High-resolution structure of the *Escherichia coli* ribosome

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Protein synthesis by the ribosome is highly dependent on the ionic conditions in the cellular environment, but the roles of ribosome solvation have remained poorly understood. Moreover, the functions of modifications to ribosomal RNA and ribosomal proteins have also been unclear. Here we present the structure of the *Escherichia coli* 70S ribosome at 2.4-Å resolution. The structure reveals details of the ribosomal subunit interface that are conserved in all domains of life, and it suggests how solvation contributes to ribosome integrity and function as well as how the conformation of ribosomal protein uS12 aids in mRNA decoding. This structure helps to explain the phylogenetic conservation of key elements of the ribosome, including post-transcriptional and post-translational modifications, and should serve as a basis for future antibiotic development.

The structure and function of the ribosome in protein synthesis is exquisitely sensitive to ionic conditions. Bacterial ribosomes carry a formal charge of nearly −4,000 because ribosomal proteins neutralize very little of the charge in the phosphodiester backbone in rRNA. In the bacterium *E. coli*, ribosomes harbor large numbers of different cations, including potassium, magnesium and polyamines. These ions influence ribosome dynamics, including intersubunit rotation, as well as the mechanisms of mRNA decoding and translocation. Furthermore, the ribosome is highly hydrated, owing to the relatively open nature of RNA secondary and tertiary structure.

In addition to high levels of solvation and counterion binding, both rRNA and ribosomal proteins undergo numerous post-transcriptional and post-translational modifications, respectively. The degree of these modifications increases with the complexity of the organism, thus suggesting important functional roles. The most common post-transcriptional modification is isomerization of uridine to pseudouridine, which bears a free imino group that can stabilize RNA structure through base-stacking interactions and the use of its N1 imino group as a hydrogen-bond donor. Further modifications of the *E. coli* ribosome include methylation of the ribose 2′-hydroxyl group, methylation of nucleobases and reduction of the nucleobase uridine to dihydrouridine. Modification of RNA nucleotides leads to altered electronic and steric effects that result in altered base-pairing potentials, preferred sugar puckers and different base-stacking properties.

Notably, post-transcriptional and post-translational modifications cluster in or near key functional sites of the ribosome, such as the decoding center, the peptidyl transferase center (PTC), the exit tunnel and the intersubunit bridges. However, the functional roles of individual modifications have remained poorly understood. Although some modifications are nearly universal in bacteria the inhibition of specific rRNA modifications often has little effect on cell growth; hence, the function of each individual rRNA modification is not clear. Notably, ribosomes lacking all pseudouridines cause severe growth defects in yeast. Furthermore, even if individual rRNA modifications may be dispensable, their presence leads to a competitive advantage for growth. In addition, some ribosomal modifications are incorporated as an environmental response and can have widespread effects on the translation capacity of the ribosome.

To date, it has not been possible to accurately model solvation characteristics of the intact ribosome, owing to the generally low resolution of previous ribosome X-ray crystal structures, most of which have a resolution lower than ~2.8 Å. Recent high-resolution structures of the *Thermus thermophilus* 70S ribosome used crystallographic data to 2.55-Å (ref. 21) and 2.4-Å (ref. 22) resolution, but the resulting structural models do not yet include solvation beyond inclusion of inner-sphere and outer-sphere coordinated Mg ions, and they do not include post-transcriptional and post-translational modifications. We therefore set out to solve the structure of the bacterial ribosome in order to elucidate its solvation characteristics and the structural effects of ribosomal modifications. Here, we present a high-resolution structure of the *E. coli* ribosome, which enabled us to greatly improve the model of the bacterial ribosome by removing many previous modeling errors. The structure reveals unprecedented views of solvation as well as post-transcriptional and post-translational modifications of the ribosome in key functional and antibiotic-binding sites. Comparisons to biochemical, genetic and phylogenetic data allow us to propose functional roles for a number of the structural features newly seen at high resolution.

