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Crystallization and preliminary X-ray crystallographic analysis of eIF5BΔN and the eIF5BΔN–eIF1AΔN complex

Aiping Zheng,[a] Reo Yamamoto,[a] Masaaki Sokabe,[b]‡ Isao Tanaka[a,b] and Min Yao[a,b]*

[a]Graduate School of Life Sciences, Hokkaido University, Sapporo 060-0810, Japan, and [b]Faculty of Advanced Life Science, Hokkaido University, Sapporo 060-0810, Japan

‡ Present address: Department of Biochemistry and Molecular Medicine, School of Medicine, University of California, Davis, CA 95616, USA.

Correspondence e-mail: yao@castor.sci.hokudai.ac.jp

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The binding between two universally conserved translation initiation factors, eIF5B and eIF1A, is important in the initiation step of eukaryotic protein synthesis on the ribosome. Through this interaction, eIF1A assists in recruiting eIF5B to the initiating 40S subunit; eIF5B then encourages the joining of the 60S subunit to form an initiating 80S ribosome. Here, the expression, purification, crystallization and preliminary X-ray analyses of eIF5BΔN and the eIF5BΔN–eIF1AΔN complex from *Saccharomyces cerevisiae* are reported. The crystal of eIF5BΔN diffracted to 2.45 Å resolution and belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 130.0$, $c = 71.7$ Å. The asymmetric unit was estimated to contain one molecule. The initial phase was obtained by Se-SAD. The crystal of the eIF5BΔN–eIF1AΔN complex diffracted to 3.3 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 101.9$, $b = 120.9$, $c = 132.8$ Å. The asymmetric unit was estimated to contain two complex molecules.

1. Introduction

Initiation of protein synthesis is a crucial process in the cells of all organisms during which the initiation codon is base-paired with the anticodon and the small and large ribosomal subunits are joined to form an elongation-competent 70S/80S ribosome. This process requires three initiation factors (IF1, IF2 and IF3) in bacteria and at least eight initiation factors (eIFs) in eukaryotes (Jackson *et al.*, 2010). Only IF1 and IF2 are universally conserved throughout all three kingdoms: their corresponding orthologues in eukaryotes are eIF5B and eIF1A, respectively (Kapp & Lorsch, 2004).

eIF1A plays multiple roles in eukaryotic translation initiation. It stabilizes binding of Met-tRNA$_i^{Met}$ to the 40S subunit and cooperates with eIF1 in promoting ribosomal scanning and start-codon selection (Pestova *et al.*, 1998). Compared with its bacterial homologue IF1, which has a conserved oligonucleotide/oligosaccharide-binding (OB) domain, eIF1A additionally possesses a helical subdomain in the C-terminus of the OB domain and N- and C-terminal unstructured tails (Battiste *et al.*, 2000). The positively charged N-terminal tail and the negatively charged C-terminal tail have opposite effects on the fidelity of start-codon selection (Fekete *et al.*, 2007). The position of eIF1A on the 40S subunit was mapped by directed hydroxyl-radical probing: the OB domain binds in the A site, similar to the binding of IF1 to the 30S subunit as shown by X-ray crystallography (Carter *et al.*, 2001), whereas the additional helical subdomain contacts the head region of the 30S subunit, creating a bridge over the mRNA channel. The N- and C-terminal tails both thread under the initiator tRNA, reaching into the P-site (Yu *et al.*, 2009).

Eukaryotic eIF5B was first identified in *Saccharomyces cerevisiae* (Choi *et al.*, 1998). Like its eubacterial homologue IF2, eukaryotic eIF5B mediates joining of the ribosomal subunits and functions as a GTPase that is activated by the presence of both ribosomal subunits. The hydrolysis of eIF5B-bound GTP is not essential for subunit joining, but is needed to release eIF5B itself from assembled 80S ribosomes (Pestova *et al.*, 2000). Compared with its archaeal homologue, which forms an extended 'chalice-shaped' structure (Roll-

Mecak *et al.*, 2000), eIF5B has an additional N-terminal domain which is not required for function (Lee *et al.*, 1999). eIF5B occupies the intersubunit cleft on the 80S ribosome and the GTP-binding domain is positioned near the GTPase-activating centre of the 60S subunit (Unbehaun *et al.*, 2007), which is similar to the position of IF2 as observed by cryo-EM reconstitution (Allen *et al.*, 2005).

