Late steps in bacterial translation initiation visualized using time-resolved cryo-EM

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The initiation of bacterial translation involves the tightly regulated joining of the 50S ribosomal subunit to an initiator transfer RNA (fMet-tRNA\textsuperscript{fMet})-containing 30S ribosomal initiation complex to form a 70S initiation complex, which subsequently matures into a 70S elongation-competent complex. Rapid and accurate formation of the 70S initiation complex is promoted by initiation factors, which must dissociate from the 30S initiation complex before the resulting 70S elongation-competent complex can begin the elongation of translation\textsuperscript{1}. Although comparisons of the structures of the 30S\textsubscript{2–5} and 70S\textsubscript{4,6–8} initiation complexes have revealed that the ribosome, initiation factors and fMet-tRNA\textsuperscript{fMet} can acquire different conformations in these complexes, the timing of conformational changes during formation of the 70S initiation complex, the structures of any intermediates formed during these rearrangements, and the contributions that these dynamics might make to the mechanism and regulation of initiation remain unknown. Moreover, the absence of a structure of the 70S elongation-competent complex formed via an initiation-factor-catalysed reaction has precluded an understanding of the rearrangements to the ribosome, initiation factors and fMet-tRNA\textsuperscript{fMet} that occur during maturation of a 70S initiation complex into a 70S elongation-competent complex. Here, using time-resolved cryogenic electron microscopy\textsuperscript{9}, we report the near-atomic-resolution view of how a time-ordered series of conformational changes drive and regulate subunit joining, initiation factor dissociation and fMet-tRNA\textsuperscript{fMet} positioning during formation of the 70S elongation-competent complex. Our results demonstrate the power of time-resolved cryogenic electron microscopy to determine how a time-ordered series of conformational changes contribute to the mechanism and regulation of one of the most fundamental processes in biology.

Initiation of translation is a fundamental step in gene expression that is essential for the overall fitness and viability of cells. In bacteria, the dynamic initiation reaction is kinetically controlled by three initiation factors (IF1; the guanosine triphosphatase IF2; and IF3), which collaborate to ensure accurate selection of fMet-tRNA\textsuperscript{fMet} and its pairing with the mRNA start codon\textsuperscript{10–13}. Canonical initiation begins with assembly of the 30S complex (IC), followed by IF2-catalysed joining of the 50S subunit to the 30S IC to form a 70S IC, and finally maturation of the 70S IC into a 70S elongation-competent complex (EC)\textsuperscript{11,14,15}. Given the essential nature of this process, structural intermediates that form during initiation in bacteria represent promising targets for the development of next-generation antibiotics\textsuperscript{16–18}. Ensemble rapid kinetic and single-molecule studies have led to the identification and characterization of several intermediate steps during the late stages of initiation. These studies have shown that subunit joining triggers the rapid hydrolysis of GTP by IF2\textsuperscript{1,12,14–21}, dissociation of the initiation factors\textsuperscript{1,12,19}, transition of the ribosomal subunits into their non-rotated inter-subunit orientation\textsuperscript{22,23}, and accommodation of fMet-tRNA\textsuperscript{fMet} into the peptidyl-tRNA binding site of the peptidyl transferase centre (PTC)\textsuperscript{24}. In addition, structures of various 30S\textsubscript{2,5–18} and 70S ICs\textsubscript{4,6–8} obtained by cryogenic electron microscopy (cryo-EM) have revealed intermediate ICs that vary in the conformation of the ribosome, initiation factors and fMet-tRNA\textsuperscript{fMet}. Nonetheless, notable discrepancies in the inter-subunit orientation of the ribosome and position of fMet-tRNA\textsuperscript{fMet} in several of the available structures of the 70S IC have made it difficult to arrive at a consensus structural model for initiation\textsuperscript{4,6–8}. Furthermore, the 70S ICs represented by the available structures were formed using a 70S ribosome and an IF2 bound to a non-hydrolysable GTP analogue (for example, GDPNP) that results in a biochemically trapped 70S IC, rather than by mixing the 50S subunit with a 30S IC that carries a native, GTP-bound IF2 and results in the formation of a 70S IC, which subsequently matures into a 70S EC. Consequently, the available 70S IC structures do not provide information about how the various structural intermediates that have been observed evolve over the course of the initiation reaction. Therefore, these structural studies have been unable to distinguish on-pathway intermediates formed during canonical initiation from spurious, off-pathway intermediates.