**RESULTS**

We substantially improved the diffraction power of *E. coli* 70S ribosome crystals by extensively optimizing the cryostabilization

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**Table 1 Data collection and refinement statistics**

| Data collection        | Merged data |
|------------------------|-------------|
| Space group            | P2₁2₁2₁     |
| Cell dimensions        |             |
| a, b, c (Å)            | 212.1, 433.9, 624.3 |
| Resolution (Å)         | 69.39–2.11 (2.17–2.11) |
| Rmerge                 | 16.9 (205.2) |
| I / σ(I)               | 5.1 (0.28)  |
| CC₁/₂ (%)              | 99.6 (13.7) |
| Completeness (%)       | 93.2 (80.9) |
| Redundancy             | 4.15 (2.09) |
| Refinement             |             |
| Resolution (Å)         | 69.39–2.1   |
| No. reflections        | 3,003,826   |
| Rmerge / Rfree         | 21.85 / 23.38 |
| No. atoms (without H)  | 295,744     |
| Protein/RNA            | 286,907     |
| Ligand/ion             | 1,083       |
| Water                  | 7,754       |
| B factors (Å²)         |             |
| Protein/RNA, ribosome I| 69.89       |
| Protein/RNA, ribosome II| 122.98     |
| Ligand/ion             | 64.13       |
| Water                  | 53.25       |
| r.m.s. deviations      |             |
| Bond lengths (Å)       | 0.008       |
| Bond angles (°)        | 1.270       |

Values in parentheses are for highest-resolution shell. 109 crystals were used to measure the data.

procedure before crystal freezing and by using detector geometry and crystal orientation to reduce diffraction overlap (Online Methods). Because of radiation damage caused by the X-ray beam, we merged diffraction data from more than 100 high-quality crystals for the structure presented here. The 70S ribosome crystallized with two 70S ribosomes in the crystallographic asymmetric unit (Table 1), with the small (30S) subunit of ribosome I rotated to an intermediate position relative to the large (50S) subunit and with ribosome II in an unrotated state. The electron density maps for most parts of ribosome I were of high quality, except for peripheral elements including protein L9, the dynamic L1 arm, the stalk proteins L10, L11 and L7–L12, sections of the GTPase center and the aminoacyl (A)-site finger in the large subunit (Fig. 1a). Ribosome II was more dynamic in this crystal form, thus resulting in electron density maps of lower quality in most regions (Supplementary Fig. 1a).

**Ribosome modeling in high-resolution electron density maps**

We determined the 70S ribosome crystal structures at a resolution of approximately 2.4–2.5 Å (I/σI = 1). Using the recently introduced cross-correlation parameter (CC₁/₂), we extended the scaled and merged X-ray diffraction data measured from the above ribosome crystals to 2.11-Å resolution, at which the value of CC₁/₂ was still significant (13.7%, Supplementary Table 1); therefore, we included these data in the structure refinement. Owing to the inherent dynamics of the ribosome and regions of disorder, the quality of the model varied by region, as reflected by the range of atomic displacement parameters (Fig. 1a and Supplementary Fig. 1a). In the following description, we accordingly focused our modeling and interpretation primarily on the regions that are well ordered in ribosome I (Online Methods).

Consistently with the CC₁/₂ resolution, the electron density maps were highly detailed in many regions of the ribosome (Online Methods and Supplementary Fig. 2). The directionality of nonbridging phosphate oxygen atoms in the rRNA was resolved, and ribose 2'-hydroxyl groups were clearly visible, as were nucleotide exocyclic amines and carbonyl groups (Supplementary Figs. 2 and 3). In well-ordered ribosomal proteins, the peptide backbone and methyl groups of valine, leucine and isoleucine side chains, as well as the guanidino groups of arginines, were clearly resolved (Supplementary Fig. 3). This level of resolution allowed us to greatly improve the ribosomal protein models relative to a prior 2.8-Å E. coli ribosome X-ray structure, as judged by MolProbity (Supplementary Tables 2–5). The data also allowed placement of more than 8,800 solvent molecules and ions. At this resolution, we could readily distinguish magnesium ions from water and ammonium ions, and we could see specific polyamines. However, water and ammonium ions were difficult to tell apart, and we therefore modeled them as waters throughout the structure.

