Structure of hibernating ribosomes studied by cryoelectron tomography in vitro and in situ

Julio O. Ortiz, Florian Brandt, Valério R.F. Matias, Lau Sennels, Juri Rapsilber, Sjors H.W. Scheres, Matthias Ebauer, F. Ulrich Hartl, and Wolfgang Baumeister

1Department of Molecular Structural Biology and 2Department of Cellular Biochemistry, Max Planck Institute of Biochemistry, Martinsried 82152, Germany
3Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh EH9 3JR, Scotland, UK
4Biocomputing Unit, Centro Nacional de Biotecnología (CSIC), Cantoblanco, 28049 Madrid, Spain

Ribosomes arranged in pairs (100S) have been related with nutritional stress response and are believed to represent a “hibernation state.” Several proteins have been identified that are associated with 100S ribosomes but their spatial organization has hitherto not been characterized. We have used cryoelectron tomography to reveal the three-dimensional configuration of 100S ribosomes isolated from starved Escherichia coli cells and we have described their mode of interaction. In situ studies with intact E. coli cells allowed us to demonstrate that 100S ribosomes do exist in vivo and represent an easily reversible state of quiescence; they readily vanish when the growth medium is replenished.

Introduction

An early description of E. coli 70S ribosomes reported that these particles were frequently found associated in pairs, later referred to as 100S ribosomes (Tissieres and Watson, 1958). It was subsequently observed that the formation of 100S ribosomes occurs in stationary phase (Wada, 1998). The 100S ribosomes are distinct from disomes (two ribosomes linked by mRNA) in that they are not translationally active, but represent a storage form or hibernation state (Wada et al., 1995). The ribosome modulation factor (RMF), and the hibernation promoting factor (HPF, previously known as YhbH), are known to be involved in the formation and maturation of these ribosome dimers, respectively, whereas YfIA (protein Y) is suggested to bind 70S ribosomes in stationary phase (Wada et al., 1990; Maki et al., 2000). Some aspects of the interaction of these factors with the translational machinery have been explored in recent years (Ueta et al., 2005, 2008; Yoshida et al., 2009), but the interpretation of these data has been hampered by the lack of a detailed 3D structure of 100S ribosomes.

X-ray crystallography and EM single-particle analysis have provided unprecedented insights into the molecular architecture of ribosomes and have been instrumental in elucidating key events during translation (for review see Schmeing and Ramakrishnan, 2009). Both techniques rely, more or less, on homogeneous samples of ribosomes, ideally trapped in a particular functional state. Other higher-order structures, such as polysomes, display a degree of plasticity in their supramolecular organization, which makes them intractable to x-ray crystallography or single-particle analysis. Cryoelectron tomography (CET; Lucic et al., 2005) can complement these techniques by allowing the visualization of variable or flexible molecular structures both in vitro and in situ (Ortiz et al., 2006), i.e., in the functional environment of intact cells. Although the resolution is considerably lower than that achieved by x-ray and single-particle cryo-EM, computational analysis of subtomogram volumes opens the way for hybrid approaches to interpret the tomograms in the light of preexisting high resolution structures. In the past, we have used such an approach to study the native 3D organization of E. coli ribosomes in densely packed polysomes (Brandt et al., 2009). Here, we report a structural study of ribosomal dimers (100S) by CET, both in ribosome-enriched fractions from starved E. coli cells and in intact cells cultured in minimal medium.
This number might well be an underestimate of the abundance studies of stained samples (Wada, 1998; Yoshida et al., 2002). via their small subunits (30S), in agreement with earlier 2D the 70S ribosomes formed dimers (Fig. 1 D), which associate able number of 70S particles could be observed (Fig. 1 A). grown to starvation in which clusters of ribosomes with a vari-quired from frozen-hydrated samples derived from result in a degradation of image quality. Tomograms were ac-quired from frozen-hydrated samples derived from the examples of a dimer [B] and a trimer [C] indicated by asterisks in A. (D) Gallery of representative 100S particles detected in different tomo-grams [bar, 50 nm]. (E and F) Center-to-center 3D distance vectors [black dots] between each ribosome and its next neighbor, depicted by x-y and x-z plots. The plot corresponds to the subset of 271 ribosomes with one or more ribosomal neighbors from 1,232 identified ribosomes. Red dots represent the centers of clusters observed in densely packed polysomes (Brandt et al., 2009).

