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Supporting Online Material for

Formation of the First Peptide Bond: The Structure of EF-P Bound to the 70*S* Ribosome

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Supporting Online Material

Materials and Methods

Construction of EF-P overexpression clone and purification of EF-P

The entire gene of *efp* was PCR amplified from genomic DNA of *Thermus thermophilus* (*T. th.*) (American Type Culture Collection, Manassas, VA) with primers that introduced upstream NdeI and downstream XhoI sites. An N-terminal His Tag was appended to the gene by site directed cloning into the *pET14b* vector (Novagen, Madison, WI). The pET14b-efp construct was transformed into *E. coli* BL21(AI) cells (Invitrogen, Grand Island, NY). Cells were grown in LB medium in the presence of 100 mg/L of ampicillin to an absorbance of 0.6 at 600 nm before inducing protein overexpression with 1 mM IPTG and 0.2 % (w/v) arabinose for four hours. The harvested cells were resuspended in lysis buffer (20 mM Hepes (pH 7.6), 10 mM $MgCl_2$, 50 mM KCl, 10 mM NH_4Cl), lysed by multiple passes through a microfluidizer (15 000 psi, Microfuidics, Newton, MA) and centrifuged to remove the cell debris (30 minutes at 25 000 rcf). After a 20-minute heat treatment (65ºC) the lysate was centrifuged (90 minutes 250 000 rcf) and loaded onto a HisTrap HP column (GE-Healthcare, Piscataway, NJ). EF-P was eluted from the HisTrap column by a linear gradient from 0 to 500 mM imidazole. The fractions containing EF-P were combined, concentrated and loaded onto a HiLoad 26/60 Superdex 75 column (GE-Healthcare, Piscataway, NJ) equilibrated in 20 mM Hepes (pH 7.6), 10 mM $MgCl_2$, 50 mM KCl, 10 mM NH_4Cl, 2 mM DTT). The purified EF-P fractions from the gel filtration column were pooled, and concentrated by ultrafiltration (Amicon-Ultra 15, 10 kDA MWCO, Millipore, Philadelphia, PA) to 20 mg/ml as determined by the Bradford assay (BioRad, Melville, NY) using BSA as a standard. EF-P was flash frozen in liquid nitrogen in small aliquots and stored at –80°C until further use in crystallization experiments.

mRNA and fMet-tRNA$_i^{fMet}$

A short piece of mRNA, Tth 32 MNF, was synthesized chemically by Integrated DNA Technologies (Coraliville, IA). The mRNA sequence was modified from (*S1*) and included a Shine Dalgarno sequence and an AUG start codon (5′ C AAG GAG GUA AAA AUG CAG UUC 3′). The fMet-tRNA$_i^{fMet}$ was prepared as described previously (*S2*).

Purification of *Thermus thermophilus* 70*S*

Thermus thermophilus 70*S* were prepared as described previously (*S1*). The final 70*S* pellets were resuspended in 5 mM Hepes-KOH (pH 7.6), 10 mM $MgCl_2$, 50 mM KCl, 10 mM NH_4Cl, 2 mM βME, at a concentration of approximately 500 A260 units/ml and stored in small aliquots at –80^{o}C until use in crystallization experiments.

Complex Formation

The EF-P ribosome complex was formed by programming 4 μM 70*S* ribosomes with 8 μM 32 Tth 32 MNF mRNA and 8 μM of fMet tRNA$_i^{Met}$ followed by an incubation with 20 μM EF-P. Each step was allowed to reach equilibrium by a 15- to 30-minute incubation at 55°C. A precipitate formed immediately after addition of EF-P to the 70*S* reaction mixture, which dissolved completely during the final incubation. The final ionic conditions contained 5 mM Hepes-KOH (pH 7.6), 10 mM $MgCl_2$, 50 mM KCl, and 10

mM NH_4Cl. The formed complex was stored at room temperature and was briefly centrifuged prior to use in crystallization experiments.

