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Purification and crystallization of *Bacillus subtilis* NrnA, a novel enzyme involved in nanoRNA degradation

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The final step in RNA degradation is the hydrolysis of RNA fragments five nucleotides or less in length (nanoRNA) to mononucleotides. In *Escherichia coli* this step is carried out by oligoribonuclease (Orn), a DEDD-family exoribonuclease that is conserved throughout eukaryotes. However, many bacteria lack Orn homologs, and an unrelated DHH-family phosphoesterase, NrnA, has recently been identified as one of the enzymes responsible for nanoRNA degradation in *Bacillus subtilis*. To understand its mechanism of action, *B. subtilis* NrnA was purified and crystallized at room temperature using the hanging-drop vapor-diffusion method with PEG 4000, PEG 3350 or PEG MME 2000 as precipitant. The crystals belonged to the primitive monoclinic space group $P2_1$, with unit-cell parameters $a = 50.62$, $b = 121.3$, $c = 123.4$ Å, $\alpha = 90$, $\beta = 91.31$, $\gamma = 90^\circ$.

1. Introduction

The last step of RNA decay is the degradation of nanoRNA (RNA oligomers five nucleotides or less in length) to mononucleotides and is carried out by specialized enzymes. In *Escherichia coli* and many other eubacteria, and in all eukaryotic genomes examined to date, this function is performed by a highly conserved enzyme called oligoribonuclease (Orn; Zhang *et al.*, 1998; Zuo & Deutscher, 2001; Fiedler *et al.*, 2004). However, *Bacillus subtilis* and many other bacteria lack an *orn*-like gene. In these organisms, this function has recently been attributed to an unrelated protein, NrnA, also known as nanoRNase A (Mechold *et al.*, 2007).

NrnA belongs to the DHH family of phosphoesterases, which includes the 5′→3′ RecJ exonuclease, inorganic pyrophosphatases, *Drosophila* and human prune proteins and yeast exopolyphosphatase (Aravind & Koonin, 1998). Proteins belonging to this family have a conserved N-terminal domain with four distinct sequence motifs and an associated C-terminal domain that can vary between family members (Aravind & Koonin, 1998). Each motif contains at least one conserved aspartate residue. Motif 3 contains the DHH sequence, a conserved aspartate followed by two histidines, after which this family of proteins is named (protein family PF01368 in the Pfam database; Finn *et al.*, 2010). The conserved DHH residues form the active site and are believed to be involved in the coordination of two metal ions. NrnA contains a C-terminal DHHA1 domain (DHH-associated domain 1; Pfam domain PF02272) that is also present in alanyl-tRNA synthetases and may play a role in RNA binding.

NrnA is likely to play an important role in nanoRNA degradation in *B. subtilis*. NrnA displays a preference for oligonucleotides three nucleotides in length and a manganese cofactor (Mechold *et al.*, 2007). NrnA also acts as a 3′ pAp phosphatase, a function carried out by a separate enzyme, CysQ, in *E. coli*. *B. subtilis* lacks both the *cysQ* and *orn* genes and is likely to utilize NrnA to accomplish these functions, since *nrnA* complements both an *E. coli orn* conditional mutant and a *cysQ* deletion. Finally, *nrnA* is not essential in *B. subtilis* under normal growth conditions and other enzyme(s) have been suggested to play supporting roles in nanoRNA degradation (Fang *et al.*, 2009).

2. Materials and methods

2.1. Expression and purification of native NrnA

The full-length *nrnA* gene (GenBank accession No. BSU29250) was PCR-amplified from *B. subtilis* 168 genomic DNA (ATCC No. 23857D-5) using the forward primer GCTCTAGAATGAAAACAGAATTGATCAGAACCATATC and the reverse primer CGGGATCCTTACTACTCGTGTTCTTTACATAATGTTTC. The gene was cloned between the *Nhe*I and *Bam*HI restriction sites of pET28a-TEV vector to yield full-length N-terminally 6×His-tagged NrnA protein. The sequence and orientation of the insert were confirmed by DNA sequencing. The pET28a-TEV vector was constructed in our laboratory by mutating the thrombin cleavage site of the original pET28a vector (Novagen) to a TEV cleavage site (C. Recinos & A. Malhotra, unpublished work).

