Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystals of X29, a *Xenopus laevis* U8 snoRNA-binding protein with nuclear decapping activity

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Received 23 April 2004
Accepted 30 June 2004

Eukaryotic ribosome biosynthesis requires modification (methylation, pseudouridylation) and nucleolytic processing of precursor ribosomal RNAs in the nucleolus. The RNA components of the small nucleolar RNPs (snoRNAs) are essential for many of these events. One snoRNP, called U8, is necessary for maturation of 5.8S and 28S rRNA in vertebrates. In *Xenopus laevis*, U8 snoRNA was found to bind specifically and with high affinity to a protein called X29. X29 is a Nudix hydrolase, a nucleotide diphosphatase that removes the m^7G and $m^{227}G$ caps from U8 and other RNAs. X29 requires an RNA as substrate and cap analogues are not substrates or inhibitors of cleavage. To study the determinants of X29 activity and its specificity for U8 RNA substrate, X29 was crystallized in an orthorhombic crystal form that diffracts to 2.1 Å resolution.

1. Introduction

Ribosome biogenesis in eukaryotes is mediated in part by a large number of small nucleolar ribonucleoprotein particles (snoRNPs) that direct site-specific modification (methylation and pseudouridylation) and nucleolytic processing of pre-rRNAs (Venema & Tollervey, 1999). Two classes of modifying snoRNAs have been identified based on conserved sequence elements: the C/D-box class, which primarily direct methylation of ribose (Peculis & Mount, 1996), and the H/ACA class, which direct pseudouridylation (Peculis, 1997). The RNAs of these particles act as guide RNAs by base pairing with target pre-rRNA sequences to specify the site of modification. As such, they may also function as RNA chaperones to modulate pre-rRNA folding.

Most snoRNPs are involved with site-specific modification of pre-rRNAs, but a small number are involved in the nucleolytic processing reactions necessary for maturation of pre-rRNA. U8 is a C/D-box snoRNP that is uniquely essential for processing both 5.8S and 28S rRNA in vertebrates (Peculis & Steitz, 1993). Several proteins are known to bind to U8 RNA, either directly or through interactions with other RNA-bound proteins. A 15.5 kDa protein binds specifically to a higher order structure in U8 RNA that is conferred by the conserved sequence elements called box C/D sequences (Nottrott *et al.*, 1999; Watkins *et al.*, 2000). Three other proteins (Nop56, Nop58 and Nop1/fibrillarin) bind to the RNA-bound 15.5 kDa protein to form the core complex of C/D-box snoRNAs including U8 (Peculis & Steitz, 1993; Watkins *et al.*, 2000).

It has previously been shown by gel-mobility shift assays (Tomasevic & Peculis, 1999) that a nucleolar protein from *Xenopus* ovary extract with an apparent molecular weight of 29 kDa on SDS acrylamide gels binds directly and specifically to U8 snoRNA. Subsequently, we showed that this protein, called X29, and its putative vertebrate homologs carry a NUDIX domain characteristic of nucleotide diphosphatases (Ghosh *et al.*, 2004). *In vitro*, X29 and the human protein homolog were found to have a nucleotide diphosphatase activity that releases m^7GDP and $m^{227}GDP$ from capped U8 and other RNAs. This cleavage is metal-dependent and requires the presence of a significant part of the RNA substrate, suggesting that X29 has specificity for sequence and/or higher order structure in its RNA substrate. Despite its specificity for a large RNA substrate, no RNA-binding motif was detected in X29. X29 is the first identified nuclear decapping protein and the only decapping protein demonstrating RNA-substrate specificity. We proposed (Ghosh *et al.*, 2004) that X29 functions *in vivo* to regulate levels of nuclear RNAs, including snoRNAs, by means of this decapping activity. A high-resolution crystal structure would provide much valuable information on the relationship of X29 to other NUDIX hydrolases, on the possible nature of the RNA-binding site and on the specificity of the enzyme for the cap substrates. Additionally, it would provide a foundation for structure-based mutagenesis and modeling studies. We have crystallized X29 in a form that is amenable to high-resolution structure determination and describe the preparation and characterization of these crystals here.