To circumvent this problem, and to capture transient, on-pathway intermediates that are created during canonical translation initiation, we have used mixing-spraying time-resolved cryo-EM\textsuperscript{9,25–27}. Previously, we have used this method to study the association of vacant 30S and 50S subunits to form 70S ribosomes\textsuperscript{25}, and to visualize transient structural intermediates formed during the ribosome recycling process\textsuperscript{26}. Having demonstrated that passage through the microfluidic device does not damage the 30S IC (Methods and Extended Data Fig. 1a, b), here we have used mixing-spraying time-resolved cryo-EM to investigate the initiation-factor-catalysed joining of the 70S IC with the 50S subunit to form a transient 70S IC that matures into a 70S EC. Using this approach, we have visualized, in real time and with near-atomic spatial resolution, the conformational rearrangements of the 30S and 70S ICs that promote and control subunit joining, initiation factor dissociation, and fMet-tRNA\textsuperscript{fMet} positioning during 70S EC formation.

Ensemble rapid kinetic studies suggest that transient intermediates formed during initiation are populated on the sub-second timescale\textsuperscript{16,18,14,19–23}. Using published rate constants\textsuperscript{19}, we developed a kinetic model and analysed how the populations of the expected structural species were predicted to vary as a function of time during subunit joining reactions in which 50S subunits were mixed with 30S ICs (Extended Data Fig. 1c and Supplementary Methods). The analysis predicts that the size of the population of 70S ICs carrying a GTP-bound or GDP-P\textsubscript{i}-bound IF2 is maximized at approximately 150 ms, and that joining of the 50S subunit to the 30S IC to form a mature 70S EC is around 65% complete within 600 ms. Using a set of microfluidic chips designed\textsuperscript{27} to provide reaction times of approximately 20 ms, 80 ms, 200 ms and 600 ms in our mixing-spraying time-resolved cryo-EM apparatus (Extended Data Fig. 2), we therefore mixed 50S subunits with 30S ICs and collected images at each time point. At each time point, two-dimensional (2D) classification of the images yielded 30S subunit-like, 50S subunit-like, and 70S ribosome-like particle classes. Subsequently, the particles from the 20-ms, 80-ms, 200-ms and 600-ms time points were combined into two datasets. The first
dataset, containing the 30S subunit-like particles, was subjected to 3D classification. The second dataset, containing 50S subunit-like and 70S ribosome-like particles, was subjected to a combination of 3D and 2D classification to sort out the compositional and conformational heterogeneity (Methods and Extended Data Fig. 3). This classification scheme yielded the structures of five distinct classes: (1) a complex containing the 30S subunit, mRNA and tRNA\(^{\text{Met}}\), but lacking IF1 and IF2; (2) the 30S IC; (3) the 50S subunit; (4) the 70S IC; and (5) the 70S EC. Execution of an independent, masked classification strategy failed to find any additional rare and/or low-population intermediate conformations of the 70S IC or 70S EC, confirming that our classification scheme did not miss any such states (Methods and Extended Data Fig. 4).

Notably, the sizes of populations of the 50S subunit, 70S IC and 70S EC (Table 1) obtained from our classification strategy qualitatively follow the predicted kinetics (Fig. 1a and Extended Data Fig. 1c), with the population of the 50S subunit decreasing as the population of the 70S EC increases from around 20 ms to 600 ms (Extended Data Fig. 1c). Moreover, the particle populations reported in Fig. 1a and Extended Data Fig. 1c and the structures of the corresponding particle classes are robust to the inclusion of up to 20% noise particles falsely picked from the background (Extended Data Fig. 5, Supplementary Methods, Supplementary Tables 1 and 2).