In bacteria, IF2 has been cross-linked to IF1 on the ribosome (Boileau *et al.*, 1983) but no direct interaction has been reported in solution. In eukaryotes, it was reported that eIF1A and eIF5B bind to each other even off the ribosome (Choi *et al.*, 2000). A previous NMR study suggested that the unstructured extreme C-terminal five residues DIDDI of human eIF1A pack into a hydrophobic groove formed by helices H13 and H14 in the C-terminal domain IV of eIF5B (Marintchev *et al.*, 2003). This interaction is unique to eukaryotes because the unstructured C-terminal tail of eIF1A is not found in bacteria or archaea and bacterial IF2 also lacks the corresponding binding pocket. There is a great deal of evidence that the interaction between eIF5B and eIF1A is required for efficient ribosomal subunit joining. Disruption of the interaction by changing either the isoleucine residue in the eIF1A C-terminal sequence DIDDI or the deletion of helix 14 in eIF5B reduces both the GTP-hydrolysis and subunit-joining activities of eIF5B (Acker *et al.*, 2006). It has been proposed that eIF1A facilitates the binding of eIF5B to the 40S subunit to promote subunit joining in the rotated conformation (Allen *et al.*, 2005). Following 80S complex formation, GTP hydrolysis by eIF5B drives the ribosome back into the unrotated conformation (Myasnikov *et al.*, 2005), as does IF2 in bacteria (Marshall *et al.*, 2009), and enables the release of both eIF5B and eIF1A (Fringer *et al.*, 2007), with the ribosome then entering the elongation phase of protein synthesis.

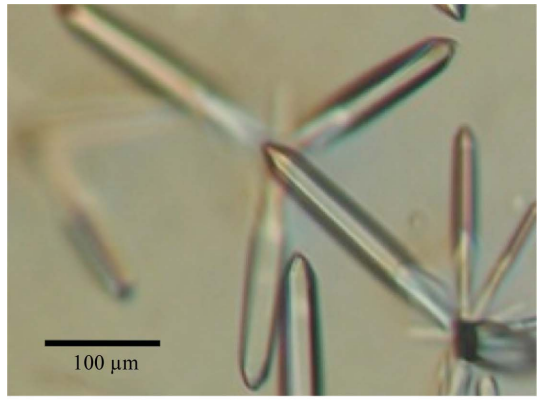
Here, we describe the expression, purification, crystallization and preliminary X-ray crystallographic analyses of eIF5BΔN and the eIF5BΔN–eIF1AΔN complex from *S. cerevisiae*. The structures of eIF5BΔN and the eIF5BΔN–eIF1AΔN complex will provide details of the interaction between eIF5B and eIF1A, which are important for understanding ribosomal subunit joining.

2. Materials and methods

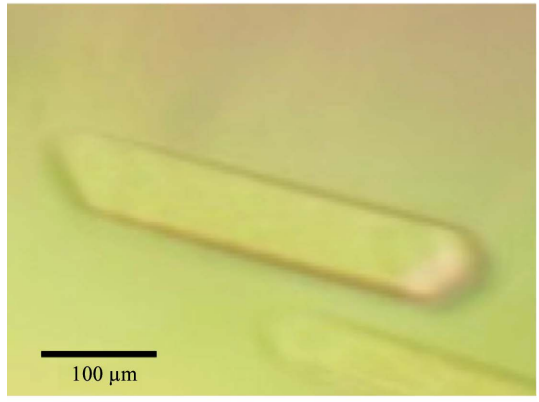
2.1. Expression and purification

As the N-terminal 402 residues of *S. cerevisiae* eIF5B are not required for its function, only the C-terminal 602 residues were expressed in *Escherichia coli* and are referred to here as eIF5BΔN. The N-terminal unstructured tail of *S. cerevisiae* eIF1A, which has 26 residues, was also deleted and the remainder is referred to as eIF1AΔN. The genes encoding eIF5BΔN and eIF1AΔN were individually subcloned with a hexahistidine tag (His_6) into the pET-28a vector. The resulting plasmid for eIF5BΔN was transformed into *E. coli* B834-CodonPlus(DE3)-RIL and the plasmid for eIF1AΔN was transformed into *E. coli* B834(DE3)-pRARE2. The eIF5BΔN transformant was cultivated in Luria–Bertani (LB) medium containing 25 µg ml^{-1} kanamycin and 34 µg ml^{-1} chloramphenicol at 310 K until the optical density at 600 nm (OD_{600}) reached 0.5–0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 m*M* to induce gene expression, which continued for 20 h at 298 K. The eIF1AΔN transformant was cultivated according to the same procedure as described for eIF5BΔN.