The recombinant plasmid pET28a-NrnA was transformed into competent *E. coli* BL21 Star cells (Invitrogen). 50 ml starter culture was grown overnight in LB medium [Difco LB Broth, Miller (Luria–Bertani)] containing 30 µg ml^{-1} kanamycin with vigorous shaking (210 rev min^{-1}) at 310 K. Subsequently, the starter culture was used to inoculate 2 l of the same medium, which was further incubated at 310 K until an optical density at 600 nm of 0.6–0.8 was reached. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at 1 m*M* to induce expression of the recombinant protein at 310 K for 3 h. After induction, cultures were centrifuged at 5000*g* for 10 min at 277 K and 15 g (wet weight) of pellet was resuspended in 35 ml buffer Ni-*A* (40 m*M* Tris–HCl, 150 m*M* NaCl, 20 m*M* imidazole pH 8.0). Cells were lysed at 69 MPa using an EmulsiFlex-C3 homogenizer (Avestin, Ontario, Canada). Before cell lysis, one Complete Mini EDTA-free protease-inhibitor tablet (Roche) and 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) were added to the resuspended cells. The lysate was centrifuged at 20 000*g* for 30 min at 277 K and Polymin P was added to 0.3% by volume (Burgess, 1991). The solution was stirred on ice for 15 min and centrifuged at 20 000*g* for 10 min at 277 K in order to precipitate nucleic acids.

The supernatant fraction was loaded onto an immobilized metal-affinity column (IMAC) with Poros MC-20 beads (Boehringer Mannheim) which was pre-equilibrated with buffer Ni-*A*. The column was washed with buffer Ni-*A* and bound protein was eluted with an imidazole gradient from 20 to 500 m*M* using an ÄKTA FPLC chromatography system (GE Healthcare). Protein elution was monitored at 280 nm and the resulting fractions were analyzed by SDS–PAGE on Gradient 8–25% PhastGel (GE Healthcare). Fractions containing NrnA were pooled and dialyzed against buffer MQ-*A* (40 m*M* Tris–HCl, 2 m*M* EDTA, 1 m*M* DTT, 150 m*M* NaCl pH 8.0). The sample was applied onto a MonoQ HR anion-exchange chromatography column (GE Healthcare) pre-equilibrated with buffer MQ-*A*. Elution was performed using a gradient to 1 *M* NaCl. Relevant fractions were concentrated to 2.5 ml using an Amicon Ultra centrifugal filter (Millipore) and applied onto a Superdex S-200 size-exclusion column (GE Healthcare) pre-equilibrated with buffer S200 (40 m*M* Tris–HCl, 1 m*M* DTT, 150 m*M* NaCl, 10% glycerol pH 8.0). The S200 column was run at a flow rate of 1 ml min^{-1}. Fractions containing purified His-tagged NrnA, as confirmed by SDS–PAGE, were pooled and concentrated to 20 mg ml^{-1}. In a typical purification run, 2 l culture gave 7 g wet cell paste and yielded 21.7 mg purified NrnA.