The quality of the electron density in many regions revealed the contribution of solvent molecules to key functional sites of the ribosome, including the subunit interface, the PTC and the exit tunnel. Furthermore, the electron density allowed modeling of many post-translational and post-transcriptional modifications, including those in three ribosomal proteins, and 35 of 36 post-transcriptional modifications except for the substoichiometric 2-thiocytidine 2501 modification in 23S rRNA; this modification was also allowed placement of more than 8,800 solvent molecules and ions. At this resolution, we could readily distinguish magnesium ions from water and ammonium ions, and we could see specific polyamines. However, water and ammonium ions were difficult to tell apart, and we therefore modeled them as waters throughout the structure.

**Solvation of the ribosome at the subunit interface**

Single-molecule Förster resonance energy transfer (smFRET) experiments demonstrated that lower Mg2+ concentrations promote rotated ribosome configurations in which tRNAs adopt hybrid-site binding (Fig. 1b and Supplementary Fig. 1b). In line with the smFRET data, lower Mg2+ concentrations favored crystallization of one ribosome in the crystallographic asymmetric unit in an intermediate rotated state (ribosome I, Fig. 1a). We included putrescine and spermidine in the crystallization and cryoprotection solutions because of their essential roles in cellular physiology and in the fidelity of translation. These cellular polyamines are also required for 70S-complex stability in smFRET experiments, although their contributions to ribosome dynamics are less clear.

In the new high-resolution structure of the ribosome, many of the RNA-rich intersubunit bridges in ribosome I showed clear evidence for water-mediated interactions between the two subunits, but there were few if any examples of clear magnesium ions or polyamines at the subunit interface (Supplementary Table 8). We observed electron density consistent with bound polyamines in the large subunit near the subunit interface in bridge B3 and near the base of helix H69 in 23S rRNA (Supplementary Fig. 3), but few other polyamines were clearly visible in the electron density. These results suggested that charge neutralization occurs primarily through diffusely bound ions, or possibly through ions bound in the RNA major groove; these ions may help to promote ribosome subunit association while also allowing for intersubunit rotation.

Bridge B3, considered the ‘pivot point’ of intersubunit rotation, involves a type I A-minor-motif interaction involving the minor-groove faces of two adenines in tandem sheared G-A base pairs in 16S rRNA helix h44 of the 30S subunit, which contact the minor groove.
Figure 1  E. coli 70S ribosome I in an intermediate rotated state. (a) Ribosomal subunits of ribosome I colored by atomic displacement factor from 20 to 150 Å². The views are from the perspective of the subunit interface. Features in the 50S subunit include the central protuberance (CP), L1 arm (L1), protein L9 (L9), L7–L12 region (L12), A-site finger (ASF) and the GTPase center (G). In the 30S subunit, these include the head (H), body (B) and platform (PL). (b) Single-molecule imaging of the modulation of ribosome dynamics by magnesium ions. Occupancy of unrotated (red) and rotated (black) states of the ribosome as measured by smFRET between fluorophores on uS13 and uL1, shown ± s.d. of three technical replicates (Online Methods).

We identified three water molecules that buttress this A-minor motif; thus increasing the number of base-pairing planes that interact between the small and large subunits in bridge B3 from three to four (Fig. 2a–c). Whereas bridge B3 probably adopts a fairly constant conformation during intersubunit rotation, the remaining bridges change position12,34,36. Although we observed ordered solvent molecules in other bridges (Supplementary Table 8), bridge B6 is nearly devoid of ordered solvent, although the space between the two RNA helices that make up the bridge leaves room for several water molecules13. This is true in both copies of the 70S ribosome in the crystallographic asymmetric unit, in which bridge B6 is well ordered in terms of the RNA and for which we could model solvent in the vicinity of the bridge.