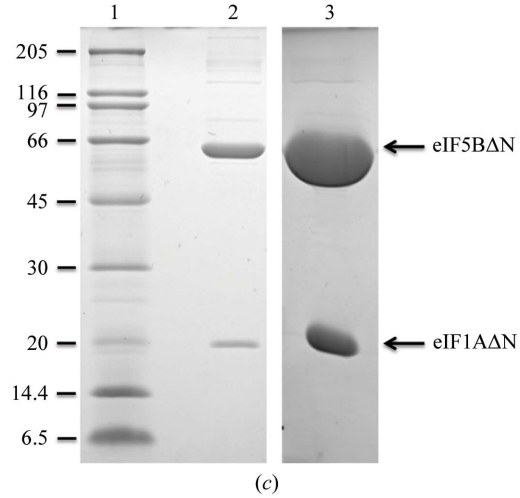
Recombinant eIF5BΔN and eIF1AΔN were purified separately according to similar procedures. The harvested recombinant cells expressing both proteins were disrupted by sonication and cell debris was removed by centrifugation at 40 000*g* for 1 h. The supernatant was subjected to 5 ml HisTrap HP (GE Healthcare) and eluted with a gradient of 0–400 m*M* imidazole in buffer *A* (25 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 10% glycerol and 5 m*M* β-mercaptoethanol). The eluted fractions of both proteins were treated with thrombin to cleave the His tag and were again applied onto the HisTrap HP column. eIF1AΔN was then further purified using a Superdex 75 pg 26/60 gel-filtration column (Amersham) equilibrated with buffer *A*; eIF5BΔN



(*a*)



(*b*)



(*c*)

Figure 1
(*a*) Crystals of eIF5BΔN obtained by the hanging-drop vapour-diffusion method at 293 K. (*b*) Crystal of the eIF5BΔN–eIF1AΔN complex obtained by the sitting-drop vapour-diffusion method at 293 K. (*c*) Coomassie Blue-stained 15% reducing SDS–PAGE gel confirming the formation of the eIF5BΔN–eIF1AΔN complex and the contents of dissolved crystals of the eIF5BΔN–eIF1AΔN complex. Lane 1, molecular-weight markers (kDa); lane 2, dissolved crystals; lane 3, eIF5BΔN–eIF1AΔN complex sample used for crystallization trials.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the last resolution shell.

	SeMet-eIF5BΔN		
	Data set 1	Data set 2	eIF5BΔN–SeMet-eIF1AΔN
Space group	$P4_12_12$	$P4_12_12$	$P2_12_12_1$
Radiation source	BL41, SPring-8	BL41, SPring-8	BL41, SPring-8
Unit-cell parameters (Å)	$a = b = 130.0$, $c = 71.7$	$a = b = 129.4$, $c = 70.3$	$a = 101.9$, $b = 120.9$, $c = 132.8$
Wavelength (Å)	0.979	1.000	0.980
No. of frames	180	360	269
Resolution range (Å)	50–2.6 (2.69–2.60)	50–2.35 (2.43–2.35)	50–3.3 (3.42–3.30)
No. of unique reflections	19466	24980	24828
$\langle I/\sigma(I)\rangle$	12.7 (2.3)	9.7 (2.1)	21.9 (2.0)
$R_{\rm merge}$† (%)	9.4 (46.7)	7.8 (64.5)	7.1 (49.2)
Multiplicity	5.9 (5.1)	4.7 (4.6)	9.4 (5.8)
Completeness (%)	99.8 (99.5)	98.3 (99.9)	98.5 (92.9)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

