Supplementary Information:

All data was acquired using a Tecnai F20 Twin transmission electron microscope operating at 120 kV, using a dose of ~10 e-/Å² and a nominal underfocus ranging from 1.0 to 2.5 µm, the exception being the tomographic tilt series. For each dataset the nominal magnification was 50,000X with a pixel size of 0.163 nm at the specimen level. All images were recorded with a Tietz F415 4k x 4k pixel CCD camera (15 µm pixel) utilizing the Leginon data collection software (Suloway et al., 2005). The details of each processing run are summarized below:

Common Lines:

350 images were collected of *E. coli* 50S subunits preserved in vitreous ice (Dataset 3). Ribosomal particles were picked with DogPicker (Voss et al., 2009) using a diameter of 290 Å. CTF parameters were estimated using ACE2 (a variation of the algorithm in (Mallick et al., 2005)), and a stack of 31,825 particles was created using from the CTF-corrected dataset. The stack was subjected to Xmipp maximum likelihood alignment (Scheres et al., 2005a; Scheres et al., 2005b) to create 15 initial 2-D references. This was followed by a single iteration of IMAGIC multi-reference alignment (van Heel et al., 1996) using the generated references to obtain a more precise fit. SPIDER correspondence analysis (Frank and van Heel, 1982) in combination with hierarchical ascendance classification was used to create 200 class averages. From these, 108 were discarded based on a manual inspection focused on eliminating circular averages. The resulting 92 class averages were used for construction of a common lines model in EMAN and a 3d0 model in IMAGIC (Figure 8), as implemented within Appion. The latter was further subjected to 5 iterations of angular reconstitution batch refinement. For this step, the number of class averages used for Euler angle assignment was incrementally increased to 400, the 1-D angular sinogram sampling was decreased to 1°, and the 3-D projection angle for references comprising the anchor set was decreased to 5°. The rest of the parameters were set to their default values within Appion. The final model achieved a resolution of 16.2 Å by $FSC_{0.5}$.

Tomography:

A tilt series of 117 images of negatively stained *E. coli* 50s subunits was collected using Leginon (Suloway et al., 2009) at an angular increment of 1 degree ranging from -59° to +56° (Dataset 2). The dose per image varied from 1.3 to 2.6 e-/Å², depending on the tilt angle. The nominal defocus was kept at 2.5 um throughout the series. From these images a 600 x 600 nm tomogram was aligned using PROTOMO (Winkler and Taylor, 2006). The transformation parameters were converted to the convention used in IMOD's global alignment and then reconstructed using the back-projection algorithm in IMOD (Kremer et al., 1996). The full-sized tomogram was uploaded into Appion and all the subsequent processing were performed within its framework.

First, the tomogram was projected along the z-axis to obtain a 2-D image for particle picking and alignment in the xy plane. Particles were picked by DogPicker (Voss et al., 2009) using a diameter of 320 Å. Maximum likelihood 2-D alignment (Scheres et al., 2005a; Scheres et al., 2005b) was applied to the 133 particles. One of the two references found by the algorithm showed a well-defined ribosomal shape, with the second being a circular average. We used the better of the two to perform a successful reference-based alignment as implemented in SPIDER (command AP MQ) (Frank et al., 1996). Kernel probability density estimator self-organization map as implemented in XMIPP (Pascual-Montano et al., 2001) was used to classify the aligned images into a 4 by 5 rectangular topology. Three of the classes showed well-defined features and had over 25 particles in each class. We combined the three classes with a total of 93 particles into a new particle stack as a link to further 3-D processing of the tomogram.

The particle xy coordinates on the projected 2-D image were used to extract the sub-tomograms from the full tomogram. Centering the particles in the z-dimension was not straightforward because side-views were noisy and the effect of missing wedge was pronounced. We used a simple approach to determine the location of the particle along the z-axis from the central slab of the sub-tomogram density above its background. The central slab was defined as a rectangular tube of the volume enclosing the center part of the particle. This volume was further compressed down to a 1-D profile along the z-axis, which was fitted with a Gaussian function. Unfortunately, slight misalignment created a negative interference pattern adjacent to the particles in the z-direction that often distorts the fitting of the positive particle density. In order to curtain the artifacts, a threshold was applied to the density profile before the Gaussian fitting, as determined by the average density at the edges of the sub-tomogram where there were neither particles nor artifacts. The centered sub-tomograms were subsequently transformed according to their in-plane alignment and averaged into a single volume.

RCT:

119 image pairs of negatively stained *E. coli* 50S subunits were collected using Leginon RCT (Yoshioka et al., 2007) (Dataset 1). Initially, a template was created from an Xmipp maximum likelihood alignment using 200 manually picked particles. The template was then used to pick 21,657 particles using FindEM (Roseman, 2004). The untilted particles were then automatically matched to their tilt pairs using the TiltPicker program (Voss et al., 2009) providing a total of 18,530 particles (9,265 pairs). The untilted particles were CTF estimated and corrected using ACE2 (a variation of the algorithm in (Mallick et al., 2005)) and boxed out into an image stack. The untilted particles were initially aligned using Xmipp maximum likelihood alignment (Scheres et al., 2005a; Scheres et al., 2005b), but they displayed a preferred orientation. They were then aligned to the original template using SPIDER reference-based alignment (AP MQ) (Frank et al., 1996). Next, 8,039 tilted particles with the best cross-correlation to the template were backprojected using Euler parameters from TiltPicker and reference-based alignment procedures. The particles were then iteratively centered using SPIDER and re-backprojected to create a new volume, which achieved a resolution of 28.2 Å by $FSC_{0.5}$. The total processing time was less than 26 hours, with most the time going toward the manual steps for the initial template creation and examination of class averages.

Projection-matching Refinement of each initial model:

1312 images were collected of *E. coli* 50S subunits preserved in vitreous ice (Dataset 4). The contrast transfer function (CTF) for each micrograph was estimated using ACE2 (a variation of the algorithm in (Mallick et al., 2005)). 93,409 particles were automatically selected from the micrographs with FindEM (Roseman, 2004), using templates representing the predominant ribosomal views, as determined by an initial maximum-likelihood alignment and classification (Scheres et al., 2005a; Scheres et al., 2005b). Particles were extracted at a box size of 200 pixels. Stacked particles were binned by a factor of 2 for the final reconstruction and contained 82,575 particles, representing only those whose CTF parameters were detemined with an accuracy above the 0.8 cutoff point specified by ACE2. The 3D reconstruction was carried out using the EMAN1 reconstruction package (Ludtke et al., 1999), as specifically described below.

A total of 12 iterations of projection-matching was carried out for each refinement, using an angular increment of 3x10°, 3x8°, 3x6°, and 3x4°. For each angular increment, we used the following EMAN parameters, as documented in http://blake.bcm.tmc.edu/eman/eman1/: “pad=126”, “mask=44”, “sym=c1”, “hard=25”, “classkeep=0.5”, “phasecls”, and “refine”. “classiter” was set to 8 for the first two iterations at each angular increment and 3 for the third.

Resolution was assessed by calculating the Fourier Shell Correlation (FSC) at a cutoff of 0.5, and by Rmeasure (Sousa and Grigorieff, 2007).