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Expression, crystallization and preliminary X-ray analysis of the *Pyrococcus abyssi* protein homologue of *Saccharomyces cerevisiae* Nip7p

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Saccharomyces cerevisiae Nip7p is a nucleolar protein required for accurate processing of the 27S precursor of the 25S and 5.8S ribosomal RNAs. Nip7p homologues are found in eukaryotes and archaea. The *Pyrococcus abyssi* homologue of Nip7p (PaNip7) was cloned, expressed in *Escherichia coli* and purified for crystallization. X-ray diffraction data were collected from native crystals and an iodide derivative using synchrotron radiation. PaNip7 native crystals diffract to 1.8 Å and belong to space group *C*2, with unit-cell parameters $a = 88.49$, $b = 90.28$, $c = 63.35$ Å, $\beta = 134.29^\circ$. The PaNip7 structure was solved using the SIRAS method.

1. Introduction

Ribosome biogenesis requires a fine balance between synthesis of the ribosomal proteins and transcription, processing and modification of the rRNA precursors (pre-rRNA). In eukaryotes, pre-rRNA processing and modification take place simultaneously with assembly of the ribosomal proteins (r-proteins) onto the rRNA, forming large ribonucleoprotein particles in the nucleolus. Over 170 *trans*-acting factors involved in rRNA biosynthesis have already been identified and include endo- and exonucleases, putative ATP-dependent RNA helicases, rRNA-modifying enzymes and an increasing number of small nucleolar ribonucleoprotein (snoRNP) complexes (Kressler *et al.*, 1999; Venema & Tollervey, 1999; Fromont-Racine *et al.*, 2003).

The yeast *Saccharomyces cerevisiae* has been an important model organism for the study of eukaryotic ribosome biogenesis. The *nip7* gene was isolated by complementation of the *S. cerevisiae* temperature-sensitive strain *nip7-1* (Zanchin *et al.*, 1997). The *nip7*-encoded protein Nip7p is essential for cell viability and 60S subunit formation. Its depletion causes a series of pre-rRNA processing defects that include accumulation of the 35S pre-rRNA transcript, presence of a 23S aberrant precursor, decreased 20S pre-rRNA levels and accumulation of 27S pre-rRNA. Delayed processing of 27S pre-rRNA is the cause of reduced synthesis of 25S and 5.8S rRNAs relative to 18S rRNA in cells deficient in Nip7p (Zanchin *et al.*, 1997). Nip7p interacts physically with the proteins Rrp43p and Nop8p (Zanchin & Goldfarb, 1999*a*,*b*). Rrp43p is a subunit of the exosome complex and was initially reported to be necessary for accurate 5.8S rRNA 3′-end formation (Mitchell *et al.*, 1997), but its deficiency mainly affects 40S subunit biosynthesis (Zanchin & Goldfarb, 1999*a*); it is also required for 18S rRNA biosynthesis and for degradation of the excised 5′ external transcribed sequence. Yeast cells depleted of Nop8p show an imbalance in ribosomal subunits with reduced levels of 60S subunits. However, whereas deficiency of Nip7p results in slower processing of the 27S pre-rRNA, deficiency of Nop8p leads to rapid degradation of this pre-rRNA, indicating that Nip7p and Nop8p are required for different 27S pre-rRNA processing steps. The molecular function of both proteins, however, still remains to be determined. Human homologues of both Nop8p (named Nop132) and Nip7p have been isolated and their physical interaction has also been demonstrated (Zanchin *et al.*, 1997; Sekiguchi *et al.*, 2004), further confirming that the mechanism of ribosome biosynthesis is highly conserved in eukaryotes.

Archaea are prokaryotic organisms in regard to the absence of a nucleus, but certain cellular processes show a resemblance to those of eukaryotes. Homologues of the eukaryotic nucleolar proteins Nop1p and Nop56p/Nop58p have been identified in all archaeal genomes sequenced (Amiri, 1994; Omer *et al.*, 2000). These proteins are found in box C/D snoRNPs that are responsible for ribose methylation on the rRNA (Decatur & Fournier, 2003). Small RNA homologues of the eukaryotic small nucleolar RNAs, which function as guides to the methylation sites of snoRNP, are also found in archaea (Dennis *et al.*, 2001; Omer *et al.*, 2003). A protein showing sequence similarity to Nip7p was identified in archaea and *Archaeoglobus fulgidus* contains the most similar archaeal Nip7p homologue, showing 25% and 45% amino-acid identity and similarity, respectively, to the yeast protein. The *Pyrococcus abyssi* Nip7 homologue shares 17.5% amino-acid identity and 38% similarity

