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FOR THE RECORD

Cloning, expression, and crystallization of a hyperthermophilic protein that is homologous to the eukaryotic translation initiation factor, eIF5A

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Abstract: A gene coding for a protein homologous to a translation initiation factor of eukaryotes, eIF5A, was cloned from *Methanococcus jannaschii*, a hyperthermophile with an optimum growth temperature of 85 °C. The protein was overexpressed, purified and crystallized. The crystals were obtained by vapor diffusion method with 8% PEG 4000 as precipitant and belong to space group $P4_122$ with unit cell dimensions $a = b = 45.52$ Å and $c = 155.59$ Å. These crystals diffract to at least 2.2 Å resolution.

Keywords: hyperthermophile; *Methanococcus jannaschii*; translation initiation factor; X-ray crystallography

Eukaryotic translation initiation factor 5A, eIF5A, is a universal protein found in all eukaryotic cells (Park et al., 1993a). It is the only protein which is known to have the unusual amino acid residue hypusine [N^ϵ-(4-amino-2-hydroxybutyl)-L-lysine] (Cooper et al., 1984). The protein was initially isolated in the process of ribosome purification and has been considered as the enzyme involved in the formation of the first peptide bond (Kemper et al., 1976). However, it is known that the complete depletion of eIF5A does not cause major changes in the rate of protein biosynthesis *in vivo* (Kang & Hershey, 1994). Since the necessity for hypusine-modified eIF5A for cell proliferation is well established (Park et al., 1993b) and both protein and its hypusine residue are essential for cell viability (Schnier et al., 1991), the protein's exact role *in vivo* may extend beyond its role in translation initiation. It is also known that the inhibition of hypusine formation correlates with the G1-S transition of the cell cycle (Hanauske-Abel et al., 1994).

Recently, eIF5A was found to be the cofactor of Rev protein of human immunodeficiency virus type 1 (HIV-1) and of Rex protein of human T-cell leukemia virus type I (HTLV-I). Because Rev/Rex activity mediates the translocation of viral mRNAs from the nucleus to the cytoplasm, it seems possible that eIF5A might interact with a nuclear RNA export system (Ruhl et al., 1993; Katahira et al., 1995).

We have cloned the *Methanococcus jannaschii* gene, MJ eIF5A, encoding the homolog of eIF5A by polymerase chain reaction (PCR) amplification. The protein coded by MJ eIF5A has 31% amino acid sequence identity with human eIF5A and 49.6% with that from *Sulfolobus acidocaldarius*, another Archaea. The well-conserved sequences surrounding the lysine modification site strongly suggest a common ancestry between this archaeal protein and eukaryotic eIF5A. In the conserved region of 36 amino acids, one finds 58% identity between *M. jannaschii* and human eIF5A.

We have chosen a hyperthermophile as a source for the protein because the proteins from hyperthermophiles are usually very stable, easy to purify, and often crystallize easily. *M. jannaschii* belongs to Archaea with an optimum growth temperature near 85 °C. To understand the structural basis for the function of eIF5A and for the extreme stability of the MJ eIF5A protein, we have purified and crystallized the eIF5A homolog from *M. jannaschii* for the purpose of crystal structure determination.

Results and discussion: *Cloning:* The MJ eIF5A gene was amplified by PCR using *M. jannaschii* genomic DNA as template and two oligonucleotide primers (A and B). Upstream primer A (5′-A AGG ATC CAT ATG GTG ATA ATA ATG CCA GGA ACA AAA C-3′) contains an NdeI (underlined) restriction site at its 5′ end. The ATG within the NdeI site is utilized as the translation start site and adds an extra methionine to the N-terminus of MJ eIF5A. Downstream primer B (5′-AA GGA TCC CTC GAG TCA CTT TCC ACC AAT AAC TCT TG-3′) contains a BamHI (underlined) restriction site. The blunt-ended 335 bp PCR product was purified using a Qiaquick PCR Purification kit (Qiagen), ligated into the SmaI site of Bluescript SK+ (Stratagene) and transformed into *Escherichia coli* XL-1-Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′proAB lacI^{q}ZdM15 Tn10 (Tet^r)]] with selection on Xgal plates containing ampicillin (50 μg/mL). White colonies were picked and the MJ eIF5A gene was sequenced to confirm that no mutations were present. The Bluescript clone was

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then restricted with NdeI and BamHI and the gene was gel isolated and ligated into NdeI/BamHI-restricted pET21a vector (Novagen). The ligation mixture was transformed into BL21 (DE3) pSJS1240 (a gift from Dr. S. Sandler), a strain that carries a plasmid that expresses the rare *E. coli* tRNA codons for arginine (AGA) and isoleucine (ATA). Clones were checked for expression of the MJ eIF5A gene.

