Materials and Methods

Ribosome preparation

E.coli MRE600 cultures were grown at 37°C until OD_{600}= 0.5. The cells were cooled down on ice before pelleting them at 5,000 rpm for 10 minutes in a Sorvall-H6000 rotor at 4°C. The cell pellets were once washed in buffer A (20 mM Tris-HCl – pH 7.5, 200 mM NH_4Cl, 20 mM $MgCl_2$, 5 mM 2 – Mercaptoethanol, 0.5 mM EDTA) and pelleted again at the same speed for the same time. The pellets were resuspended in 25 ml of buffer A containing ~40 µg PMSF and 10 Units of RNase free DNase (Promega) per 1 liter of cell culture before lyses. The lysate was centrifugated twice at 17,000 rpm for 30 minutes to get rid of the cell debris. The clear supernatant was layered on a sucrose cushion in buffer B (20 mM Tris-HCl - pH 7.5, 500 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM 2-Mercaptoethanol, 0.5 mM EDTA, 1.1 M Sucrose), followed by a centrifugation step at 36,000 rpm in a Ti45 rotor for 18 hours. The crude 70S pellet was resuspended in buffer C (20 mM Tris-HCl - pH7.5, 60 mM NH_4Cl, 6 mM $MgCl_2$, 5 mM 2-Mercaptoethanol, 0.5 mM EDTA) at 4°C while slightly shaking. 15% - 40% (w / w %, 15% = 0.464 M, 40% = 1.375 M) sucrose gradients in buffer C were prepared onto which 10 - 12 mg/ml of ribosomes (OD_{260} with 320-240 nm Spectrum ([70S] = 57 µg / ml) were loaded. The gradients were centrifuged at 28,000 rpm in a SW28 rotor for 15 hours. The 70S fractions were collected using a gradient collector before being washed and concentrated in an Amicon,

Millipore, (pressure concentration) with buffer C using a Ultrafiltration membrane (Millipore, MWL: 30,000 Dalton, Dia: 44.5 mm, cellulose).

Ribosomes for crystallization trials were stored at 4°C whereas the ones for biochemical assays were frozen at -80°C.

Protein Y purification

The gene yfiA encoding Protein Y was cloned into the pET15b vector using the XhoI and NdeI sites, resulting in a His-tag at the C-terminus. The plasmid was transformed into Bl21-pLysS cells. Cells were grown to OD_{600}= 0.8 before they were induced with 0.4 mM IPTG (final concentration) for 2 more hours. Cells were pelleted at 5,000 rpm for 10 minutes in a Sorvall-H6000 rotor at 4°C before lysis in lysis-buffer (20 mM Tris-HCl - pH 7.5, 500 mM NH_4Cl, 5 mM Imidazol) containing 40 µg of PMSF per 1 liter of cell culture. The lysate was centrifuged for 30 minutes at 30,000 rpm in a Ti45 rotor in order to remove the cell debris. A Nickel - chelate column was equilibrated in lysis - buffer before the addition of the supernatant. The column was washed with 5 - 6 column volumes using wash-buffer-I (20 mM Tris-HCl - pH 7.5, 500 mM NH_4Cl,10 mM Imidazol), followed by 5 - 6 steps of washing with wash-buffer-II (wash buffer I + 1M NaCl). The elution of the Protein was performed using 5 - 6 volumes of elution buffer (20 mM Tris-HCl, pH 7.5, 500 mM NH_4Cl, 300 mM Imidazol). Protein Y was dialysed into its storage buffer (20 mM Tris-HCl - pH 7.5, 60 mM NH_4Cl, 6 mM $MgCl_2$, 5 mM 2 - Mercaptoethanol, 0.1 mM EDTA, 15% glycerol) over night at 4°C, and stored at 4°C for crystallization trials and at -80°C for biochemical experiments.

Initiation factor purification

The plasmids of the His-tagged Initiation Factor -1, -2, and -3 were a kind gift from Dr. Daniel N Wilson from Dr. K.H. Nierhaus' laboratory. Their genes were all cloned into pET14b plasmids.