with the yeast Nip7p. Comparative sequence analysis identified a putative RNA-binding domain at the C-terminal region of Nip7p (Aravind & Koonin, 1999). This domain is found in a number of RNA-modifying enzymes and was named PUA after pseudouridine synthases and archaeosine-specific transglycosylases. The PUA domain comprises approximately 78–83 amino acids, showing a predicted β-strand-rich structure (Aravind & Koonin, 1999). These observations indicate that Nip7p shows a conserved RNA-binding activity and led us to investigate the three-dimensional structure of *P. abyssi* Nip7 by X-ray crystallography.

Table 1
Data-collection statistics.

Values in parentheses are for the outer resolution shell.

	Native crystal	Iodide derivative
Beamline	LNLS D03B-CPR	LNLS D03B-CPR
Wavelength (Å)	1.4270	1.4270
Space group	*C*2	*C*2
Unit-cell parameters (Å, °)	a = 88.49, b = 90.28, c = 63.35, β = 134.29	a = 87.33, b = 88.99, c = 63.59, β = 133.36
Resolution limits (Å)	31.6–1.80 (1.90–1.80)	32.1–1.90 (2.00–1.90)
Total observations	235642	458129
Unique reflections	33002	27392
Completeness (%)	99.9 (100.0)	98.5 (96.9)
Multiplicity	7.1 (7.0)	16.7 (16.6)
R_{sym} (%)	4.9 (30.1)	6.2 (26.7)
Mean $I/\sigma(I)$	8.6 (1.7)	6.4 (2.4)
Anomalous completeness (%)		98.4 (97.1)
Anomalous R factor (%)		7.3 (13.0)
Isomorphous R factor (%)		38.3 (43.6)

2. Methods

2.1. Cloning of the *P. abyssi* Nip7 homologue

DNA cloning and analysis were performed according to previously described standard procedures (Sambrook *et al.*, 1989) using *Escherichia coli* strain DH5α, which was maintained in LB medium containing 50 µg ml^{-1} ampicillin. The *P. abyssi* Nip7 homologue is a 166-amino-acid protein with a molecular weight of 19 291.38 Da and was identified by database searches for proteins showing sequence similarity to the *S. cerevisiae* Nip7p protein. A 502 base-pair DNA fragment comprising the coding sequence of the *P. abyssi* Nip7 homologue (PAB0176; PaNip7) was amplified by polymerase chain reaction (PCR) from genomic DNA using oligonucleotides containing *NdeI* and *Bam*HI restriction sites (forward, 5′-AGGAAGCATATGAGTGGTGAGCTGAGG-3′; reverse, 5′-CCGGATCCAGTTAGGCAAGAAAACAAG-3′). The PCR product was cloned using the pGEM-T system (Promega) and subsequently subcloned into the pCYTEXP3 (Schneppe *et al.*, 1994) expression vector, producing vector pCYTEX-PaNip7. The nucleotide sequence of the PCR product was verified by DNA sequencing analysis using an Applied Biosystems ABI Prism 377 DNA-sequence analyzer.

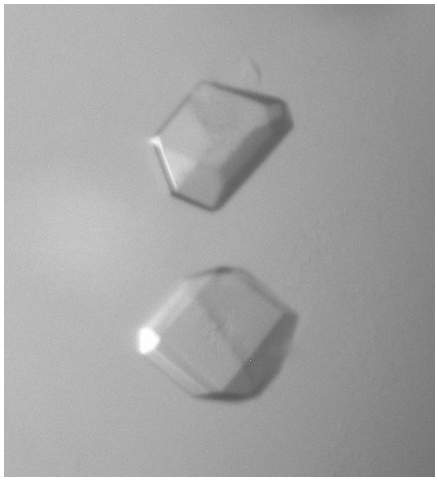

Figure 1
Crystals of *P. abyssi* Nip7 obtained by vapour-diffusion equilibration against a reservoir solution consisting of 4.1 *M* NaCl and 100 m*M* HEPES pH 7.2. Maximum crystal dimensions were 150 × 150 × 75 µm.