Figure 1. Variability in the spatial relationship of identified ribosomes within tomograms of starved E. coli lysates. (A) XY slice of tomogram (bar, 100 nm). White arrows indicate 100S particles and black arrows ribosomal trimers. Isosurfaces of a reference ribosome (30S, yellow; 50S, blue) were placed in relative orientations found by template matching for the two 30S subunits of each dimer are in close proximity, but their relative orientation is more variable than in stalled polysomes where the densely packed ribosomes are found in two clearly preferred relative orientations (Fig. 1, E and F; Brandt et al., 2009). The association of ribosome pairs isolated from starving cells seems to have a higher degree of translational and rotational freedom around the contact sites, which are located between the entry and exit sites of the mRNA on the 30S subunits.

Variability of isolated ribosomal assemblies

Initially, 100S ribosomes were investigated in vitro, i.e., in di-luted cell lysates in which nonspecific interactions are reduced. Cell lysates can be examined in relative thin (≤50–200 nm) ice layers compared with the much thicker ice layers (>0.5 µm) needed for embedding cellular structures. The thicker ice layers result in a degradation of image quality. Tomograms were ac-
quired from frozen-hydrated samples derived from E. coli cells grown to starvation in which clusters of ribosomes with a vari-able number of 70S particles could be observed (Fig. 1 A). Visual inspection of the tomograms showed that 20–25% of the 70S ribosomes formed dimers (Fig. 1 D), which associate via their small subunits (30S), in agreement with earlier 2D studies of stained samples (Wada, 1998; Yoshida et al., 2002). This number might well be an underestimate of the abundance of 100S ribosomes, given the fact that the 100S particles easily dissociate into monomeric ribosomes. The remaining ribosomal particles were individual 70S ribosomes or clus-
ters of three or more ribosomes in close proximity. The ribo-
somes forming trimers also appeared to associate via the 30S subunits.

We performed a 3D analysis of the tomographic data using template matching for an initial determination of the relative po-
positions and orientations of the 70S ribosomes (Frangakis et al., 2002). A reference density map derived from the crystal structure of an E. coli ribosome (Schwirth et al., 2005) was used as a template to detect and align individual ribosomes (Fig. S1). Replacement of individual ribosomes by the template in the orientation determined for the given ribosomes confirmed the proximity of small ribosomal subunits (Fig. 1, B and C). The position and orientations determined by template matching were used for the 3D analysis of center-to-center vector distances between neighboring ribosomes. This analysis provided an objective characterization of the spatial organization of ribosomes. The two 30S subunits of each dimer are in close proximity, but their relative orientation is more variable than in stalled polysomes where the densely packed ribosomes are found in two clearly preferred relative orientations (Fig. 1, E and F; Brandt et al., 2009). The association of ribosome pairs isolated from starving cells seems to have a higher degree of translational and rotational freedom around the contact sites, which are located between the entry and exit sites of the mRNA on the 30S subunits.

When all the subtomograms containing monomeric, di-
meric, as well as trimeric ribosomes were aligned with a 70S ribosome reference and averaged, the resulting density map showed a 70S particle with some additional blurred density around the 30S subunit, but no well-defined density corresponding to a second 70S particle as would be expected for a rigid and well-defined ensemble (Fig. 2 A). But this is not surprising given the low abundance of dimeric ribosomes in the sample (see above) and the flexible nature of the linkage between monomers, discernible already in the raw data.