The proteins were purified following the purification protocol for Protein Y with the following changes. Cells were induced at OD_{600} = 0.6 with 1mM IPTG (final concentration) for 4 hours. The following buffer systems were used: lysis-buffer (50 mM Tris-HCl – pH 8.0, 100 mM NaCl, 1 mM EDTA, 10 mM 2-Mercaptoethanol), wash-buffer-I (10mM Tris-HCl – pH 8.0, 5mM 2-Mercaptoethanol, 5% glycerol), wash-buffer-II (10 mM Tris-HCl – pH 8.0, 5 mM 2-Mercaptoethanol, 5% glycerol, 1 M NaCl), elution buffer (10 mM Tris-HCl – pH 8.0, 5 mM 2-Mercaptoethanol, 5% glycerol, 200 mM NaCl, 250 mM imidazol), storage buffer (20 mM Tris-HCl – pH 8.0, 1 mM DTT, 10% glycerol, 0.2 mM EDTA). The proteins were stored at -80°C.

tRNA purifications

a) $tRNA^{Phe}$

$tRNA^{Phe}$ was purified by T7-transcription using a plasmid (courtesy of K.H.Nierhaus) bearing a T7 promotor region upstream of the gene and a FOK I restriction site downstream. After amplification of the plasmid by using a Qiagen mega-prep kit, a restriction digest of the plasmid using 0.2 ml of FOKI (per 1 mg of plasmid at 37°C for 2-3 hours followed. The enzyme was removed by Phenol / Chloroform Extraction and the linear DNA Ethanol precipitated at -80°C for 1

hour. The T7 transcription was performed in the reaction-buffer containing 30 mM Tris-HCl - pH8.1, 1 mM DTT, 0.1 % Triton X - 100, 2 mM Spermidine using 0.09 mg of T7 polymerase (kind gift of Kai-Hong in Dr. Jennifer Doudna's laboratory) per 0.3 mg of DNA for 5 hours at 37°C. The reaction was incubated with RNAse free DNAse (Promega, 1U per 1 µg of plasmid DNA) for 30 minutes on ice before gel purification on a denaturing 8M UREA gel. The yield was 2 mg of $tRNA^{Phe}$ out of 2 mg DNA.

b) bulk tRNA enriched in $tRNA^{fMet}$

The pUC13-plasmid encoding $tRNA^{fMet}$ was a kind gift from UL RajBhandary. The tRNA was prepared in the following way:

Cells were grown for 5 hours in 2 x YT medium containing 200 µg / ml ampicillin before pelleting them for 15 minutes at 5000 rpm in a Sorvall-H6000 rotor at 4°C. The cell pellet was resuspended in cold (4°C) buffer containing 10 mM Tris – HCl - pH7.5 and 10 mM EDTA. 1/3 volume of phenol (pH 7.5) were added. The nucleic acid was extracted by mixing the contents on a shaker for 15 minutes at room temperature. The mixture was centrifuged for 15 minutes at 7,000 rpm at room temperature in a Sorval GSA rotor in order to separate the phases.

1/10 volume of 3 M sodium acetate (pH 5.4) together with 2.5 volumes of Ethanol were added to the aqueous (upper) phase. The nucleic acid was precipitated at -20°C over night and spun down at 7,000 rpm for 15 minutes at 4°C in a Sorval GSA rotor. The pellet was washed with 70 % ice-cold Ethanol before resuspending it in buffer I (100 mM Tris-HCl - pH7.5, 100 mM LiCl, 1 mM EDTA).

The nucleic acid solution was loaded on 10ml of DEAE cellulose (Whatman DE - 52, pre-swollen anion exchanger) that was pre-equilibrated in buffer I. All operations were done in a sterile 50ml filter-tube (Scienceproducts.corning.com, 0.22 µm Cellulose acetate) under vacuum at room temperature. After loading the nucleic acid solution vacuum was applied carefully so that the "column" would not dry out. The "column" was washed 5 times with 10 ml of buffer I before eluting the tRNA with buffer II (100 mM Tris-HCl - pH7.5, 1 M LiCl, 1 mM EDTA) in three 10ml fractions. Vacuum was applied after each single washing and elution step. Each fraction was run on a 1% agarose gel for 45 minutes at 100V in order to figure out which fractions contained bulk tRNA. The fractions were combined and added to 2.5 volumes of Ethanol and 1/10 volume of 3 M sodium acetate (pH 5.4) before incubating the mixture at -20°C over night.