was further purified using a Superdex 200 pg 26/60 gel-filtration column (Amersham) equilibrated with buffer *A* and a Resource Q anion-exchange column (GE Healthcare) eluted with a gradient of 0–0.8 *M* NaCl in buffer *B* (25 m*M* Tris–HCl pH 7.5, 10% glycerol and 5 m*M* *β*-mercaptoethanol). The eIF5BΔN–eIF1AΔN complex was obtained in two steps. Firstly, eIF5BΔN and eIF1AΔN were mixed in a 1:1.3 ratio in 25 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl for 30 min at room temperature. The mixed sample was then purified using a Superdex 200 pg 16/60 gel-filtration column (Amersham) equilibrated with buffer *C* (25 m*M* Tris–HCl pH 7.5 and 0.5 m*M* DTT). The purified complex was concentrated to 42 mg ml^{-1} and used for crystallization.

Cells producing selenomethionine-labelled eIF1AΔN (SeMet-eIF1AΔN) or eIF5BΔN (SeMet-eIF1AΔN) were grown at 298 K in minimal medium containing selenomethionine and the proteins were purified using the same protocol as used for unlabelled eIF1AΔN or eIF5BΔN.

2.2. Crystallization

Initial crystallization conditions for native eIF5BΔN were obtained by sparse-matrix screening (Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, Index and PEG/Ion from Hampton Research and Wizard I and II from Emerald BioStructures) using the sitting-drop vapour-diffusion method. 1 µl 36 mg ml^{-1} protein solution (25 m*M* Tris–HCl pH 7.5, 5 m*M* *β*-mercaptoethanol) and 1 µl reservoir solution [0.2 *M* trisodium citrate, 20%(*w*/*v*) PEG 3350] were mixed together and equilibrated against 100 µl reservoir solution at 293 K. Small crystals were obtained after two weeks. Improved crystals were obtained by the seeding method. To prepare the seed stock, 1 µl of a drop containing small crystals was diluted with 50 µl reservoir solution and crushed using a homogenizer pestle in a microtube. 0.3 µl of a serial dilution of seed stock, 1 µl 36 mg ml^{-1} protein solution and 1 µl reservoir solution were then mixed and equilibrated against 500 µl reservoir solution using the hanging-drop vapour-diffusion method. After two rounds of seeding, crystals that diffracted to 3.1 Å resolution were obtained. Crystals of SeMet-eIF5BΔN were obtained using 100 m*M* HEPES pH 6.0, 0.2 *M* trisodium citrate and 19%(*w*/*v*) PEG 3350 and the diffraction limit was much better than that of the native protein (Fig. 1*a*).

Initial crystallization trials for the native eIF5BΔN–eIF1AΔN complex were carried out by the sitting-drop vapour-diffusion method in 96-well plates using commercial kits including JCSG Core Suites I–IV from Qiagen. 1 µl 42 mg ml^{-1} complex was mixed with an equal volume of reservoir solution and the protein–reservoir solution mixture was equilibrated against 75 µl reservoir solution at 293 K. Initial crystals of eIF5BΔN–eIF1AΔN were obtained using a condition consisting of 0.1 *M* Bicine pH 9.0 and 20%(*w*/*v*) PEG 6000 after two weeks. Further optimization gave larger (∼0.6 × 0.15 × 0.1 mm) but fragile crystals which diffracted to at best 3.6 Å resolution. When the eIF1AΔN in the complex was labelled with SeMet, further optimization gave crystals of better quality with smaller size (∼0.4 × 0.07 × 0.05 mm) using 100 m*M* Tris–HCl pH 8.2 and 12.5%(*w*/*v*) PEG 3350 as the reservoir solution (Fig. 1*b*). To confirm that the crystals contained the eIF5BΔN–eIF1AΔN complex, the crystals were washed with reservoir solution three times, dissolved in SDS–PAGE sample buffer and analyzed by SDS–PAGE. Fig. 1(*c*) shows that both proteins were detected on Coomassie Blue staining.