Among the five particle classes that we obtained, we selected the 30S IC, 70S IC and 70S EC for further structural analysis (Fig. 1b, c, 2a). The 70S IC structure reported here is obtained by mixing the 50S subunit with a 30S IC carrying a native, GTP-bound IF2, and the 70S EC structure is obtained directly from a 70S IC formed by an initiation-factor-catalysed initiation reaction. The resolutions of the 30S IC, 70S IC and 70S EC were estimated to be 4.2 Å, 4.0 Å and 3.9 Å, respectively, according to a resolution-estimating protocol that avoids overfitting and uses the Fourier shell correlation (FSC) with the 0.143 criterion\(^b\) (Extended Data Fig. 6). Molecular dynamics flexible fitting (MDFF)\(^b\) was then used to generate structural models of the 30S IC and 70S IC, and rigid-body fitting of previously published structures of the 30S and 50S subunits (Protein Data Bank (PDB) codes 2AVY and 2AW4, respectively) was used to generate a structural model of the 70S EC (Methods).

Analysis of the 70S ICs that are formed within the first 20–80 ms after mixing 50S subunits with 30S ICs shows that all inter-subunit bridges are formed. Moreover, we find that IF1 has also dissociated from these 70S ICs (compare Fig. 2a and b). This observation is important because IF1 occupies a binding site between the cleft of 16S rRNA helix (h) 44, h18, and ribosomal protein uS12 on the 30S subunit that enables turn 1 of IF1, consisting of residues 18–21, to establish contacts with the minor groove of h44. Consequently, dissociation of IF1 relieves a turn of IF1, consisting of residues 18–21, to establish contacts with IF1 during 70S IC formation increases the conformational freedom of IF1 during 70S IC formation.

By the time 80 ms has elapsed, the population of the 70S IC has reached its maximum and, by 200 ms, IF2 has dissociated from a notable fraction of this 70S IC population, resulting in the formation of mature 70S ECs, a process that continues through the 600-ms time point and beyond. Notably, the 70S IC that is captured in this study

Table 1 | Populations of the 50S subunit, 70S IC and 70S EC obtained after 3D classification

|          | 50S (%) | 70S IC (%) | 70S EC (%) |
|----------|---------|------------|------------|
| 20 ms    | 58.0±0.7| 35.0±0.8   | 7.1±0.8    |
| 80 ms    | 36.0±3.4| 43.4±1.7   | 20.6±1.7   |
| 200 ms   | 30.8±5.6| 12.7±3.4   | 56.4±3.4   |
| 600 ms   | 28.4±2.1| 8.1±2.0    | 63.6±2.0   |

Standard deviations were obtained by repeating the 3D classification procedure three times for each time point.

Fig. 1 | Structural and time-resolved population analyses of the 50S subunit, 70S IC and 70S EC. a, The populations of the 50S subunit, 70S IC and 70S EC at the 20 ms, 80 ms, 200 ms and 600 ms time points as obtained by 3D classification of the imaged particles. Error bars represent standard deviations obtained by repeating the 3D classification procedure three times for each time point. b, c, The cryo-EM reconstruction (that is, cryo-EM–derived Coulomb potential maps\(^c\)) of the 70S IC (b) and 70S EC (c).
and orange, respectively), and IF2 and fMet-tRNA\textsuperscript{fMet} from the 70S IC (dark during 70S IC formation.

Ribosome, initiation factor and fMet-tRNA\textsuperscript{fMet} dynamics during 70S IC formation. a, b, Cryo-EM reconstructions viewed from the inter-subunit faces of the 30S IC (with the 30S subunit shown in yellow) (a) and the 30S subunit (pale yellow), IF2 and fMet-tRNA\textsuperscript{fMet} from the 70S IC (b). c, Superposition of the 30S subunits of the 30S IC and the 70S IC and analysis of the conformations of the 30S subunit and IF1 (shown in magenta) from the 30S IC and the 50S subunit from the 70S IC. The analysis reveals that rapid dissociation of IF1 after joining of the 30S subunit to the 30S IC relieves a potential steric clash between IF1 and the 50S subunit that would take place during 70S IC formation. d, A magnified view of the superposition shown in c highlights the potential steric clash between turn 1 of IF1 and H69 of the 50S subunit. e, Superposition of the 30S subunits from the 30S IC and the 70S IC, and comparative analysis of the conformations of IF2 and fMet-tRNA\textsuperscript{fMet} from the 30S IC (light purple and orange, respectively), and IF2 and fMet-tRNA\textsuperscript{fMet} from the 70S IC (dark purple and green, respectively). The analysis reveals that d\textsuperscript{IV} of IF2 moves towards the inter-subunit face of the 30S subunit by approximately 10 Å and, as it rearranges from its 30S P/I to its 70S P/I configuration, the central domain of fMet-tRNA\textsuperscript{fMet} moves by around 28 Å towards the P site. A 180° rotation of the superposition shown in c highlights the untangling of the 3′ CCA fMet tail of the fMet-tRNA\textsuperscript{fMet} and its 22 Å movement into the PTC.