The tRNA was recovered by centrifugation at 7,000 rpm for 30 minutes in a Sorvall-H6000 rotor at 4°C.The pellet was washed with 70 % EtOH before resuspending it in 10 mM Tris – HCl - pH8.0 and 1 mM EDTA. The yield is about 500 A_{260} of bulk tRNA of which 20% contain initiator tRNA $tRNA_2^{fMet}$ (Mandal and RajBhandary 1992).

5' end labeling of RNA (mRNA, tRNA)

12000 pmol of RNA in a total volume of 50 µl ddH_2O were incubated for 5 minutes at 75°C before the addition of 40 µl CIP (calf intestinal alkaline phosphatase (CIP), New England Biolabs, 1 U / µl) and 10 µl of 10 x CIP buffer that will remove the 5' phosphate group of the RNA during an incubation at 50°C

for 45 minutes. The reaction is phenol/chloroform extracted 2 times before ethanol precipitation at -20°C over night. The RNA will be spun down at 4°C for 30 minutes at 15,000 rpm and resuspended in about 50 µl of ddH_2O. 18 µM of ^{33}P labeled ATP (Amersham, 10µCi/µl), 0.5 µl of T4-polynucleotide-kinase (New England Biolabs), 9 µl of T4-polynucleotide kinase reaction-buffer (10 x) will be added to a final volume of 100 µl before incubating the mixture at 37°C for 20 minutes. 4 µl of 33 mM ATP, 1 µl of 10 x reaction-buffer, 1 µl T4-polynucelotide kinase and 4 µl of ddH_2O are added to the reaction followed by an incubation step of 45 minutes at 37°C.

The reaction is purified over a G25-micro spin column (Amersham Bioscience, apply 25-50 µl reaction) before ethanol precipitating it at 20°C over night.

Charging assays for bulk tRNA enriched in $tRNA^{fMet}$

Bulk tRNA enriched in $tRNA_2^{fMet}$ (Mandal and RajBhandary 1992) prepared using the plasmid for $tRNA^{fMet}$ (described above) was used to charge $tRNA_2^{fMet}$ with ^{35}S - methionine. 105 µl of ^{35}S-methionine (Amersham Biosciences, 10 µCi / µl) diluted with cold methionine to a final concentration of 1 mM (specific activity 351.9 cpm / pmol), 30 µl ATP [200mM], 150 µl folic acid, 150 µl methinonyl-tRNA-synthetase (MetRS) [5.7 µg / µl], 300 µl methionyl-tRNA-formyltransferase (MTF) [0.6 µg / µl] (Ramesh, Gite et al. 1997) were used in a final reaction volume of 1.5 ml to charge 300 µl of bulk tRNA (enriched in $tRNA^{fMet}$) in the following final buffer concentrations 20 mM imidazol pH7.5, 0.1 mM EDTA, 7 mM $MgCl_2$, 150 mM NH_4Cl, 1 ug / ml BSA. The reaction was

incubated at 37°C for 1 hour before phenol/chloroform extracting and ethanol precipitating it adding 1/10 volume 3 M NaOAc – pH 5.4. The tRNA pellet was resuspended in 600 µl 5 mM NaOAc – pH 5.4 and the tRNA concentration determined by measuring OD_{260} (1 OD_{260} = 40 µg / ml tRNA). To make sure that the tRNA was charged a sample was loaded on the HPLC (Shimadzu SCl-1CA). Samples were loaded onto a HPLC column packed with reverse phase matrix C4-Butyl (Vydac, 300Å, 4.6 mm ID x 25 mm) equilibrated with Buffer I (1 M NaCl, 20 mM NH_4Cl, 10 mM $MgCl_2$). A gradient of Buffer I and 5-55% Buffer II (Buffer I + 30% MeOH) was run at a speed of 1 ml / min for 35 minutes. Charging of the tRNA resulted in a shift about 5 minutes later in the gradient compared to the uncharged tRNA.