Solvation of antibiotic-binding sites in the exit tunnel

Macrolides, ketolides and streptogramin B are clinically important antibiotic classes that partially block the nascent peptide–exit tunnel and thus lead to bacterial cell death37,38. In the exit tunnel, nucleotide A2058 of 23S rRNA is critical for antibiotic binding, and antibiotic resistance results from mutation or methylation of this nucleotide39. In the high-resolution ribosome structure, a number of solvent molecules in the proximity of A2058 overlap with the binding sites of macrolides, ketolides and streptogramin B antibiotics (Fig. 2d and Supplementary Fig. 5a). These solvent molecules (here interpreted as water) form a layer between nucleotides A2058 and pseudouridine (Ψ) 746 and extend toward the base pair formed by A752 and U609, which stacks on the alkyl-arly arm of the ketolide telithromycin40,41 (Fig. 2d). These solvent molecules would have to be displaced upon antibiotic binding, the effects of which could be included in future computational docking methods.

The narrow ‘constriction site’ of the nascent peptide–exit tunnel is only about 10 Å wide and is confined by residues of ribosomal proteins uL4, uL22 and A751 of 23S rRNA42,43. Notably, clear electron density for 2-methyl-2,4-pentanediol (MPD), a moderately hydrophobic molecule used in the crystallization solution, stacks on A751 and partly blocks the exit tunnel (Supplementary Fig. 5b). Cryo-EM studies44 and molecular-dynamics simulations45 of the SecM-stalled ribosome previously identified that stacking of the hydrophobic side chain of amino acid Trp155 of SecM on nucleotide A751 is critical for antibiotic binding, the effects of which could be included in future computational docking methods.

Ribosomal modifications near the PTC

A number of post-transcriptional and post-translational modifications are clustered near the PTC (Fig. 3a). In the classical state, nucleotides C74 and C75 in the 3′ CCA acceptor arm of the peptidyl (P)-site tRNA base-pair with G2252 and Gm2251 of the P loop of 23S rRNA, respectively; this helps to orient the peptidyl-tRNA for the peptidyl-transfer reaction21,49. In the E. coli ribosome structure, Gm2251 forms a C3′-endo sugar pucker, which may be stabilized by the 2′-O-methylation, because a C2′-endo pucker could lead to altered positioning of the nucleobase50. Notably, the remaining nucleotides of the E. coli ribosome carrying 2′-O-methylations (Supplementary Table 6) also adopt a C3′-endo pucker. The nonbridging phosphate oxygen of Gm2251 contacts the guanidinium group of Arg81 of uL16, which itself is modified with a hydroxyl group at the β-carbon (Fig. 3a,b). In the present structure, the hydroxyarginine 81 is present in two alternate conformations, and its absolute configuration at the β-carbon is R, in agreement with the hydroxylation catalyzed by ribosomal oxygenase ycfD27. On the opposite side of Arg81, Gm2251 is positioned next to the only dihydrouridine in the E. coli ribosome, D2449. A dihydrouridine nucleotide disrupts base-stacking interactions, owing to the nonplanar nature of the dihydrouridine nucleobase, and it can stabilize its ribose and that of the 5′ neighboring nucleotide in C2′-endo pucker51, as observed in the E. coli ribosome structure (Fig. 3c). In proximity to the dihydrouridine, the methyl group on the exocyclic amine of m2G2445 adopts an s-trans position and occupies a cavity created by neighboring nucleobases including D2449 (Fig. 3a and Supplementary Table 7).