2. Experimental

2.1. Expression and purification of X29

The X29 cDNA was cloned into pET19b (Invitrogen) and sequenced to ensure the reading frame was maintained with the N-terminal His tag. The plasmid was transformed into BL21(DE3)pLysS cells from which cultures were grown to OD = 0.5 in LB plus ampicillin and induced with 0.6 m*M* IPTG. After 2 h of growth, cells were harvested by centrifugation and the cell pellets frozen at 193 K. The frozen cell pellet (700 ml culture) was suspended in 20 ml lysis buffer [50 m*M* Tris pH 8.5, 1 *M* NaCl, 15% glycerol plus protease inhibitor (Sigma) added prior to use]. The cells were passed twice through a French pressure cell, another 30 ml of lysis buffer were added and the lysate was spun at 10 000 rev min^{-1} for 10 min (SS34 rotor) to yield cleared lysate, which was loaded onto a 6 ml Ni–NTA Superflow (Qiagen) column. The column was washed with four volumes of low-imidazole buffer (20 m*M* Tris pH 8.5, 250 m*M* NaCl, 20 m*M* imidazole, 15% glycerol) and eluted in high-imidazole buffer (20 m*M* Tris pH 8.5, 250 m*M* NaCl, 350 m*M* imidazole, 15% glycerol). SDS gel analysis determined the fractions to be pooled. For some preparations, the cysteine residues were alkylated by reducing the X29 pooled fractions in 1 m*M* EDTA, 20 m*M* Tris pH 8.5, 30 m*M* DTT for 1 h at room temperature, followed by addition of iodoacetamide to 300 m*M* with incubation for 1 h. The pooled X29 protein (unalkylated or alkylated) was diluted with two volumes of buffer 1 (20 m*M* Tris pH 8.5, 1 m*M* EDTA, 1 m*M* DTT, 2% glycerol) and loaded onto an 8 ml Heparin-Sepharose CL-6B (Pharmacia) column. The column was washed with four volumes of buffer 2 (20 m*M* Tris pH 8.5, 1 m*M* EDTA, 2% glycerol) plus 60 m*M* NaCl. The acetylated His-tagged X29 protein was eluted with a linear gradient of 60–400 m*M* NaCl in buffer 2. Fractions containing X29 were pooled, diluted with an equal volume of buffer 2 plus $CaCl_2$ added to 2 m*M,* and incubated with enterokinase (NEB) overnight at room temperature. An equal volume of buffer 2 was added to the cleaved protein, which was then loaded onto a MonoQ 5/5 column (Pharmacia) and washed with five volumes of buffer 3 (20 m*M* Tris pH 8.5) plus 60 m*M* NaCl. The X29 protein was eluted from the column with a 90–300 m*M* NaCl gradient in buffer 2. The >99% pure X29 (determined by staining of an overloaded gel) eluted at a concentration of 6–8 mg ml^{-1} in 20 m*M* Tris pH 8.5, 150 m*M* NaCl. The yield was approximately 15 mg of X29 per litre of culture as determined by BCS assay (Pierce). Purified X29 was obtained in the unalkylated (native) form by the same procedure, except that elution from the MonoQ column took place in 20 m*M* Tris pH 8.5, 1 m*M* DTT with a 90–400 m*M* NaCl salt gradient. No difference was seen in RNA-binding or decapping activity using the alkylated or native protein when assayed for decapping and by gel-mobility shift analysis.

2.2. Crystallization and diffraction intensity data from crystals of X29

X29, alkylated with iodoacetamide and purified as described above, was used directly from the MonoQ column to screen for crystallization conditions by the hanging-drop method and the Hampton Research Grid Screen PEG 6000 kit. Two different crystal forms were initially obtained from 5% PEG 6000, 0.1 *M* NaCl pH 6.0 (rhomboids) and pH 8.0 (thin swords) at room temperature. Further refinement of the crystallization conditions in seeded sitting drops gave well shaped tablet prisms and rhomboids (up to 0.5 mm) within a few days from drops that consisted of approximately 4–5 mg ml^{-1} X29, 0.025 *M* HEPES pH 7.68, 3.75% PEG 6000 set against a reservoir of the same composition. Both crystal forms had the same unit-cell parameters. Unalkylated X29 could be crystallized under the same conditions in the presence of DTE.

Crystals were cryoprotected in stages of 5% increments of glycerol in reservoir solution with equilibration for 10 min at each step to 30% glycerol and flash-cooled in a nitrogen stream. Diffraction data were collected at liquid-nitrogen temperature on an R-AXIS II image-plate detector with Cu $K\alpha$ radiation from a Rigaku rotating-anode generator equipped with Osmics confocal optics run at 50 kV and 100 mA. Intensity data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) or *MOSFLM* (Leslie, 1992) and *SCALA* (Kabsch, 1988).

3. Results and discussion

X29 was expressed in soluble N-terminal His-tagged form from pET19b vector in BL21 cells with a yield of about 15 mg per litre of culture. The protein was initially alkylated on cysteines to minimize aggregation during crystallization, but it was later found that the unalkylated form crystallized isomorphously in the presence of DTE as a reducing agent.

Crystals of X29 were orthorhombic, space group $P2_12_12_1$, with unit-cell parameters a = 49.19, b = 82.99, c = 111.06 Å. Crystallographic data are given in Table 1. Although X29 runs with a molecular weight of 29 kDa on SDS gel, its nucleotide sequence specifies a molecular weight of about 25 kDa. V_M for the X29 crystals is consistent with a dimer per asymmetric unit, as are light-scattering measurements at concentrations comparable to those used for the crystallization. However, gel-permeation chromatography under somewhat different but physiological conditions gives no indication of species larger than a monomer. A self-rotation search gave no significant peaks, indicating that the putative dyad axis of this dimer is parallel to one of the twofold screw axes in the lattice. The high quality and resolution of the X29 diffraction data are favorable for structure determination, which is currently in progress.

Table 1
Crystallographic data.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.5418
Lattice type	Orthorhombic
Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	49.19
b (Å)	82.99
c (Å)	111.06
α, β, γ (°)	90
Volume of unit cell (Å^3)	453379
Molecules per AU	2
Resolution (Å)	26.8–2.1
Observations	185780 (12420)
Unique reflections	27062 (1909)
Completeness (%)	99.7 (99.7)
Multiplicity	6.8 (6.7)
R_{sym}† (%)	5.4 (36.0)
$I/\sigma(I)$	7.1 (2.1)
Solvent content (%)	45.3
V_M (Å^3 Da^{-1})	2.27

† $R_{sym} = \sum_i \sum_h |I_h - I_{hi}| / \sum_i \sum_h I_h$, where I_h is the mean intensity of reflection h.

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