2.2. Expression and purification of SeMet-labeled NrnA

Selenomethionine-labeled NrnA was prepared by suppressing the endogenous methionine-biosynthesis pathway in the expression strain (Doublié, 1997), which was grown using the auto-induction procedure developed by Studier (2005). The following stock solutions were prepared and filter-sterilized [the buffers are named using the nomenclature of Studier (2005); *X* refers to the *X*-fold concentrations used in the stock solutions]. P buffer [1*X* P = 50 m*M* KH_2PO_4, 50 m*M* Na_2HPO_4, 25 m*M* $(NH_4)_2SO_4$] was prepared as a 20*X* P stock solution by mixing 6.6 g $(NH_4)_2SO_4$, 13.6 g KH_2PO_4, 14.2 g Na_2HPO_4 and water to a final volume of 100 ml. 5052 solution (1*X* 5052 = 0.5% glycerol, 0.05% glucose, 0.2% α-lactose) was prepared as a 50*X* stock solution by mixing 25 g glycerol, 2.5 g glucose, 10 g α-lactose and water to a final volume of 100 ml and stored at 277 K. Methionine 2000*X* and selenomethionine 167*X* were prepared as 10 mg ml^{-1} stock solutions and stored at 277 K. The 17-amino-acid (no Cys, Tyr and Met) 50*X* stock solution was prepared by mixing 500 mg of each amino acid and adding water to 50 ml (1*X* = 200 µg ml^{-1} of each of the 17 amino acids) and stored at 277 K. Vitamin B_{12} 783*X* stock solution was at 100 µg ml^{-1} (1*X* = 100 n*M*). The 10 000*X* trace-metal solution was prepared by the Thomas K. Harris laboratory according to the protocol of Studier (2005). Next, 1 l PASM-5052 medium was assembled by mixing 50 ml 20*X* P buffer solution, 20 ml 50*X* 5052 solution, 1 ml 1 *M* $MgSO_4$ solution, 100 µl 10 000*X* trace-metals solution, 20 ml 50*X* 17-amino-acid mixture, 0.5 ml 2000*X* methionine solution, 6 ml 167*X* selenomethionine solution and 1.27 ml 783*X* vitamin B_{12} solution.

5 ml LB medium [Difco LB Broth, Miller (Luria–Bertani)] supplemented with 30 µg ml^{-1} kanamycin was inoculated with *E. coli* BL21 Star containing the recombinant pET28a-NrnA plasmid and incubated at 310 K with shaking for ∼18 h. Next, this culture was used to inoculate 1 l PASM-5052 medium supplemented with an increased concentration of kanamycin (100 µg ml^{-1}) to account for the sensitivity of *E. coli* BL21 Star to phosphate buffers (Studier, 2005). We reduced the amount of SeMet and Met in the medium to 60 µg ml^{-1} (125 µg ml^{-1} recommended) and 5 µg ml^{-1} (10 µg ml^{-1} recommended), respectively, to improve cell growth. The culture was grown at 310 K with shaking at 180 rev min^{-1} for 48 h until an A_{600} of ∼3.0 was reached. The cells were harvested and lysed and SeMet-NrnA was purified using a protocol similar to that used for the native protein (Fig. 1). From 1 l culture approximately 5 g wet cells was obtained, yielding 25 mg pure SeMet-NrnA.

2.3. Crystallization of NrnA

The purified native protein was concentrated to 20 mg ml^{-1} and sent for high-throughput screening at the Hauptman–Woodward Institute (Buffalo, New York, USA) using the microbatch-under-oil

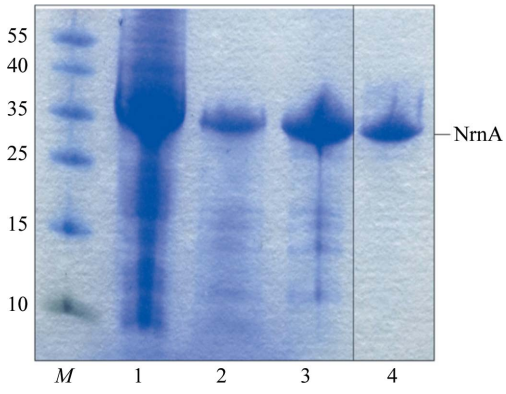


Figure 1
SDS–PAGE (8–25% PhastGel) analysis of the SeMet-NrnA purification procedure. Lane *M*, molecular-weight standards (labeled in kDa); lane 1, cell extract; lane 2, Ni^{2+} column; lane 3, Mono Q column; lane 4, Superdex 200 column.