Conformation of uS12 near the mRNA-decoding center

Protein uS12 forms key parts of the mRNA-decoding center of the small ribosomal subunit, with proline 45 of uS12 positioned less than 4 Å away from the third nucleotide of the mRNA A-site codon21,22 (Fig. 3d,e). Interestingly, Pro45 clearly adopted a cis-peptide conformation in the present high-resolution structure (Fig. 3d,e), a conformation that persists through mRNA decoding22, even though cis-prolines occur at a low frequency in proteins52. This stable conformation could have functional implications in mRNA decoding and mRNA and tRNA translocation. For example, in the eukaryotic homolog of uS12, rpS23, the corresponding proline residue, Pro62 in humans and Pro64 in yeast, is mono- or dihydroxylated, respectively, depending on environmental or stress conditions17–19. Interestingly,
Solvation at the ribosomal subunit interface and in the nascent peptide–exit tunnel. (a–c) Water molecules that bridge the 16S (blue) and 23S (gray) rRNA in bridge B3 of ribosome I. 2′-hydroxyls involved in the water interactions are marked with asterisks. The location of bridge B3 is indicated in the inset in (c). The feature-enhanced maps are contoured at 2.5 s.d. from the mean. (d) Solvation at the entrance of the nascent peptide–exit tunnel. Labeled residues of 23S rRNA are shown in orange. The feature-enhanced map is contoured at 2.0 s.d. from the mean.

the hydroxylation state of uS12’s Pro64 in yeast has been shown to affect stop-codon readthrough in a sequence- and context-dependent manner. Hydroxylation or dihydroxylation of this proline would change its interaction from a van der Waals contact to the mRNA to one mediated by a hydrogen bond with the O3′ of the third position of the A-site codon (Fig. 3f), and this may increase the binding energy of the ribosome–mRNA complex by about 1–2 kcal/mol, corresponding to the energy of a hydrogen bond.

In E. coli, uS12, aspartate 89 is post-translationally modified to β-methylthioaspartate, with an absolute configuration of 3R in the present structure (Fig. 3d,e). In this configuration, the side chain carboxylate is within hydrogen-bonding distance of the guanidinium group of Arg50 of uS12, thus neutralizing its charge, and the methylthio group points toward the positively charged m7G-psi of the 3rd position of the A-site codon. Although mutations of Asp89 are lethal, modification on Asp89, specific to bacteria, is introduced by RimO and enhanced maps are contoured at 2.5 s.d. from the mean. (d) Solvation at the entrance of the nascent peptide–exit tunnel. Labeled residues of 23S rRNA are shown in orange. The feature-enhanced map is contoured at 2.0 s.d. from the mean.

**Syn pyrimidines in the ribosome**

Although the favorable conformation of the nucleobase is anti with respect to the ribose, purines can assume sterically less favorable syn conformations. We observed 120 examples of syn purines with good electron density in the E. coli ribosome (defined with a χ-angle in the range of −90° to +90°; Supplementary Table 10). However, syn pyrimidines are rare, owing to a larger energetic penalty resulting from the steric clash of the O2 of pyrimidines with the ribose compared to the N3 in purines. Syn nucleotides seem to occur more frequently in functional sites of RNA structures, where the energetic cost for adopting the syn conformation may be compensated for by favorable effects on RNA structure and function. In highly ordered regions of the E. coli ribosome structure, we observed 15 pyrimidines in the syn conformation (Supplementary Table 11 and Supplementary Fig. 6).

**Pseudouridines in the ribosome**

Pseudouridine has been proposed to stabilize RNA structures by presenting an extra hydrogen-bond donor in the major groove, relative to uridine. For six of the seven well-ordered pseudouridines in the present high-resolution structure (Supplementary Table 9), we observed a water-mediated contact between the pseudouridine N1 imino group in the major groove and the rRNA phosphate backbone. This is consistent with the model that these pseudouridines stabilize the local ribosomal structure by locking the nucleobase in place with respect to the rRNA backbone. One example in the 50S-subunit rRNA P site involves a network of water-mediated interactions including G954 and υ955 of the 23S rRNA helix H39 (Fig. 4a).