To verify the identification of ribosomes in selected sub-

tomograms and their correct localization in 3D space, a subset of subtomograms was aligned in a completely unsupervised manner using a novel, reference-free maximum-likelihood refinement al-
gorithm for 3D images with missing data regions in Fourier space (Scheres et al., 2009). The refined model was almost indistin-
guishable from the one obtained using alignment based on tem-
plate matching (Fig. 2 F). The similarity between the models proves that the use of a reference in the template-based approach does not introduce a bias in this case.

Next, we applied successive steps of classification to the data using constrained correlation (Förster et al., 2008; Fig. 2, B–E). Dimer-only class averages created from independent sam-
ple pools clearly showed density for both 70S particles (Fig. 2, C and D). It was reassuring to see that the result obtained with template matching and the nonsupervised alignment and
In a 70S ribosome, the surface of the 30S subunit that is opposed to the inter-subunit space is commonly referred to as the cytosolic face (Fig. S1, “front” view). In the refined structure of the 100S ribosome, the cytosolic faces of the two 30S subunits are juxtaposed (Fig. 3 A). We denote this arrangement as “front-to-front” (f-f; Fig. 3 B). The pairing in the structure derived from hibernating ribosomes is quite different from the one found in stalled polysomes in vitro, called “top-to-top” (t-t; Fig. 3 B).

Figure 2. **Alignment and classification of identified ribosomes in tomograms from starved *E. coli* lysates.** (A–E) Reference-based alignment and classification using constrained correlation. (A) Slices of the average structure derived from 1,232 subtomograms (Pool II) containing 70S ribosomes. (B) Mask used for classification excluding the aligned central ribosome. (C and D) Slices of average structures containing 100S ribosomes with the two ribosomes in a preferred orientation derived from 43 classified subtomograms from Pool II and 35 classified subtomograms from Pool I, respectively. (E) Schematic slices of the two ribosomes oriented as in C [30S, blue; 30S, yellow; light colors for central ribosome excluded during classification; dark colors adjacent particle revealed after classification]. (F and G) Unsupervised alignment and classification using a maximum likelihood approach. (F) Isosurface representation of 3D maps obtained during iterative reference-free alignment of 601 subtomograms (Pool I) that were windowed to contain only a single ribosome particle. Iteration 0 (iter 0) corresponds to the initial, unbiased reference that was obtained by averaging over all sub-tomograms in random orientations. The final map (iter 15) has a resolution of 38 Å according to the FSC = 0.5 criterion and is readily identified as a 70S ribosome particle. (G) Simultaneous alignment and classification of the same particles used in F, but without windowing in order to include neighboring ribosomes. Three initial reference structures (class 1–3, iter 0) were refined simultaneously during 25 iterations. Note that during the first 5 iterations similarity between the three references was imposed. The final averages (iter 25) were interpreted as class 1 trimers (or larger clusters, 74 particles), class 2 dimers (158 particles), and class 3 monomers of 70S ribosome (369 particles).

classification yielded very similar results (Fig. 2 G; Fig. S4). The dimers have an approximate twofold symmetry axis. However, differences in the composition and conformation of the ribosomal subunits in each dimer that may exist as some variability in the 3D averages were observed depending on which monosome from each pair was aligned to the reference (nonredundant averages). Thus, an unambiguous assignment of each ribosome in a pair to a position in the dimer was not possible.
Rigid-body docking of two copies of the E. coli 70S ribosome structure into the dimer density map revealed possible interacting ribosomal components (Fig. 3, C and D). Two major contact regions between the small subunits of the dimers could be distinguished (Fig. 3 E). The first (I) involves proteins S9, S10, and the 16S rRNA helix 39 (Fig. 3, F and G). The second region (II) appears to involve the S2 protein because this protein is localized in the docked crystal structure adjacent to the density in the region II and it was found outside the density map envelope (Fig. 3, H and I). It has been suggested that S2 contacts the mRNA during movements associated with translation, and therefore displays a high degree of flexibility (Yusupova et al., 2006).