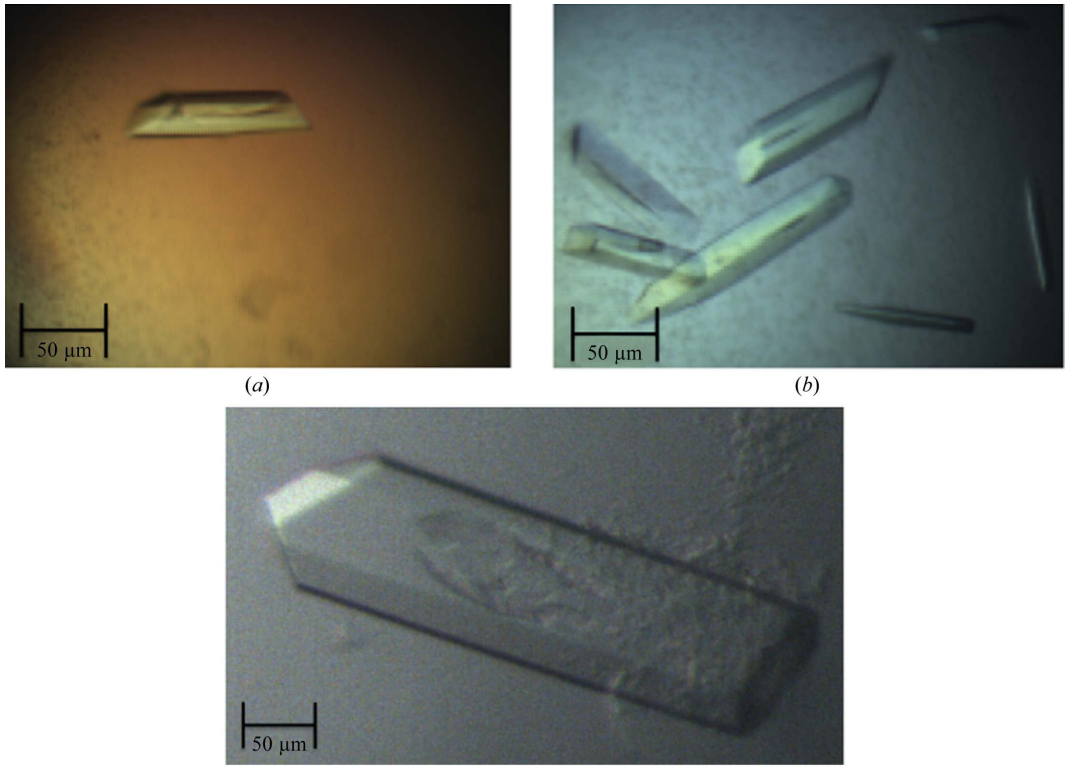
technique (Luft *et al.*, 2003). Crystals were obtained using 0.17 *M* ammonium acetate, 0.085 *M* sodium acetate trihydrate pH 4.6, 25.5% PEG 4000 and 15%(*v*/*v*) anhydrous glycerol. Crystals were reproduced and optimized in-house *via* hanging-drop vapor diffusion. Both glycerol and ammonium acetate were required for crystallization, and PEG MME 2000 significantly increased the crystal size. The final native crystals were obtained using 5 mg ml^{-1} native protein solution mixed with the reservoir solution in a 1:1 ratio. $MnCl_2$ or $MgCl_2$ (10 m*M*) were soaked into native crystals for 1–1.5 h with 1.9*X* concentrated crystallization solution. For cocrystallization studies, a nanoRNA model substrate 2′-*O*-methylated at its 3′ end (pAAA-2′-*O*-Me) was pre-incubated with NrnA for 30 min prior to crystallization. These crystals were then soaked for at least 1 h with 10 m*M* $MnCl_2$ or $MgCl_2$ prior to flash-freezing in liquid nitrogen.