2.2. Expression and purification of PaNip7

E. coli DH5α harbouring plasmid pCYTEX-PaNip7 was incubated overnight at 303 K in LB medium containing ampicillin (50 µg ml^{-1}). This pre-culture was used to inoculate 4 l of fresh LB–ampicillin medium and incubated at 303 K until an optical density at 600 nm (OD_{600}) of approximately 0.8–0.9 was reached. PaNip7 expression was induced by transferring the culture to 315 K for 4 h. Cells were harvested by centrifugation, resuspended in buffer *A* [10 m*M* Tris–HCl pH 7.0, 50 m*M* NaCl, 1 m*M* EDTA, 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF)] and treated with lysozyme (50 µg ml^{-1}) for 30 min on ice. Subsequently, cells were disrupted by sonication using a Branson sonifier (Branson Ultrasonics Co). The cell extract was initially incubated at 338 K for 1 h for thermal denaturation of *E. coli* proteins. The suspension was submitted to centrifugation at 20 000*g* for 30 min at 277 K and the soluble fraction was applied onto a heparin-Sepharose column (Amersham-Pharmacia) equilibrated with buffer *A*. The column was eluted with a linear 50 m*M* to 1 *M* NaCl gradient. The purified protein was dialyzed against a buffer containing 10 m*M* Tris–HCl pH 7.0 and 5 m*M* β-mercaptoethanol for 48 h at 277 K and concentrated using an Ultrafree 4 concentrator (Millipore). This expression system yielded approximately 2 mg of protein per litre of culture.

2.3. Crystallization assays

PaNip7 was crystallized by the hanging-drop vapour-diffusion method at a concentration of 12 mg ml^{-1} in 10 m*M* Tris–HCl pH 7.0 and 5 m*M* β-mercaptoethanol at 293 K. Initial crystallization trials were performed using Crystal Screen, Crystal Screen II (Hampton Research), Wizard I and Wizard II (Decode Genetics). 24-well plates were used and 300 µl reservoir solution was used in each well. Equal volumes (2 µl) of protein sample and reservoir solution were mixed for crystallization. Small needle-shaped crystals were observed in most wells 24 h after the assay was begun. Monocrystals were only observed in a Crystal Screen II well containing 4.3 *M* NaCl and 100 m*M* HEPES pH 7.5 after 2 d. This crystallization condition was refined by changing the NaCl concentration and the pH of the HEPES buffer. Crystals were observed in wells containing reservoir solutions with NaCl concentration in the range 3.9–4.3 *M* and pH in the range 7.2–8.2. Larger crystals were obtained using 4.1 *M* NaCl and 100 m*M* HEPES pH 7.2 after 2 d at 293 K. The crystals grew to dimensions of 150 × 150 × 75 µm (Fig. 1).

2.4. Data collection and processing

X-ray diffraction data were collected at the protein crystallography beamline D03B-CPR of the Brazilian Synchrotron Light Laboratory, LNLS, Campinas, Brazil. Native crystals were cryoprotected with 20% glycerol prior to flash-cooling in a 100 K nitrogen-gas stream. Iodide-derivative (I^-) crystals were obtained by the rapid cryo-soaking technique (Dauter *et al.*, 2000; Nagem *et al.*, 2001). The soaking solution

consisted of 20% glycerol, 100 m*M* HEPES pH 7.2, 3.1 *M* NaCl and 1.0 *M* NaI (1.0 *M* NaCl of the mother liquor was replaced by NaI in order to facilitate iodide-anion incorporation). Crystals were incubated in the soaking solution for 2 min prior to flash-cooling. Complete data sets were collected from native and derivative crystals using a MAR CCD detector. The beamline wavelength was set to 1.427 Å. In order to obtain highly redundant data from the derivative crystal, 700 oscillation images ($\Delta\varphi = 1^\circ$) were taken. Data processing and scaling was performed using the programs *MOSFLM* (Leslie, 1992) and *SCALA* (Kabsch, 1988; Blessing, 1995) from the *CCP*4 package (Collaborative Computational Project 4, Number 4, 1994).

3. Results and discussion

PaNip7 native crystals diffract to 1.8 Å resolution and belong to space group *C*2. I^--derivative crystals diffract to 1.9 Å resolution and belong to the same space group with similar unit-cell parameters. Table 1 summarizes the data-collection statistics. According to Matthews (1968), a solvent content of 48% corresponds to V_M = 2.4 Å^3 Da^{-1}, indicating the presence of two monomers in the asymmetric unit.