The f-f dimer average has additional density corresponding to a mass of approximately 25 kD associated with the small ribosomal subunit, in a region close to the mRNA tunnel exit and the so-called platform (Fig. 3, A and C). At the present resolution, it was not possible to assign any of the factors reported as being associated with 100S ribosomes, such as RMF (6 kD) and HPF (10.8 kD), unambiguously to these densities. It is in fact unlikely that the additional density associated with the 30S subunit is due to these factors. RMF has been shown to bind to the large ribosomal subunit (50S) in a region close to the peptidyl transference center (Yoshida et al., 2002, 2004; Fig. 3, F and G). HPF shares 40% sequence homology with YfiA (12.7 kD; Ueta et al., 2005) and adopts an overall structure similar to YfiA (Sato et al., 2009). HPF has been suggested to bind to the peptidyl-tRNA site (P-site) and aminoacyl-tRNA site (A-site) of the 30S subunit, similar to where YfiA is known to dock (Vila-Sanjurjo et al., 2004; Fig. 3, F and G).

Additional proteins associated with 100S ribosomes

In an attempt to identify additional nonribosomal proteins bound to dimers, we analyzed crude ribosomal fractions isolated from starved E. coli by mass spectroscopy. Several candidate proteins were detected, including RMF, HPF, and YfiA, and several other ribosome-binding proteins, such as the stationary phase–induced ribosome-associated protein (SRA, 5 kD), the ribosome-dependent endonuclease RelE, and initiation and elongation factors (Table S1). At this stage, we have not yet been able to discriminate between factors that associate with 100S ribosomes, but not with 70S ribosomes, nor could we assign any of the candidate proteins to the extra density found on the 30S subunits. Future studies with tagged ribosomes allowing for a better purification and cross-linking approaches in conjunction with mass spectrometry should enable us to identify specific interactors and correlate them with the observed density.

Consistent with the suggested function of HPF in blocking tRNA binding in the A- and P-sites, we have not found densities in the canonical tRNA positions, even at low intensity thresholds. Given that the platform has been described as a center for translational regulation (Marzi et al., 2007), we consider a potential participation of structured mRNAs or other RNA molecules in the 30S-associated density present in the 100S ribosomes.

While this manuscript was under revision, Kato et al. (2010) published an article describing the structure of isolated...
Thus, we aimed at identifying 100S particles in situ by CET of intact \textit{E. coli}. Cells grown in rich media have an average diameter of \( \sim 1 \mu \text{m} \), which is too thick for a reliable identification of most macromolecules. Slow growth in minimal medium results in cells with a diameter of \( \sim 400 \text{ nm} \) (Woldringh et al., 1977), improving tomogram quality significantly and thereby facilitating the recognition of macromolecular complexes. We recorded tomograms of \textit{E. coli} cells grown in minimal media up to mid-exponential and stationary phases (Fig. 4, A–E) and analyzed the resulting ribosome distribution (Fig. 4, F–G; Fig. S2). We found that only cells grown to stationary phase have a fraction (10–20\%) of identified ribosomes with neighboring 70S particles in the spatial arrangement described above as characteristic for 100S ribosomes (Fig. 4 G vs. Fig. 1 E). Interestingly, the clustering found in the cellular tomograms is indicative of a lower degree of variability in the relative orientation of the two monomers of the 100S ribosome in situ than in the in vitro preparations. Shear forces acting upon the particles during isolation might well cause distortions of the weakly connected monomers. Under conditions of exponential growth in minimal media we observed neither clustering indicative of dimers nor of \textit{E. coli} 100S ribosomes. They used cryo-EM in conjunction with single-particle analysis to generate a structural model. To reduce the risk of dissociation of the 100S particles into monomeric 70S particles they applied a mild chemical fixation and to cope with the flexible linkage between the two monomers the alignment was restricted to only one of them but including the small subunit of the adjacent monomer. This allowed them to determine the relative orientation of the monomers and to generate a synthetic 100S model from the individually reconstructed 70S particles. Despite of the different approaches taken (electron tomography vs. single-particle analysis), the resulting structures are in good agreement: the monomers interact flexibly via the 30S subunits and this interaction appears to involve protein S2; an additional mass not present in individual 70S particles was observed on the 30S subunits of the 100S ribosomes, but no density attributable to tRNAs was found.