Crystallization

Initial crystalline needles were obtained by screening around previously published ribosome crystallization conditions (*S1*, *S3*–*S5*). Crystals were grown by in hanging drops, with 1–2 μL of the ribosome complex were mixed with 1–2 μL of reservoir solution (0.1–0.2 M Arg-HCl, 0.1 M Tris-HCl (pH 7.6), 2.5%[w/v] PEG 20K, 7–12%[v/v] MPD, 0.5 mM βME). Within 2–5 days the crystals reached typical dimensions of 50 x 100 x 1000 μm. After slowly increasing the concentration of MDP to a final concentration of 30%, the crystals were flash frozen in liquid nitrogen or liquid propane.

Data Collection and Refinement

Diffraction data were collected at the beamlines X29 at Brookhaven National Laboratory (Upton, NY) and 24ID at the Advanced Photon Source (Argonne, IL). A complete dataset was collected from seven separate regions of the same crystal. The raw data were processed and scaled with both the HKL (*S6*) and the XDS program package (*S7*). The crystal belongs to the orthorhombic space group $P2_12_12_1$ with unit cell dimensions of a = 210, b = 451, c = 622 Å and has two ribosomal complexes in the asymmetric unit cell.

The structure was solved by molecular replacement using the program Phaser from the CCP4 program suite (*S8*) with the small and the large ribosomal subunit from the previously published structure of *T. th.* 70*S* ribosome with bound mRNA and tRNA (*S1*). Both the mRNA and the tRNA were excluded from the search model. The molecular replacement solution was refined by rigid body refinement using multiple domains for each subunit (*S9*). Clear density for the fMet-$tRNA_i^{Met}$, EF-P, and mRNA was visible in the $F_{obs} - F_{calc}$ difference map calculated after the initial round of refinement. The structure was further refined in Phenix to generate the $F_{obs} - F_{calc}$ difference map used for building (*S10*).

Structure Building

The initial model of EF-P was from derived the published crystal structure of EF-P from *Thermus thermophilus* [(*S11*) PDB ID: 1ueb]. All three beta barrel domains of EF-P were clearly visible in the initial $F_{obs} - F_{calc}$ difference map, with the exception of a loop at the end of domain III (residues 140–146). The overall shape of EF-P bound to the 70*S* ribosome is quite similar to the *apo* crystal structure (*S11*). Superposition of the N-terminal domain of the *apo* with the ribosome bound EF-P structure (rmsd of 0.81 Å for 66 c-alpha atoms) revealed that domains two and three rotate 10 degrees away from domain I upon binding.

The initial coordinates for the ribosomal protein L1 were derived from the published crystal structure of *T. th.* L1 [(*S12*) PDB ID: 1ad2] and manually fit into the initial $F_{obs} - F_{calc}$ difference map. The majority of the rRNA for the L1 stalk had to be rebuilt, because of large conformational changes. The initial model of the initiator tRNA was derived from the previously published structure of the *T. th.* 70*S* ribosome with bound mRNA and tRNA (*S1*). Only the AUG start codon and two upstream bases were built and refined, because the rest of the mRNA was disordered. The final model was generated by multiple rounds of model building in Coot (*S13*), followed by refinement with CNS (*S14*, *S15*) and PHENIX (*S10*).

Table S1: Data Collection and Refinement Statistics

Data Collection	
Space group	$P2_12_12_1$
Cell dimensions	209.5 x 447.0 x 622.8 (Å)
Resolution (Å)*	50–3.3 (3.4–3.3)
$I/\sigma(I)$	4.7 (1.2)†
R_{sym} (%)	23.6 (97.1)‡
No. of unique reflections	989777
Completeness (%)	96 (75.8)
Redundancy	4.4 (2.8)
Refinement§	
Resolution	50–3.3 (3.4–3.3)
R_{work}/R_{free} (%)	25.7/30.3 (32.7/35.6)
No. of atoms	289467
RMS deviations	
Bond lengths	0.009
Bond angles	1.901

*Parentheses indicate statistics from the outer shell.
†$I/\sigma(I) = 2$ at 3.5 Å.
‡The value for R_{sym} is higher than normal, most likely because the data were collected with a small degree of oscillation from multiple wedges that were scaled together, and is in the range for R_{sym} values reported for other 70*S* structures.
§ Refinement was carried out in PHENIX and CNS.

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