The non-hydrolysable GTP-analogue-form of IF2 reported in all of the other 70S IC structures that have been published\textsuperscript{4,6–8} suggests that when IF2 hydrolyses GTP, it does not immediately undergo a conformational change. This indicates that the transition from the 70S IC to the 70S EC is largely regulated by the release of P\textsubscript{i} from IF2 and/or the subsequently rapid release of the GDP-form of IF2 from the 70S IC.

As the 70S IC matures into a 70S EC, dissociation of IF2 disrupts the IF2–ribosome interactions that stabilize the semi-rotated inter-subunit orientation of the 70S IC. Disruption of these IF2–ribosome interactions therefore triggers the reverse rotation of the 30S subunit by approximately 3° (Fig. 3a), which allows the 70S ribosome within the 70S EC to occupy the non-rotated inter-subunit orientation. Dissociation of IF2 also disrupts the contact between d\textsuperscript{IV} of IF2 and fMet-tRNA\textsuperscript{fMet}—an event that, simultaneously with the reverse rotation of the 30S subunit (at least at our time resolution), enables the central domain and 3′ CCA-fMet tail of fMet-tRNA\textsuperscript{fMet} to move by around 28 Å and 22 Å, respectively, from the 70S peptidyl/initiation (P/I) configuration of fMet-tRNA\textsuperscript{fMet} that is observed in the 70S IC to the peptidyl/peptidyl (P/P) configuration of fMet-tRNA\textsuperscript{fMet} that is observed in the 70S EC (Fig. 3b–d). This rearrangement of fMet-tRNA\textsuperscript{fMet} is accompanied by an ‘untangling’ of the 3′ CCA-fMet tail that allows the fMet moiety to acquire its peptidyl-transfer-competent position within the P site of the PTC (Fig. 3c, d). Given the simultaneous nature of these conformational changes, at least at our time resolution, we propose that the transition of the 70S ribosome into its non-rotated inter-subunit orientation is coupled to the rearrangement...
of fMet-tRNA\textsuperscript{Met} into its P/P configuration in the 70S EC, along with the untangling of the 3\textsuperscript{′}CCA–fMet tail and positioning of the fMet moiety into the PTC.

Here, we have shown how mixing-spraying, time-resolved cryo-EM is able to capture physiologically relevant, short-lived, structural intermediates in a biomolecular reaction, and have used this approach to determine the molecular mechanism of bacterial translation. On the basis of our collective observations, we propose a structure-based model for the late steps of bacterial translation initiation (Fig. 4). Notably, we did not observe formation of the minor population of the 70S IC reported previously (that is, 70S–IC I\textsuperscript{8}), suggesting that this conformation of the 70S IC might represent an off-pathway intermediate that is formed only when the 70S IC is trapped when using the GDPNP-form of IF2 and/or prepared using a steady-state approach. By contrast, because the conformation of the 70S IC that we observe here was obtained using the native, GTP-bound form of IF2 under pre-steady-state conditions, we can be certain that it represents an intermediate that is formed on the initiation reaction pathway. Mixing-spraying, time-resolved cryo-EM is a new and powerful structural biology technique that we expect will be used to follow the formation and maturation of reaction intermediates and elucidate the molecular mechanisms of fundamental biomolecular reactions such as DNA replication, transcription, precursor mRNA processing and splicing, and mRNA and protein degradation.