**Figure 2** Solvation at the ribosomal subunit interface and in the nascent peptide–exit tunnel. (a–c) Water molecules that bridge the 16S (blue) and 23S (gray) rRNA in bridge B3 of ribosome I. 2′-hydroxyls involved in the water interactions are marked with asterisks. The location of bridge B3 is indicated in the inset in (c). The feature-enhanced maps are contoured at 2.5 s.d. from the mean. (d) Solvation at the entrance of the nascent peptide–exit tunnel. Labeled residues of 23S rRNA are shown in orange. The feature-enhanced map is contoured at 2.0 s.d. from the mean.

**Figure 3** Post-transcriptional and post-translational modifications in functional centers of the ribosome. (a) View of the PTC with C75 of the P-site tRNA (orange) modeled from PDB 4V6F. Hydroxyarginine 81 (magenta) is in protein uL16, and Gm2251, dihydrouridine (DHU) 2449 and m2G2445 are in 23S rRNA (gray). (b) R configuration of hydroxylated Arg81 of uL16 at the C8 position. The feature-enhanced map is contoured at 2.5 s.d. from the mean. (c) C2-endo sugar pucker of dihydrouridine 2449 and A2448. Feature-enhanced electron density map shown as in b. (d) Geometry of protein uS12 (cyan) near the mRNA-decoding center. Pro45 of uS12 in the cis-peptide conformation would be positioned less than 4 Å away from the third nucleotide in the mRNA A-site codon (green). mRNA is modeled from PDB 4W2F. β-MeS, β-methylthio modification. (e) Pro45 in uS12 with a cis-peptide bond and β-methylthioaspartate in the R configuration at position 89, shown with feature-enhanced maps contoured at 2.5 and 2.2 s.d. from the mean, respectively. (f) Model of mRNA (PDB 4W2F) superimposed on the E. coli high-resolution structure, in which protein uS12 in the decoding site is modeled with a trans-3-hydroxyproline at position 45. The 3-hydroxy group of Pro45 would be within hydrogen-bonding distance of the O3′ of the third position of the A-site codon.
Intriguingly, many of these are supported by phylogenetic analysis as being nearly universal in bacteria or across all domains of life (Supplementary Table 11). For example, U960 of 16S rRNA adopts a syn conformation to form a reverse U-U base pair with U956 that stabilizes a tight triloop located at the base of helix h31 (Fig. 4b,c); this loop plays an active part in the movement of A-site tRNA into the P site on the small ribosomal subunit during translocation56. In the 50S subunit, U1779 assumes a syn conformation to form an unusual reverse Hoogsteen A-U base pair with A1784 in the loop capping helix H65 of 23S rRNA. U1781 of this loop forms part of the peptido-exit tunnel and lines the binding pocket for several antibiotics (Fig. 4d).

**DISCUSSION**

The new high-resolution structure of the *E. coli* 70S ribosome presented here provides a foundation for unraveling key mechanisms shared by ribosomes in all domains of life. For example, the geometry of ribosomal protein uS12 near the mRNA-decoding site suggests a model for how hydroxylation of Pro45 could influence the energetics of mRNA decoding and mRNA and tRNA translocation. Sites of syn-pyrimidines, rare in most RNA contexts, reveal conserved base-pair patterns that could contribute to rRNA tertiary folding and key ribosomal functions, such as tRNA translocation56. Structural knowledge of clustered rRNA and protein modifications near functional centers can now be used to test the mechanisms of fine-tuning translation that are likely to confer a selective advantage to microbes in their natural habitats.