**Existence of 100S ribosomes in situ**

The existence of ribosome dimers in vivo has remained a controversial issue because it cannot be ruled out that the ionic conditions of the working buffer could favor nonspecific interactions. Thus, we aimed at identifying 100S particles in situ by CET of intact \textit{E. coli}. Cells grown in rich media have an average diameter of \( \sim 1 \mu \text{m} \), which is too thick for a reliable identification of most macromolecules. Slow growth in minimal medium results in cells with a diameter of \( \sim 400 \text{ nm} \) (Woldringh et al., 1977), improving tomogram quality significantly and thereby facilitating the recognition of macromolecular complexes. We recorded tomograms of \textit{E. coli} cells grown in minimal media up to mid-exponential and stationary phases (Fig. 4, A–E) and analyzed the resulting ribosome distribution (Fig. 4, F–G; Fig. S2). We found that only cells grown to stationary phase have a fraction (10–20\%) of identified ribosomes with neighboring 70S particles in the spatial arrangement described above as characteristic for 100S ribosomes (Fig. 4 G vs. Fig. 1 E). Interestingly, the clustering found in the cellular tomograms is indicative of a lower degree of variability in the relative orientation of the two monomers of the 100S ribosome in situ than in the in vitro preparations. Shear forces acting upon the particles during isolation might well cause distortions of the weakly connected monomers. Under conditions of exponential growth in minimal media we observed neither clustering indicative of dimers nor of...
polysomes (Fig. 4 F), possibly because initiation events are rare under these conditions. It is known that the inhibition of ribosomes by RMF and consequent ribosome dimerization is reversible, as the 100S ribosomes dissociate back into 70S ribosomes within 2 min upon transfer of starved cells into fresh media (Wada et al., 1990; Yoshida et al., 2002). We tested whether the ribosomal clustering observed in stationary phase would vanish when supplying fresh nutrients. Indeed, no clustering of ribosomes was detected when cells previously grown to stationary phase in minimal media were supplemented with amino acids or transferred to rich media (Fig. 4 H). Moreover, a low resolution model of the f–f ribosome dimer was generated by averaging a subset of 70S ribosomes from tomograms of cells in stationary phase identified as having a second ribosome attached (Fig. 4, I and J). The results confirm the physiological relevance of the f–f ribosome dimers observed in fractions from cell lysates and further support their association with nutritional stress.

Discussion

Our understanding of ribosomal clustering coupled to growth-phase transitions is summarized in Fig. 5. Stalling of ribosomes during translation is not unusual, and it can be caused, among other reasons, by secondary mRNA structures, rare codons, or amino acid scarcity (Buchan and Stansfield, 2007). Stalled ribosomes can be recycled by at least two different mechanisms: in the case of truncated messengers, by the tmRNA system or in the case of ribosomes stalled at the stage of elongation, by the initiation factor 3 (IF3) and the ribosome recycling factor (RRF; Singh et al., 2008). The synthesis of stable RNA species in many E. coli strains is sharply decreased by deprivation of amino acids. This response is predominantly regulated by the guanosine pentaphosphate or tetraphosphate [(p)ppGpp]. The level of (p)ppGpp in bacteria is controlled by two ppGpp synthetases (PSI/PSII), encoded by the relA and spoT genes (Murray and Bremer, 1996). The binding of uncharged tRNAs to the ribosomes is a signal for activation of the ribosome-bound PSI. The accumulation of (p)ppGpp reduces the synthesis of additional ribosomes and thereby reduces the consumption of amino acids; this in turn allows the remaining ribosomes to function at a higher rate (Bremer and Dennis, 2008). The transcription of the rmf gene is positively regulated by (p)ppGpp (Izutsu et al., 2001).