**Online content**

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1. Antoun, A., Pavlov, M. Y., Andersson, K., Tenson, T. & Ehrenberg, M. The roles of initiation factor 2 and guanosine triphosphate in initiation of protein synthesis. *EMBO J.* 22, 5593–5601 (2003).
2. Hussain, T., Iacon, J. L., Wimberly, B. T., Kieft, J. S. & Ramakrishnan, V. Large-scale movements of IF3 and tRNA during bacterial translation initiation. *Cell* 167, 133–144.e13 (2016).
3. Julían, P. et al. The Cryo-EM structure of a complete 30S translation initiation complex from *Escherichia coli*. *Proc. Natl Acad. Sci. USA* 109, 15656–15661 (2013).
4. Simonetti, A. et al. Structure of the 30S translation initiation complex. *Nature* 455, 416–420 (2008).
5. Simonetti, A. et al. Structure-based kinetic model for late steps in bacterial translation initiation. *Cartoon depicting the timing of structural and mechanical events that occur during the late stages of bacterial translation initiation. Within the first 20 ms after mixing 50S subunits and 30S ICs, 50S subunits (blue) reversibly join to most 30S ICs (yellow) to form transient pre-70S ICs. Conversion of most of these pre-70S ICs into 70S ICs takes place within 20–80 ms after mixing of 50S subunits and 30S ICs and begins with the rapid hydrolysis of GTP on IF2 (light purple). GTP hydrolysis is followed by the dissociation of IF1 (magenta), repositioning of dIV of IF2 (dark purple), and formation of IF2–ribosome interactions and inter-subunit bridges that stabilize the ribosome in its semi-rotated inter-subunit orientation and the fMet-tRNA\textsuperscript{Met} in its 70S P/I configuration. Within the next several hundred milliseconds, most 70S ICs mature into 70S ECs in a process that begins with the release of P\textsubscript{i} from IF2 and dissociation of the GDP-form of IF2 from the 70S IC—events that enable the rotation of the ribosomal subunits into their non-rotated inter-subunit orientation, rearrangement of fMet-tRNA\textsuperscript{Met} into its P/P configuration, untangling of the 3\textsuperscript{′}CCA–fMet tail of fMet-tRNA\textsuperscript{Met}, and relocation of the fMet moiety of fMet-tRNA\textsuperscript{Met} into the PTC in preparation for formation of the first peptide bond after delivery of the first aminoacyl-tRNA into the ribosomal aminoacyl-tRNA binding (A) site.*
28. Chen, S. et al. High-resolution noise substitution to measure overfitting and validate resolution in 3D structure determination by single particle electron cryomicroscopy. Ultramicroscopy 135, 24–35 (2013).

29. Trabuco, L. G., Villa, E., Mitra, K., Frank, J. & Schulten, K. Flexible fitting of atomic structures into electron microscopy maps using molecular dynamics. Structure 16, 673–683 (2008).

30. Wang, J., Liu, Z., Frank, J. & Moore, P. B. Identification of ions in experimental electrostatic potential maps. IUCrJ 5, 375–381 (2018).

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METHODS
Preparation, purification and validation of IC components and the 30S IC. 30S and 50S subunits were purified from the MRE600 Escherichia coli strain as previously described, with minor modifications.15,16,18,19,20 Tight-coupled 70S ribosomes were isolated by ultracentrifugation of crude ribosomes through a 10–40% sucrose density gradient prepared in ribosome storage buffer (10 mM Tris(hydroxymethyl) aminomethane (Tris)-acetate buffer (pH 7.5 at 4 °C), 60 mM NH4Cl, 7.5 mM MgCl2, 0.5 mM EDTA, 6 mM 2-mercaptoethanol (BME)). To maximize the purity of our tight-coupled 70S ribosomes and minimize contamination by free 50S subunits, a second round of ultracentrifugation through a 10–40% sucrose density gradient prepared in ribosome storage buffer was added to our standard ribosome purification protocol. Highly pure, tight-coupled 70S ribosomes were buffer-exchanged into ribosome dissociation buffer (10 mM Tris-acetate (pH 7.5 at 4 °C), 60 mM NH4Cl, 1 mM MgCl2, 0.5 mM EDTA, 6 mM BME) using a centrifugal filtration device (Amicon Ultra, Millipore) with a 100-kDa molecular mass cut-off to promote the dissociation of ribosomes into 30S and 50S subunits. 30S and 50S subunits were isolated from the dissociated tight-coupled 70S ribosomes by ultracentrifugation through a 10–40% sucrose density gradient prepared in ribosome dissociation buffer. To ensure high purity, 30S and 50S subunits isolated from the first gradient were subjected to a second round of ultracentrifugation through a 10–40% sucrose density gradient prepared in ribosome dissociation buffer. Highly purified 30S and 50S subunits were concentrated and buffer-exchanged into ribosome storage buffer using a centrifugal filtration device with a 100-kDa molecular mass cut-off. After determining the concentration of the 30S and 50S subunits, small aliquots were prepared, flash frozen in liquid nitrogen, and stored at −80 °C.