Crystallization of 70S ribosomes / ProteinY

Crystals of 70S ribosomes were grown using the vapor diffusion technique at 4°C. 4-6 mM 70S ribosomes were mixed on a silanized cover slip with the well solution in a ratio 1:1 or 1: 1.5. The well solution contained 22 – 23% 2-methyl-2, 4 pentanediol (MPD), 80 mM 2-Morpholinoethanesulfonic acid (MES) – KOH – pH 6.0, 20 mM Tris-HCl – pH 7.5, 25-28 mM $MgCl_2$, 290-330 mM NH_4Cl, 0.98mM Spermine, 0.5 mM Spermidine. Crystal trays were set up at room temperature and stored in a Styrofoam box at room temperature for 24 hours before transferring them to 4°C. Crystals started to appear after about 2 weeks and were grown to a suitable size (~ 1µm) before mounting. The crystals were stabilized before cryo – cooling them in liquid nitrogen in 13.3 mM Tris-HCl - pH

7.5, 106.7 mM MES – KOH – pH 6,

34.5 mM $MgCl_2$, 175 mM NH_4Cl, 5 % Sucrose, 22.5 % MPD containing 23.2 µM his-tagged ProteinY (stored at 4°C in the following storage buffer: 20mM Tris-HCl pH 7.5, 60 mM NH_4Cl, 6 mM $MgCl_2$, 5 mM 2-Mercaptoethanol, 0.1 mM EDTA) over night. Diffraction data was measured at beamlines 8.3.1 and 8.2.2 at the Advanced Light Source.

Antón Vila-Sanjurjo solved the structure of the unit cell containing five 50S molecules per asymmetric unit in the following way:

Since the unit cell of the new crystal form is related to the previous unit cell by a five-fold increase in the length of the c axis, the structure was solved by molecular replacement in which five ribosomes from the small unit cell crystal packing (Vila-Sanjurjo, Ridgeway et al. 2003) were used as the search model. The initial molecular replacement model was then refined against structure factor amplitudes measured from crystals of the apo-ribosome using rigid body refinement in CNS (Brünger, Adams et al. 1998). An F_{obs}-F_{obs} difference electron density map, calculated using observed structure factor amplitudes and structure factor phases from the molecular replacement model (**Table 1**), was improved by performing real space averaging with RAVE (Jones, Zou et al. 1991). A truncated model of PY (PDB accession code 14LS (Ye, Serganov et al. 2002)) lacking the flexible C terminal tail was docked into the averaged density both manually and with ESSENS (Kleywegt and Read 1997) with an almost identical outcome. Transfer RNAs and mRNA from the 5.5 Å structure of the 70S ribosome from

Thermus thermophilus (accession code 1GIX (Yusupov, Yusupova et al. 2001)), and IF1 complexed with the 30S subunit (accession code 1HR0 (Carter, Clemons et al. 2001)), were superimposed with the 70S ribosome-PY complex structural model using O (Jones, Zou et al. 1991). Figures were created with PYMOL/NUCCYL (Delano 2002) and RNAVIEW (Yang, Jossinet et al. 2003).

Chemical probing (exclusively performed by Antón Vila-Sanjurjo)

1 µM 70S ribosomes were incubated with 4 µM PY or the same volume of PY storage buffer (20 mM Tris-HCl, pH =7.5, 6 mM $MgCl_2$, 60 mM, NH_4Cl, 4 mM 2-Mercaptoethanol, 0.5 mM EDTA) for 15 minutes at 37°C, followed by a second incubation in the absence (lanes 1-3) or presence (lanes 4 and 5) of 30 µg/mL poly-U mRNA and 7 µM $tRNA^{Phe}$ in 100 µl of probing buffer (25 mM $MgCl_2$, 125 mM NH_4Cl, 80 mM Hepes – KOH pH 7.2) for 15 minutes at 37°C.
Chemical probing and gel electrophoresis were performed as described (Vila-Sanjurjo, Ridgeway et al. 2003).