The present *E. coli* ribosome structure can also be used for comparisons to other structures of the 50S subunit and 70S ribosome. For example, the 2.2- to 2.4-Å structures of the archaeal *Haloarcula marismortui* 50S subunit42,57,58, which serve as a model for the bacterial A2058G mutation that confers antibiotic resistance, reveal that the A2058G mutation would substantially alter the architecture and solvation of the macrolide-, ketolide- and streptogramin B–binding sites. Additionally, Um2552 in the A loop of the PTC adopts the conformation in the *E. coli* 70S ribosome and the *H. marismortui* 50S subunit, with its 2'-O-methyl group wedged between the nucleobases of U2554 and G2553, which pairs with C75 in A-site tRNA52. This positioning suggests that this modification could have functional importance in modulating the base-pairing between 23S rRNA and A-site tRNA. It will be interesting in the future to compare the present structure to the recent 2.4- to 2.55-Å crystal structures of the *T. thermophilus* 70S ribosome21,22, which are probably at sufficient resolution to allow modeling of post-transcriptional and post-translational modifications and solvation.

Finally, the new high-resolution structure of the *E. coli* 70S ribosome can be used as a resource and reference for comparison to other RNA structures and future ribosome structures in other conformational states, for analyses of ribosome phylogenetics and for biochemical studies. For example, it should now be possible to probe the functional roles of pseudouridines and conserved syn-pyrimidines more directly. It is striking that, although some ordered solvent molecules could be identified in key locations at the subunit interface, e.g., in universally conserved bridge B3, most of the solvation at the subunit interface is seemingly diffuse in nature32,39, and this could be important for ribosome dynamics. Future studies of the ribosome by smFRET experiments and molecular-dynamics simulations should be helpful in deciphering the roles of ordered and diffuse water and ions in the structure and the dynamics of the ribosome.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Coordinates and structure factors have been deposited in the Protein Data Bank under accession code 4YBB.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

J.N. optimized crystal growth and cryo-stabilization procedures, measured the X-ray diffraction data, solved the structure and carried out refinement and structural analysis. J.H.D.C. assisted with data reduction, refinement and structural analysis. M.R.W., D.S.T., R.B.A. and S.C.B. conducted the smFRET experiments. J.N., J.H.D.C. and S.C.B. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Preparation of ribosome initiation complexes for smFRET experiments. Initiation complexes for the smFRET inter-subunit rotation assay were formed with E. coli S313 (N-Sp) 3OS and 35S subunits and initiated with unlabeled tRNA^Met^GTP^Phe-tRNAPhe^Cy5-acp3U47) (for the tRNA hybrid state assay) for 2 min in Tris-polyvinyl buffer, as previously described.4 Cy3 fluorophores on either uS13 or P-site tRNA were illuminated with a 532 nm diode-pumped solid-state laser (Opus, LaserQuantum). Fluorescence emission from Cy3 and Cy5 fluorophores was collected with a 1.27 N.A. PlanApo water-immersion objective (Nikon) and spectrally separated with a DualCam device (Photometrics) with a 640dcxr dichroic mirror (Chroma). smFRET data were acquired at 25 °C integration time and analyzed with analytical software implemented in MATLAB (MathWorks). FRET trajectories were idealized with the segmental k-means algorithm, as previously described.61 Experiments were performed with three technical replicates, i.e., with frozen aliquots of the same ribosome-complex preparations used on three separate days.