The inhibition of translation by ribosome–dimer formation during starvation has been attributed primarily to RMF binding, although the role of the RMF in the dimerization process is not yet well understood. The action of RMF might resemble the inhibition of translation by certain antibiotics (Yoshida et al., 2002). In more recent studies, it has been suggested that RMF promotes the formation of a 90S complex, which matures with the addition of HPF to form the 100S particle (Fig. 5, C and D; Ueta et al., 2005). Interestingly, the proteins S9 and S10, which are located in the contact region I of the f–f 100S dimers, have domains (C-terminal in S9 and R45-H70 in S10) extending to a site close to the probable HPF binding site (Fig. 3, F and G). A conformational change induced by HPF or YfIA at the tRNA binding sites could be propagated through the head of the 30S subunit, altering its affinity for another 30S subunit.

Our experiments proved that the 100S ribosome complex is a distinct biologically relevant ribosomal assembly in E. coli cells under conditions of nutritional stress. This is in agreement with biochemical studies reporting the appearance of ribosome dimers (Wada, 1998; Ueta et al., 2008). Mutants with a disrupted rmf gene do not form ribosome dimers and lose viability earlier than wild-type cells in stationary phase (Yamagishi et al., 1993; Wada et al., 2000). Due to the antagonistic functions of HPF and YfIA, mutants with single deletions in the corresponding genes live slightly longer than wild type (Ueta et al., 2005). These effects on cell survival have been interpreted in terms of ribosome protection against degradation, as the free 50S and
30S subunits, not the 70S particles, are the targets for endoribo-
nuclease action and ultimately degradation (Zundel et al., 2009).
Stalled ribosomes can be recovered by the tmRNA system or
IF3/RRF interactions causing the release of truncated nascent
chains. However, with reduced availability of nutrients, the in-
activation of a portion of available ribosomes by RMF/HPF
could alleviate the problem of stalling during the transition to
stationary phase.

Although active 70S ribosomes are thought to be rare in the
stationary phase, the blocking of tRNA binding sites in dimerized
ribosomes might allow an effective use of limited aminoacyl-
tRNAs for the remaining active ribosomes. Thus, ribosome dis-
merization might have a dual role in protecting ribosomes against
degradation, allowing rapid recovery of translation, and facilitat-
ing protein synthesis under stringent conditions by reducing com-
petition for scarce resources (Fig. 5 D). The association of the
100S ribosomes with a variety of factors required for translation
may allow these complexes to rapidly resume protein synthesis
once more favorable nutrient conditions have been established.
Our results provide a model for the interactions of hibernating
ribosomes, which can help to direct further biochemical experi-
ments leading to a fuller understanding of the processes underly-
ing their formation.

Materials and methods

Cell cultures

Overnight cultures of E. coli (ECOR48) cells in LB medium (0.5 ml) were
inoculated into 1 L of medium (Vogel and Bonner, 1956) supplemented
with 2% Bacto-Trypton (Becton Dickinson) and 0.5% glucose at 37°C with
shaking at 100 cycles per min during 3 d. These growing conditions are
reported to give maximal stationary phase survival for most E. coli strains
(Wada et al., 2000). For ribosome isolation, cell pellets were stored
at −80°C until use. E. coli (B/r k) was grown in M9 minimal medium sup-
plemented with 0.03% (wt/vol) alanine (as the sole carbon source) up to
mid-exponential and stationary phase (optical density at 600 nm of 0.08
and 3 mM DTT) containing RNase-free DNase I at 2 µg/ml. Lysates were
overnight cultures of E. coli (B/r k) suspensions were applied to R2/2 Quantifoil grids (Quantifoil), followed by 1 µl of colloidal gold (10 nm, protected with BSA). After blotting, with filter paper, the grids were vitrified in liquid ethane and stored until usage in liquid nitrogen. For lysates, 26 single-axis tilt series were recorded using a CM20 FEG transmission electron microscope [FEI] operating at 160 kV, a 4096 × 4096 slow-scan CCD camera, and control software for automated image tracking and focusing (TVIPS). The angular range used was ±60°, sampled with angular increments of 3°; and with a cumulative electron dose not exceeding 50 e−/Å2. Images were collected using a nomi-
3-µm underfocus, a tilt range of ±60°, and an angu-
lar increment of 1.5°. Alignment and reconstruction were performed using the IMOD software package (Kremer et al., 1996). The number of tomograms analyzed for exponential phase, stationary phase, and aa-supplemented stationary phase was 5, 6, and 4, respectively.