Bacterial expression and protein purification: The *E. coli* cells BL21 (DE3) pSJS1240 harboring pET21a/MJ eIF5A plasmid were grown at 37 °C in LB medium containing 50 μg/mL of ampicillin and 30μg/mL of spectinomycin. At an $OD_{600} = 0.9$, the MJ eIF5A protein was induced with 0.5 mM of IPTG for 2 h. The cells were harvested by centrifugation (5,000 × *g* for 6 min) and washed with buffer A (25 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM DTT, 1 mM EDTA). The cell pellet was resuspended in buffer A containing 1 mM PMSF and sonicated for 30 s three times. Broken cells were incubated at 80 °C for 30 min to precipitate heat unstable proteins from *E. coli*. The supernatant was collected after centrifugation for 20 min at 40,000 × *g* in a Beckman Ti60 rotor and applied onto a 5-mL HiTrap SP column (Pharmacia) previously equilibrated with buffer B (25 mM potassium phosphate, pH 7.0, 2 mM EDTA, 2 mM DTT). The column was washed with buffer B until OD_{280} was below 0.05. A 30-mL linear gradient of 0–0.5 M NaCl in buffer B was applied. The MJ eIF5A protein eluted at 0.3 M NaCl. Fractions were pooled and concentrated by ultrafiltration (Amicon, YM3) before loading onto a 1.5 cm × 90 cm S-200 HR gel filtration column (Pharmacia) equilibrated with buffer B containing 100 mM NaCl. The fractions containing the MJ eIF5A protein were pooled and concentrated by ultrafiltration (Amicon, YM3) and dialyzed against distilled water until the protein precipitated in the dialysis bag. Precipitated protein was collected by centrifugation and solubilized in buffer C (25 mM Hepes, pH 7.4, 2 mM EDTA, 2 mM DTT). The protein concentration measured by absorbance at 280 nm was calculated using an extinction coefficient of 0.26.

Selenomethionine substituted MJ eIF5A: The clone containing the structural gene of the MJ eIF5A homolog was transformed into *E.coli* mutant B834 [F^- ompT $hsdS_B(r_B^- m_B^-)$ gal dcm met (DE3)], a methionine auxotroph (Novagen). *E. coli* cells were grown at 37 °C in M9 minimal media containing 45 μg/mL selenomethionine, 50 μg/mL of ampicillin, and 30 μg/mL of spectinomycin. Selenomethionine containing MJ eIF5A was purified the same way as the wild-type MJ eIF5A. The difference in molecular weight between the substituted and wild type protein was checked by electrospray mass spectrometry, confirming that all six methionine sites, including the first methionine, were substituted by six selenomethionines.

Crystallization: The purity of the expressed MJ eIF5A protein was determined by SDS gel electrophoresis and electrospray mass spectrometry. Protein was prepared in buffer C at a concentration of 10 mg/mL. Crystallization conditions were tested using the sparse matrix sampling method (Jancarik & Kim, 1991) and membrane protein screen (Hampton Research) using the hanging drop vapor diffusion method with 2 μL drops (1 μL of protein + 1 μL of mother liquor) equilibrated against 500 μL of reservoir solution at 4 °C as well as room temperature. Tetragonal bipyramidal crystals were obtained in sodium acetate buffer (pH 4.6) and polyethylene glycol (PEG 350, PEG 4K, PEG 8K) conditions at room temperature. Crystals grew within a week to a size of 0.3 mm × 0.3 mm × 0.6 mm in 0.1 M sodium acetate buffer, pH 4.6, 8% PEG 4K.

Selenomethionine substituted protein did not crystallize in the same condition as wild-type protein. Initial crystallization conditions were searched using the sparse matrix sampling method at room temperature. Crystallization conditions were optimized by addition of 5 mM L-methionine and 5 mM $CaCl_2$ in the drops. Crystals in the shape of long needles grew within a week to a size of 0.15 mm × 0.15 mm × 1.0 mm in 0.1 M Tris-HCl, pH 8.5, and 8% PEG 8K.

Table 1. *Data collection statistics (the last resolution bin)*

Resolution	30.0–2.6 Å (2.69–2.60 Å)
Total observations	15,214
Tnique reflections	5,371
R_{sym}[a]	6.2% (28.3%)
Completeness	96.8% (83.0%)

[a] $R_{sym} = \Sigma(\langle I \rangle - I)/\Sigma I$.

Space group determination: Preliminary X-ray data were collected on a Rigaku R-Axis IIC imaging plate system, using Cu Kα radiation from a Rigaku RU200 B rotating anode operated at 40 kV and 100 mA with a MSC focusing mirror. Fully grown crystals were mounted in a glass capillary and data were collected at room temperature. Possible space group and unit cell parameters were predicted by DENZO data processing software (Otwinowski, 1993). The space group was determined to be $P4_122$ and the refined cell parameters are $a = b = 45.52$ Å and $c = 155.59$ Å. Assuming the molecular weight of 14,636 Da and one molecule in the asymmetric unit, a solvent content of 55% and a volume to mass ratio V_m = 2.76 Å^3/Da (Matthews, 1968) were calculated. Data were processed and integrated by DENZO and scaled by SCALEPACK (Otwinowski, 1993). Crystals diffracted to 2.2 Å at the beginning of the data collection but decayed quickly. Complete native data sets were collected to 2.6 Å with R_{sym} of 6.2% and data statistics are summarized in Table 1. The crystals from the selenomethionine substituted protein diffracted to 3.0 Å when crystals were frozen at 100 K and were indexed in a triclinic space group with cell dimensions of $a = 45.99$ Å, $b = 48.33$ Å, $c = 64.74$ Å, $\alpha = 103.0°$, $\beta = 99.9°$, and $\gamma = 111.6°$.

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