The SeMet protein failed to crystallize using the above conditions. Both the native and SeMet protein stocks were in buffers containing 1 m*M* DTT, but we hypothesized that additional reducing agents would be needed to crystallize the SeMet protein since selenomethionine residues are easily susceptible to oxidation. Several reducing agents were added to the reservoir and SeMet-NrnA crystals appeared within 1 d upon the addition of 0.1 *M* DTT to otherwise native conditions. The rate of crystallization was reduced by diluting SeMet-NrnA to 2.5 mg ml^{-1} in a storage buffer containing 30% glycerol for cryoprotection and 0.1 *M* DTT. Crystals also grew in the presence of various molecular-weight PEGs, including PEG 3350, PEG 4000 and PEG MME 2000.

Cocrystallization of the diluted SeMet protein proceeded with various RNA substrates, including A_3 with a phosphorothioate linkage (AA*A), 3′-phosphoadenosine 5′-phosphate (pAp) and sodium orthovanadate. Phosphorothioate (2 m*M*) was added directly to the hanging drop in a 1:1:1 ratio with the SeMet-NrnA protein (5 mg ml^{-1}) and the reservoir. The ligand pAp (0.2 µl of a 200 m*M* stock) was also added directly to a 2 µl drop. Sodium orthovanadate was added to the well at a final concentration of 1 m*M*. SeMet crystals were also grown in the presence of 1–2 m*M* $MgCl_2$, $MnCl_2$, $ZnCl_2$ and $CoCl_2$. Further optimization of the SeMet crystals proceeded by adding a 1/10 volume of one of the following additives to the reservoir: 30–50% DMSO, 20% glucose, 10% ethanol, 30% methanol, 30% ethylene glycol, 1 *M* MES-OH pH 6 and 1 *M* guanidine–HCl. In addition, various sugars improved the crystal size, including 50% sucrose, 10% lactose, 30% sorbitol and 1 *M* IPTG.

2.4. X-ray diffraction data collection and processing

Diffraction data were collected on beamlines 23-ID-B, 23-ID-D and 24-ID-C at the Advanced Photon Source (APS) of the Argonne National Laboratory (Argonne, Illinois, USA). Several data sets were collected, with a native NrnA crystal diffracting to a resolution of 2.0 Å and a SeMet-NrnA crystal diffracting to 2.3 Å resolution. Data were collected from crystals flash-frozen in liquid nitrogen without any additional cryoprotectant since the crystallization mother liquor included 15% glycerol. X-ray diffraction data were indexed and scaled using the *HKL*-2000 software package (Otwinowski & Minor, 1997).



(*a*) (*b*) (*c*)

Figure 2
Crystals of *B. subtilis* NrnA. Crystals were grown in 24-well plates by hanging-drop vapor diffusion. (*a*) Native crystals were grown from NrnA concentrated to 5 mg ml^{-1} and crystallized in the presence of 20% PEG MME 2000, 85 m*M* sodium acetate, 0.17 *M* ammonium acetate and 15% glycerol. Crystals grew to maximum size in 3–5 d. The longest dimension of a typical crystal was approximately 0.35 mm. (*b*) Selenomethionine-derivatized NrnA was crystallized using protein at 2.5 mg ml^{-1} with the same mother liquor as in (*a*) but supplemented with 0.1 *M* DTT. (*c*) Native crystal grown in the presence of the NrnA inhibitor pAAA-2′-*O*-Me and then soaked for 1 h at 10 m*M* $MnCl_2$.

Table 1
Summary of X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Native NrnA	SeMet-NrnA
Wavelength (Å)	0.97918	0.97918
Space group	$P2_1$	$P2_1$
Unit-cell parameters (Å, °)	a = 50.62, b = 121.3, c = 123.4, $\alpha = \gamma$ = 90, β = 91.31	a = 50.41, b = 121.3, c = 123.7, $\alpha = \gamma$ = 90, β = 91.38
Resolution range (Å)	50–2.0 (2.03–2.00)	50–2.30 (2.38–2.30)
$R_{\rm merge}$†	0.058 (0.315)	0.172 (0.577)
Reflections		
Measured	493314	316960
Unique	99703	65314
Completeness (%)	98.8 (96.9)	99.9 (99.7)
$\langle I/\sigma(I)\rangle$	35.19 (4.5)	11.54 (2.8)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity of multiple observations.

