Material and Methods

Ribosome preparation

Ribosomes were prepared in the same way as for the 70S/PY crystals (page 50) but with the following changes.

After the centrifugation step at 36,000 rpm in a Ti45 rotor for 18 hours the pellet, containing crude ribosomes was resuspended in Dissociation buffer (20mM Tris-HCl - pH7.5, 200 mM NH_4Cl, 1 mM $MgCl_2$, 2mM DTT, 2mM THP (Tris(hydroxypropyl)phosphine, Calbiochem) resulting in the dissociation of 70S ribosomes into the 30S and 50S subunit. In order to remove protein S1 (30S subunit) 20 mg of Poly-U mRNA (Amersham Bioscience) per 1.5 mg of ribosomes (OD_{260} with 320-240 nm Spectrum ([70S] = 57 µg / ml) were added and incubated for 15-120 minutes on ice before loading the subunits on 15% - 40% sucrose gradients in Dissociation buffer (prepared by making up a solution of 25 % sucrose, w/v, in dissociating buffer that was frozen in 50ml SW28 tubes at -80°C at least for 2 hours and thawed at room temperature. This results in the required gradient). 10 -16 mg of subunits were loaded on each gradient in a volume of 1ml before centrifugation at 20,000 rpm for 20 hours at 4°C in a SW28 rotor. The 30S and 50S subunit were collected separately or together and dialyzed at 4°C into Re-association buffer (20mM Tris-HCl - pH7.5, 60 mM NH_4Cl, 20 mM $MgCl_2$, 2mM DTT, 2mM THP) under pressure using an Amicon, (Ultrafiltration membrane, Millipore, MWL: 30,000 Dalton, Dia: 44.5 mm, cellulose). The re-associated 70S (70S-R) were incubated at 40°C for 45 - 60

minutes to help the re-association step to occur before loading them on 15% - 40% sucrose gradients (10-12 of 70S-R/gradient). The gradients were centrifuged at 28,000 rpm in a SW28 rotor for 15 hours before collecting them using a gradient collector. The 70S-R were dialyzed into Buffer C (20mM Tris-HCl - pH7.5, 60 mM NH_4Cl, 20 mM $MgCl_2$, 2mM DTT, 2mM THP) and concentrated in an Amicon (Millipore, MWL: 30,000 Dalton, Dia: 44.5 mm, cellulose) at 4°C.

Crystallization of *E. coli* 70S ribosome

Initially the first crystals of 70S-R ribosomes were grown using the vapor diffusion technique at 4°C. 4 - 6 mM 70S ribosomes (in Buffer C) were mixed on a silanized cover slip with the well solution in a ratio 1:1. The well solution contained 1 – 4% Plyethylenglycol (PEG) - 8000, 90 – 95 mM 2-Morpholinoethanesulfonic acid (MES) – KOH – pH 6.0, 20 mM $MgCl_2$, 220 mM NH_4Cl, 1 mM Spermine and 1.5mM 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. This approach resulted in showers of needle crystals that started to appear after about 3 weeks.

The conditions used in the vapor diffusion technique were improved using the batch crystallization technique. Using a 96 well microbatch-crystallization plate plate (Hampton Research, 30 µl volume, dimensions: 12.4 cm x 8.2 cm) 1,6 – 2,4 µM of 70S-R (in buffer C) were set up in a 1:1 ratio with the well solutions containing 350 - 550 mM NH_4Cl, 5 - 12% PEG – 8000, 30 – 50 mM $MgCl_2$, 180

mM MES - KOH – pH 6.0, 2 mM Spermine, 3 mM Spermidine (spread out into 96 different conditions). The trays were set up at room temperature, incubated on ice for 10 minutes before a temperature gradient was applied going from 4°C - 25°C - 4°C within 24 hours in a Hampton M6 - Incubator. Crystals grown to a suitable size (~1 µm) started to appear after 3 - 6 weeks.

The crystals were stabilized before cryo – cooling them in liquid nitrogen in 20% MPD, 20 mM Hepes - pH 7.5, 28 mM MgCl2, 350 mM NH4Cl, 0.98 mM Spermine, 0.5 mM Spermidine, 3% PEG 8000 and 24.1% PEG 400. The stabilizer was added step – wise to the well in the following way: A 2:1 mixture of well solution to stabilizer was added before changing to a 1:1 mixture after 12 hours. The crystals were incubated with the 1:1 mixture for another 12 hours before changing to 100% stabilizer in which they were again incubated for about 12 hours before mounting.

Diffraction data was measured at beamlines 8.3.1 and 12.3.1 at the Advanced Light Source at Lawrence Berkeley National Laboratory.

Soaking experiments

Kasugamycin (ksg) as well as the Ribosome Recycling Factor (RRF) were soaked into pre-stabilized crystals over night. The ligands were added to the final (the 3.) stabilizing step (as explained above).

In the case of *E .coli* as well as *T. thermophilus,* RRF was soaked into the crystals using a three-fold access ([4.8 – 7.2 µM] final concentration) compared to the ribosome.

In the case of kasugamycin, a final concentration of 1 mg / ml was used.