Filter binding for P-site inhibition experiments

Reactions with mRNA containing a Shine-Dalgarno sequence (5'-GUUAACUUUAGU-AGGAGGUAUCAUAUGUUCAAAAAAAAA-3') were carried out in buffer A (50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, and 1 mM DTT). 1 µM of 70S ribosomes were first incubated with or without 3 µM of PY for 15 minutes. 3 µM of $tRNA^{fMet}$ (Subriden) and mRNA

containing the Shine-Dalgarno sequence in the ribosome binding site were added to a final reaction volume of 40 µl and incubated for an additional 15 minutes. After quenching with 110 µl of reaction-buffer, reactions were applied to nitrocellulose filters (Millipore 0.45 µM HA) and washed twice with 1 ml of reaction-buffer. Reactions were carried out at either 16°C or 37°C. Reactions with leaderless mRNA (5'-pAUGUUC-AAACGUAAUAAU-3') were performed in buffer containing 20 mM Tris-HCl pH 7.5, 60 mM NH_4Cl, 24 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. 1.9 µM 70S ribosomes were incubated with or without 5.7 µM PY at 37°C for 15 minutes. The resulting mixtures were then diluted to a final ribosome concentration of 0.25 µM in 10 µl reactions containing 1.5 µM ^{33}P-labeled leaderless mRNA and 1.5 µM deacylated $tRNA^{fMet}$ (Subriden) in the same buffer and incubated for a further 15 minutes. Reactions were quenched with 300 µl of reaction-buffer, applied to nitrocellulose filters as above, and washed twice with 1 ml of reaction-buffer.

Filter binding for initiation assays

Bulk tRNA enriched in $tRNA^{fMet}$ (15% of the total tRNA) was used to label the initiator tRNA with ^{35}S-Methionine as described above and in (Jelenc and Kurland 1979). For the reactions carried out in the presence of polyamines polymix buffer (5 mM K-phosphate, pH=7.3, 5 mM $Mg(CH_3COO)_2$, 5 mM NH_4Cl, 95 mM KCl, 0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, and 1 mM DTT (dithioerythritol) (Jelenc and Kurland 1979)) was used. In the absence of polyamines a buffer system introduced by Wintermeyer and collegues was used

(Semenkov, Rodnina et al. 2000) containing 50 mM Tris-HCl-pH7.5, 70mM NH_4Cl, 30 mM KCl, 7mM $MgCl_2$, 1 mM DTT. All reactions were carried out in the same following way: 1.0 µM 70S ribosomes were first incubated with 4 µM PY for 15 minutes in reaction-buffer, followed by the addition of 1.5 µM each of IF1, IF2 and IF3, 3 µM mRNA, 1 mM GTP and 14 µg of ^{35}S-Methionine labeled bulk tRNA in a final reaction volume of 40 µl. After a second incubation of 30 minutes, reactions were quenched with 110 µl of reaction-buffer and filtered as described above. Reactions were carried out at either 16°C or 37°C.

Separation of ribosomes and ribosomal subunits

1 µM 70S ribosomes were first incubated with 3 µM IF3 for 15 minutes at 37ºC in buffer containing polyamines (20 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 60 mM NH_4Cl, 4 mM 2-mercaptoethanol, 0.5 mM EDTA, 2 mM spermidine, and 0.05 mM spermine) or lacking polyamines (50 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, and 1 mM DTT). This was followed by a second incubation with 4 µM PY for 15 minutes at 37ºC in a final volume of 75 µl. Reactions were layered on 15%-40% (w/w %) sucrose gradients containing reaction-buffer and run for 3 hours at 40,000 rpm in a Beckmann SW-41 rotor. Experiments with IF3 and IF1 were carried out as above, with the first incubation step containing 3 µM each of IF3 and IF1. The ribosomal subunit composition of each peak in the sucrose gradient was verified by agarose gel electrophoresis (not shown).