Template matching

For detection of the 70S ribosomes on recorded tomograms, down-sampled reconstructed volumes [2.24 nm]3/voxel for tomograms of ribosomal frac-
tions and [1.4] nm]3/voxel for cellular tomograms] were cross-correlated with a template structure using the program MOLMATCH (Franakis et al., 2002; Ortiz et al., 2006). The 3D template was generated from 3.5 Å cryo-
transmission electron microscope [T30 Polara; FEI] equipped with a field emission gun operated at 300 kV. Zero-loss energy filtered images were taken with a 2048 × 2048 Multiscan CCD camera (Gatan) at the end of a Gif 2000 post-column energy filter [Gatan]. Tomograms were collected at final magnification of 42105 (giving a pixel size of 0.713 nm) and using a 9 µm underfocus, a tilt range of ±60°, and an angular increment of 1.5°. Alignment and reconstruction were performed using the IMOD software package (Kremer et al., 1996). The number of tomograms analyzed for exponential phase, stationary phase, and aa-supplemented stationary phase was 5, 6, and 4, respectively.

Averaging and classification of subtomograms

Selected particles were reconstructed in subtomograms with lower binning
factor ([0.56 nm]3/voxel for tomograms of ribosomal fractions and
[0.7 nm]3/voxel for cellular tomograms) and 3D aligned against a 4-nm resolution 70S ribosome reference properly scaled as described previously (Förster et al., 2005; Brand et al., 2009). 3D alignments and 3D averaging was performed with the use of AV3 (Fürster and Hegel, 2007; Förster et al., 2008). After recognition of putative ribosomes using template matching, subsequent discrimination of false positives was addressed by classification of subvolumes using a constrained-correlation method (Fürster et al., 2008) in which the search of similarity between particle pairs was restricted to the volume to be occupied by only one centered ribosome i, i.e., employing a spheri-
cal mask of 1.4 nm radius. The number of classes was set as high as possible, such that each class contained a small number of particles (at least 20 parti-
cles in order to increase the signal of tomograms by one order of magni-
tude). Averages of classified particles were calculated for each class. Particles contributing to averages not consistent with the ribosome structure were discarded. Thus, 601 particles for tomograms of the Pool I, and 1,232 particles of Pool II, were identified as bona fide ribosomes.

30S subunits, not the 70S particles, are the targets for endoribo-
nuclease action and ultimately degradation (Zundel et al., 2009).
A second step of classification used the same constrained-correlation algorithm but considering only areas surrounding each identified (centered) ribosome \( i \) in a sphere of 30 nm. Thus, ribosomes pairs with \( F \) organization were identified. Ribosomes in each dimer with higher similarity to an initial average map were selected to contribute to the same position in the dimer during iterative alignment steps. Thus, 35 dimers (~11% of identified 70S ribosomes) from Pool II were averaged.