Ribosome crystallization and cryoprotection. Ribosomes purified from E. coli strain MRE600 (ref. 33) were crystallized as described previously.23 A highly optimized seven-step crystallization procedure was established to prepare ribosome crystals for cryocooling. This procedure ensured robust crystal-to-crystal reproducibility, allowingdiffraction data to be measured and merged from multiple crystals. Crystals were soaked in stabilization buffer containing 6–7% PEG 8000, 7% MPD, 0–24% PEG 400, 3.75–4.5 mM MgCl2, 5–5.75 mM putrescine, and 4.35–5 mM spermidine, pH 4.8–5.7. More specifically, the concentrations of the variable parameters for the individual crystallizers were as follows: stabilizer 1, 7% PEG 8000, 7% MPD, 3.75 mM MgCl2, 5 mM putrescine, 4.35 mM spermidine, 380 mM NH4Cl, and 0% PEG 400, pH 5.7; stabilizer 2, 6.7% PEG 8000, 7% MPD, 3.75 mM MgCl2, 5.75 mM putrescine, 5 mM spermidine, 380 mM NH4Cl, and PEG 400 at 6% (stabilizer 2), 9% (stabilizer 3), 12% (stabilizer 4), 15% (stabilizer 5), or 18% (stabilizer 6), pH 4.8; stabilizer 7, 6% PEG 8000, 7% MPD, 4.5 mM MgCl2, 5.75 mM putrescine, 5 mM spermidine, 380 mM NH4Cl, and 24% PEG 400, pH 5.0. Crystals were soaked in stabilizer 1 for 100 min, then soaked in each of stabilizers 2–6 for 5–10 min and stored overnight in stabilizer 7.

X-ray diffraction data measurement. Diffraction data were measured from crystals cooled to 100 K with 0.1° oscillations at beamline 8.1.3.1 at the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory (LBNL), which is equipped with an ADSC Q315r detector. In order to reduce diffraction spot overlap due to the large unit-cell size of the ribosome crystals, crystals were adjusted to a position in which the c-axis ran parallel to the detector, i.e., normal to the X-ray beam. Furthermore, the crystal-to-detector distance was increased to 650 mm, and the slit width was reduced to ~0.35 mm during X-ray diffraction data collection. With these settings and exposure times of 3 min, diffraction spots to at least 2 Å could be observed. These were captured by a face-normal offset of the detector by about 17.5°. Diffraction data frames usually spanning only a total of 0.6° were indexed, integrated and scaled with XDS65. Over 470 data sets collected from 109 crystals were used.

Structure model refinement. Structure-factor phases were obtained by molecular replacement with the phenix.refine component of PHENIX65 and the following PDB entries as model input with mRNA, tRNAs, and ribosome recycling factor removed: 3RS8, 3RT 4GD1 and 4GD2. The resulting model was manually improved with Coot68 and subsequent multiple rounds of positional, atomic displacement parameter (ADP), and TLS refinement with the phenix.refine component of PHENIX. In order to improve the model of the second ribosome in the asymmetric unit, coordinate information of the first ribosome was used as a reference model during refinement.

For initial model building, electron density maps were calculated with structure factors from PHENIX refinements sharpened by application of a negative B factor of ~50 Å2, and with the figure of merit dampened by application of a positive B factor of 50 Å2. The resulting structure factors and figures of merit were subjected to density modification in DM69, with a resolution range of 30–2.1 Å and Sayre’s equation70,71. Finally, the DM-modified structure factors and figures of merit were used to calculate sharpened Fo – Fc and Fc – Fo electron density maps. Homology models for various parts of the ribosome were used to guide manual rebuilding22–27 (including PDB 3A1P). Subsequent refinement and model building used feature-enhanced maps generated by phenix.fem in the PHENIX 1.9-1692 release, with default parameters plus the combined omit-map algorithm72. All maps presented in the figures use feature-enhanced maps.

MolProbity analyses were carried out as follows. Prior to running the MolProbity web server, nucleotides 77–93, 841–847, 999–1012 and 1016–1042 were removed from 16S RNA in the 3OS subunit, owing to disorder. From the 5OS subunit, E. coli ribosomal proteins L1, L9, L10, L11, and nucleotides 883–897, 1057–1090 and 2099–2188 of 23S RNA were removed, owing to their high degree of disorder. High-resolution ribosome 1L in the present structure and PDB 4U26 for the 2.8 Å structure73 were used for the analysis.

Phylogenetic comparisons of nucleotides in the ribosome were carried out with the Comparative RNA Web Site and Project (http://www.rna.icmb.utexas.edu/)70. The comparative RNA web (CRW) site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. BMC Bioinformatics 3, 2 (2002).