2.3. Data collection, processing and analysis

All diffraction data were collected at 100 K with a MAR CCD detector using synchrotron radiation on the BL41XU beamline at SPring-8. Prior to data collection, the crystals were cryoprotected by soaking them briefly in mother liquor supplemented with 20%(*v*/*v*) glycerol before flash-freezing them in a stream of nitrogen gas. Data sets 1 and 2 for SeMet-eIF5BΔN and the data set for the eIF5BΔN–SeMet-eIF1AΔN complex were collected with wavelengths of 0.979, 1.000 and 0.980 Å, respectively. The wavelengths for the collection of Se single-wavelength anomalous diffraction (Se-SAD) data were chosen based on the fluorescence spectrum of the Se *K* absorption edge (Rice *et al.*, 2000). All diffraction data were indexed, integrated and scaled using the *HKL*-2000 program package (Otwinowski & Minor, 1997) and the data-collection statistics are summarized in Table 1. The number of molecules in the asymmetric unit was analyzed using the Matthews coefficient (Matthews, 1968).

3. Results and discussion

As the nonfunctional N-terminus of eIF5B and the unstructured N-terminal tail of eIF1A may hamper crystallization, a C-terminal fragment of eIF5B (amino acids 403–1004) and a C-terminal fragment of eIF1A (amino acids 27–153) from *S. cerevisiae* were individually overexpressed in *E. coli* and purified. The eIF5BΔN–eIF1AΔN complex was reconstructed by mixing the two proteins and was further purified by gel filtration. The formation of the eIF5BΔN–

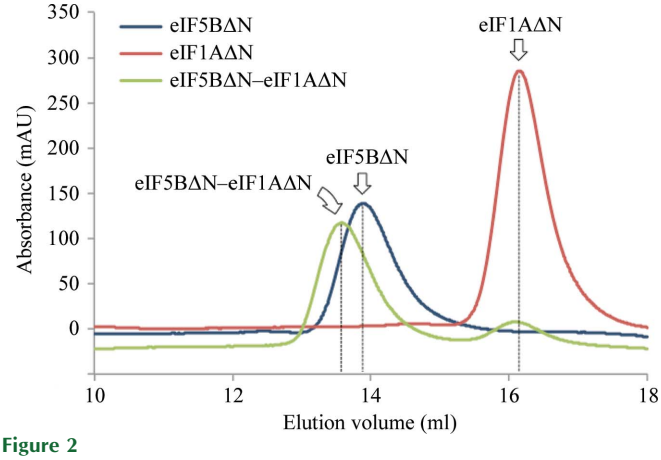


Figure 2
Size-exclusion chromatography of eIF5BΔN, eIF1AΔN and eIF5BΔN–eIF1AΔN. The elution volumes of eIF5BΔN, eIF1AΔN and the eIF5BΔN–eIF1AΔN complex are indicated by arrows.

eIF1AΔN complex was confirmed by the elution-volume shift on gel filtration and SDS–PAGE (Figs. 1*a* and 2).

The crystals of eIF5BΔN belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 130.0$, $c = 71.7$ Å. Calculation of the Matthews coefficient indicated that there is one molecule in the asymmetric unit, with a Matthews coefficient of 2.25 Å^3 Da^{-1} corresponding to a solvent content of 54.6%. Initial phasing was performed by the single-wavelength anomalous diffraction (SAD) method with the programs *SHELXD* (Sheldrick, 2010) and *SOLVE*/*RESOLVE* (Terwilliger & Berendzen, 1999; Terwilliger, 2000) using data set 1, which contains data to 2.6 Å resolution. The structure of eIF5BΔN has been built and is being manually refined using data set 2, which contains data to 2.45 Å resolution, with the programs *REFMAC*5 (Murshudov *et al.*, 2011), *PHENIX* (Adams *et al.*, 2010), *LAFIRE* (Yao *et al.*, 2006) and *Coot* (Emsley *et al.*, 2010). The X-ray diffraction data from the eIF5BΔN–eIF1AΔN complex crystal showed that it belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 101.9$, $b = 120.9$, $c = 132.8$ Å. Assuming the presence of two complexes in the asymmetric unit, the solvent content of the crystal is 50%, corresponding to a Matthews coefficient V_M of 2.45 Å^3 Da^{-1}. The data set was processed and scaled to a resolution of 3.3 Å.

Phasing was carried out by the molecular-replacement method using the program *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP*4 program suite (Winn *et al.*, 2011). As no solution was obtained using the full structure of *S. cerevisiae* eIF5BΔN as a search model, *S. cerevisiae* eIF5BΔN was divided into three parts, consisting of domains I–II, domain III and domain IV, which were used individually as search models. The correct solution was successfully obtained in this way.

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