The structure of PaNip7 was determined using the SIRAS method. LNLS D03B is a monochromatic beamline and with the experimental setup used (wavelength = 1.427 Å) the theoretical f'' value of iodide is 6.0243. Seven I^- sites with occupancies greater than 0.7 were found with the program *SHELXD* (Sheldrick & Schneider, 2001) using only anomalous data from the derivative crystal. Derivative-to-native data scaling was performed using the program *SCALEIT* (Howell & Smith, 1992) from the *CCP*4 package (Collaborative Computational Project 4, Number 4, 1994). Anomalous and isomorphous data statistics are presented in Table 1. The high value of the isomorphous *R* factor also indicated the incorporation of a large number of iodide ions. Fig. 2 shows a comparison between the anomalous and isomorphous Patterson maps obtained from experimental data and the predicted Patterson map generated using the iodide coordinates found by *SHELXD*. According to the Matthews coefficient calculation, the asymmetric unit should contain two molecules (332 residues in total); therefore, *SHELXD* results indicate the presence of at least one anomalous scatterer per approximately 47 residues. Refinement of heavy-atom parameters and calculation of phases were carried out with *SHARP* (de La Fortelle & Bricogne, 1997) using data from native and derivative crystals. An improved electron-density map was obtained by solvent flattening with *SOLOMON* (Abrahams & Leslie, 1996). Model building and refinement are currently in progress.

In conclusion, we have crystallized the first homologue of *S. cerevisiae* Nip7p and expect that the three-dimensional structure of *P. abyssi* Nip7 will provide essential information about the molecular function of this group of proteins. Together with RNA-binding assays, the PaNip7 structural analysis is expected to confirm whether the PUA domain found in these proteins is a functional RNA-binding domain.

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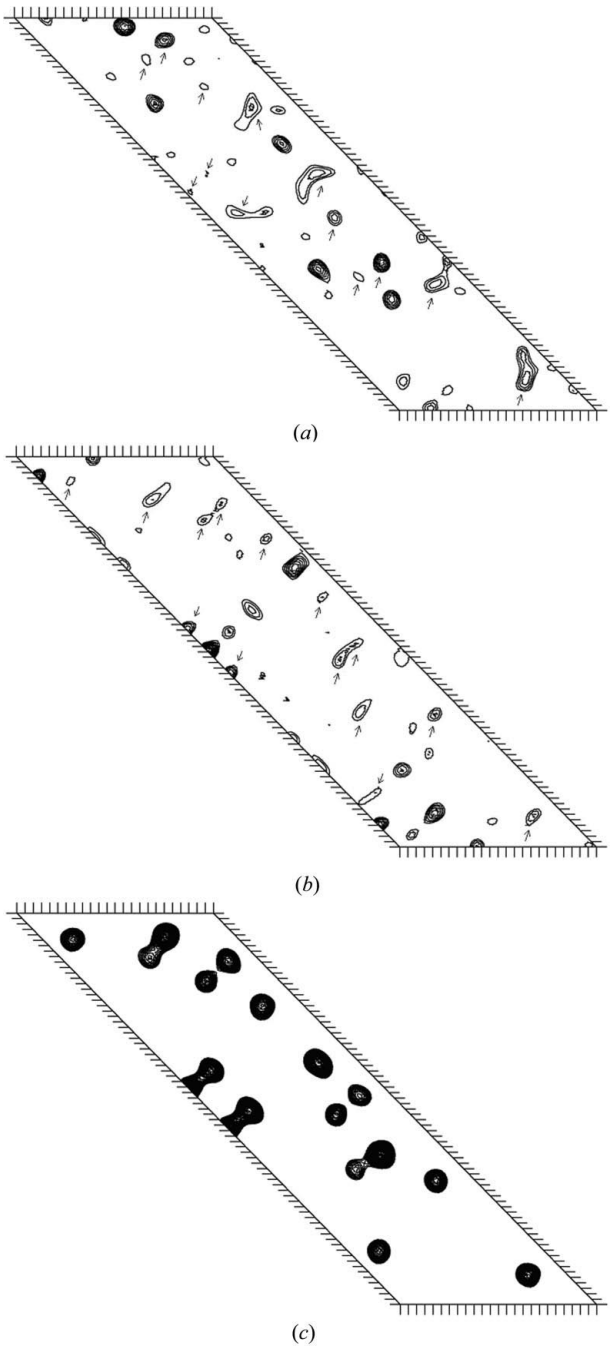

Figure 2
Harker sections of the anomalous (*a*) and isomorphous (*b*) Patterson maps (minimum contour level = 2.0σ). The peaks indicated by arrows are also found in the predicted Harker section (*c*) generated using the heavy-atom coordinates.

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