The analysis of the relative spatial relationship between particles was performed as previously for polysomes (Brandt et al., 2009) with slight modifications. In brief, distances between the center of mass of each detected ribosome \( i \) and its nearest neighbor \( j \) were calculated. A vector for ribosome \( i \) was calculated relative to the coordinates of the reference. Relative Euclidean distances and relative angles were hierarchically clustered, using the sum of pairwise dot products as metric. The relative angles were transformed into quaternion space before calculating dot products between pairs of quaternions and back-transformed into Eulerian space for display of clustered rotations. In an alternative classification approach to the constrained-correlation based, we classified particles using this hierarchically clustering of relative localization and orientation.

Resolution of density maps were determined by Fourier shell correlation of two averages each derived of half of the pertinent particles.

Reference-free alignment

The subset of 601 particles from Pool I were aligned in a completely unsupervised manner using novel, reference-free maximum-likelihood refinement algorithm for 3D images with missing data regions in Fourier space (Scheres et al., 2009). Precentered particles were windowed to 60 × 60 voxels in order to contain a single ribosome at the center of each particle. An unbiased initial model for the iterative maximum-likelihood refinement algorithm was obtained by assigning random orientations to all particles, resulting in a relatively featureless blob of density. This model was refined during 12 iterations with an angular sampling rate of 12 degrees, followed by 3 more iterations with an angular sampling rate of 9 degrees and restricting the integrations over the angles by ±32 degrees around the optimal angles from the 12th iteration.

Reference-free classification

Unsupervised classification was done using the same 3D maximum likelihood approach, but using the particles without windowing. Unbiased starting models were generated by calculating the average of three random subsets of the particles in random orientations. The resulting models were refined simultaneously against the entire dataset during 25 iterations and using an angular sampling rate of 15 degrees. During the first 5 iterations similarity among the three references was imposed as described previously (Scheres et al., 2009). A smaller spread of ribosome dimer orientations could also be obtained by subsequent reclassification of the dimer class into three subclasses using the same unsupervised maximum likelihood approach. To speed up these calculations all particles were down-scaled inside the maximum likelihood program to 40 × 40 × 40 voxels (corresponding to a voxel size of 0.84 nm).

CTF correction

Projection images were aligned (Nickell et al., 2005) and Periodogram averaging (Fernández et al., 1997) was extended over these aligned projection images from the mean defocus (Fernández et al., 2006). Defocus was refined during 12 iterations with an angular sampling rate of 12 degrees, followed by 3 more iterations with an angular sampling rate of 9 degrees and restricting the integrations over the angles by ±32 degrees around the optimal angles from the 12th iteration.

Fitting of high resolution structures

Fitting was performed in a quantitative manner using Chimera (Petersen et al., 2004). Docked crystal structures were derived from Protein Data Bank (PDB), entries 2AW7 and 2AWB (Schwirch et al., 2003), or 1VOG and 1VOR (Vila-Sanjurjo et al., 2004). For 3D visualization, the programs Chimera (Petersen et al., 2004) and 3ds max (Discret) were used.

Accession numbers

The 3D density map of the 100S ribosome in conformation H has been deposited in the Electron Microscopy Data Bank with accession code EMD-1750.

Online supplemental material

Fig. S1 shows the orientation conventions used in this study. Fig. S2 is the complete analysis of spatial relationship of in situ identified ribosomes. Fig. S3 shows the resolution improvements gained by CTF correction applied to in vitro data. Fig. S4 shows subsequent unsupervised classification of previously identified ribosome dimers. Table S1 lists the identified protein in crude ribosomal fractions from starved Escherichia coli cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201005007/DC1.

We thank S. Echells and K. Bopp for experimental help at initial steps of the project, T. den Blaauwen for providing us with the E. coli strain B/r K, and E. Villa, F. Förster and D. Thomas for critical reading of the manuscript.

This work received funding from the European Commission's seventh Framework Program (grant agreement HEALTH-F4-2008-201648/PROSPRECT), the Center for Integrated Protein Science Munich (CIPSM), by "Fondation Fourmion Guibert", and by a Marie Curie Excellence Grant.

Submitted: 3 May 2010
Accepted: 26 July 2010

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