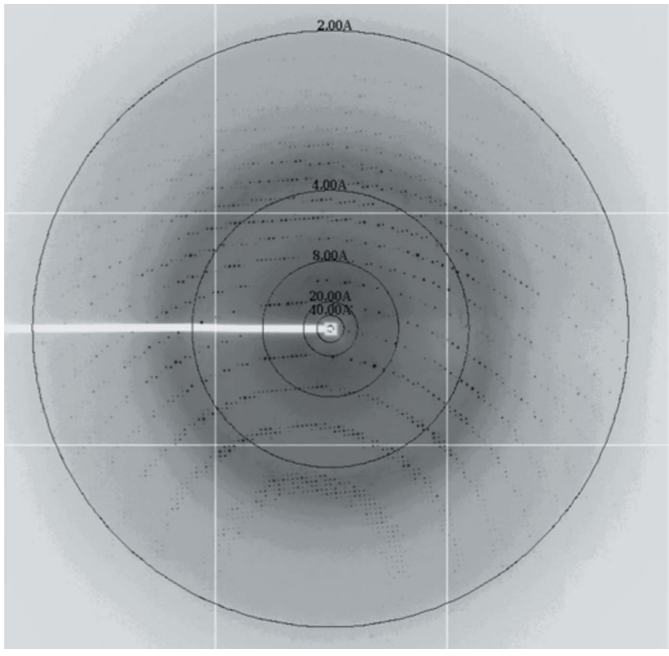


Figure 3
A typical X-ray diffraction image from a native NrnA crystal obtained using an ADSC Q315 CCD detector (Advanced Detector Systems Corporation) on the Advanced Photon Source NE-CAT 24-ID-C beamline. The exposure time was 1 s, with an oscillation range of 1°. The left and right edges are at a resolution of 1.76 Å.

3. Results and discussion

The *nrnA* gene from *B. subtilis* encodes a 313-amino-acid protein with a calculated pI of 4.88. Native and SeMet-labeled His-tagged NrnA were overexpressed and purified as described in §2. Good purification was achieved by a combination of affinity and anion-exchange chromatography followed by a size-exclusion polishing step (Fig. 1). Size-exclusion gel-filtration chromatography suggested that NrnA exists as a monomer with a molecular weight of ∼35 kDa, consistent with the predicted mass of the protein. Purity was monitored by SDS–PAGE, confirming the calculated molecular weight of 35.1 kDa (Fig. 1).

NrnA is a member of the DHH/DHHA1 family of phosphoesterases. X-ray structures are now available for several DHH-family phosphoesterases, including the 5′→3′ DNase RecJ (Yamagata *et al.*, 2002; Wakamatsu *et al.*, 2010) and *B. subtilis* inorganic pyrophosphatase (Ahn *et al.*, 2001). The active-site architecture suggests a two-metal-ion mechanism with a preference for Mg^{2+} or Mn^{2+} coordination at the conserved DHH motif. Unlike RecJ, which requires at least six nucleotides of ssDNA, NrnA prefers nanoRNAs 2–5 nucleotides in length. An examination of these different substrate specificities awaits the NrnA crystal structure.

Crystals of *B. subtilis* NrnA have been obtained in both native and SeMet forms (Figs. 2*a* and 2*b*). Well diffracting crystals were also obtained after 1 h soaks of divalent cations into crystals grown in the presence of the NrnA inhibitor pAAA-2′-*O*-Me (Fig. 2*c*). Apo crystals and those soaked with ligands diffracted to 2.0 Å resolution (Fig. 3) and are amenable to structural analysis (Table 1). SeMet-NrnA crystals diffracted to 2.3 Å resolution with a strong anomalous signal, suggesting suitability for phasing. The complete structure is currently being refined and will be reported elsewhere.

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