Hampton Research), Nucleix, JSGC+ and AmSO₄ (Qiagen) screens were trialled. Based on the results of these screens, the SaltRX screen was repeated and optimized using vapour-diffusion hanging drops by mixing 1 ml ZRANB2–RNA complex solution and 1 ml reservoir solution in a 1:1 ratio and placing the drop over 1 ml reservoir solution in 24-well plates (Linbro, ICN Biochemicals).

**2.3. Data collection and processing**

Crystals were frozen in a stream of liquid nitrogen without additional cryoprotectant. Diffraction data were recorded on a MAR345 imaging-plate detector (MAR Research) using X-rays produced by a Rigaku RU200H rotating-anode generator (Cu Ka) focused with Osnic mirrors (MSC Rigaku). The diffraction data were integrated and scaled with *HKL*-2000 (Otwinowski & Minor, 1997). Additional

![Hexagonal crystal of the ZRANB2-F2–RNA complex of 120 × 300 μm in size.](image1)

![Diffraction image of a ZRANB2-F2–RNA complex crystal taken on a laboratory source.](image2)
Table 1
Data-collection statistics.

<table>
<thead>
<tr>
<th>Data set 1</th>
<th>Data set 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>1.54178</td>
</tr>
<tr>
<td>Space group</td>
<td>P6₃2 or P6₃2</td>
</tr>
<tr>
<td>Unit-cell parameters (Å, °)</td>
<td>a = b = 54.51, c = 48.05</td>
</tr>
<tr>
<td>Resolution limit (Å)</td>
<td>1.61 (1.64–1.61)</td>
</tr>
<tr>
<td>Monoclinicity (°)</td>
<td>0.4</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.9 (100.0)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>5845</td>
</tr>
<tr>
<td>Redundancy</td>
<td>16 (14.3)</td>
</tr>
<tr>
<td>Rmerge</td>
<td>0.039 (0.201)</td>
</tr>
<tr>
<td>I/(σ(I))</td>
<td>62.5 (13.8)</td>
</tr>
</tbody>
</table>

† Rmerge = Σ(|I(hkl)| – ⟨|I(hkl)|⟩)/Σ|I(hkl)|.

3. Results and discussion

Diffraction-quality crystals were obtained from Hampton SaltRX screen condition No. 34 and subsequently from further fine screening of conditions in the range 2.0–2.3 M malic acid, 0.1 M bis-Tris propane pH 6.9–7.1. Single crystals (Fig. 1) were grown over two weeks (2.2 M malic acid, 0.1 M bis-Tris propane pH 7.0 and pH 7.2) and exhibited strong birefringence, which is commonly observed in crystals containing nucleic acids (Kettenberger & Cramer, 2006). Native diffraction data were first recorded to 1.6 Å resolution at a home source (Fig. 2), with an additional data set recorded to 1.4 Å resolution at a synchrotron source using a different crystal. Data-collection and processing statistics are summarized in Table 1. The symmetry and systematic absences indicate that the crystals belong to one of the hexagonal space groups P6₃2 or P6₃2.

Solvent-content analysis using the program MATTHEWS_COEF (Kantardjieff & Rupp, 2003) indicated that the crystal contained either one molecule of protein or one protein–RNA complex in the asymmetric unit. Assuming that a single 1:1 protein–RNA complex is present in the asymmetric unit of the crystal, the estimated solvent content of the crystal is 39.6%, with a Matthews coefficient of 1.82 Å³ Da⁻¹ (Matthews, 1968). Alternatively, assuming the crystal contains only protein and no RNA, the estimated solvent content of the crystal is 55.2%, with a Matthews coefficient of 2.74 Å³ Da⁻¹.

To clarify whether the crystals did indeed contain RNA, a crystal was washed in reservoir solution three times and dissolved in 1 μl water and the UV spectrum was recorded. The spectrum, which is identical to one recorded on the mother liquor, shows a clear absorption peak at 260 nm, which is indicative of the presence of nucleic acid (Fig. 3). Furthermore, the measured 260/280 nm ratio of 2.0 is consistent with a 1:1 ratio of protein and RNA. Currently, structure determination and refinement are in progress. Attempts to use the existing NMR structure of ZRANB2-F1 to obtain a molecular-replacement solution were unsuccessful and we propose either to make use of the anomalous scattering from either the four cysteine S atoms in our existing data set or to record additional diffraction data at the zinc edge to permit a multiple anomalous diffraction solution.

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References