Electron microscopy. The purity of our 30S and 50S subunits was confirmed by negative staining electron microscopy (EM). In brief, one aliquot of the highly purified 30S subunits was diluted to 50 nM Tris-polymer buffer (50 mM Tris-acetate (pH 7.5 at room temperature), 100 mM KCl, 5 mM ammonium acetate, 0.5 mM calcium acetate, 5 mM magnesium acetate, 0.1 mM EDTA, 6 mM BME, 5 mM putrescine dihydrochloride, and 1 mM spermidine, free base). Subsequently, 3 µl of this 50 nM 30S subunit solution was applied to a carbon-coated EM grid for 30 s. Any excess sample solution was wicked away from the EM grid using filter paper, thereby generating a thin layer of sample solution on the EM grid. Following this, 3 µl of a 2% solution of uranyl acetate in water was applied to the EM grid and the EM grid was incubated for 30 s at room temperature. Excess sample solution was wicked away from the EM grid using filter paper, once again generating a thin layer of sample solution on the EM grid. This uranyl acetate, negative staining procedure was repeated two more times and, subsequently, the negatively stained 30S subunits were imaged using a 200 kV F20 cryogenic transmission electron microscope (TEM; FEI). Visual inspection of the images that were obtained revealed a highly uniform set of particles exhibiting the characteristically elongated shape of the 30S subunit, thereby demonstrating the purity of the 30S subunits. Analogous procedures were followed to load, negatively stain and image the highly purified 50S subunits, with visual inspection of the images revealing a highly uniform set of particles exhibiting the characteristic ‘crown view’ of the 50S subunit, thereby demonstrating the purity of the 50S subunits.

IF1 and the γ-isoform of IF2 containing tobacco etch virus (TEV) protease-cleavable, N-terminal, hexa-histidine (6×His) tags were overexpressed in BL21 (DE3) cells and purified as described previously.15,16,18,19,20 In brief, 6×His-tagged initiation factors were purified by nickel nitrilotriacetic acid (Ni2+NTA) affinity chromatography using a batch-binding and elution protocol. After elution of the 6×His-tagged initiation factors, the 6×His-tags were removed by adding TEV protease to the purified initiation factors and dialysing the mixture over-night (around 12 h) at 4 °C against TEV cleavage buffer (20 mM Tris-HCl (pH 7.5 at 4 °C), 200 mM NaCl, 0.1% Triton X-100, and 2 mM BME). IF1 was further purified on a HiLoad 16/60 Superdex 75 prep grade gel filtration column (GE Biosciences, and IF2 was further purified on a HiTrap SP HP cation-exchange column (GE Biosciences). The purified initiation factors were concentrated and buffer exchanged into buffer exchanged into 468, 605, 445 and 363 micrographs were selected at 20, 80, 200 and 600 ms, respectively. The beam-induced motion of the sample captured by the images was corrected using the MotionCor2 software program.33 The contrast transfer function (CTF) of each micrograph was estimated using the CTFFIND4 software program.34 Image particles were picked using the AutoPicker algorithm included in the RELION 2.0 software program.35 These particles were first extracted using 2×binning of the images and subjected to 2D classification to separate 30S subunit-like, 50S subunit-like, and 70S ribosome-like particles from ice-like and/or debris-like particles picked by the Autopicker algorithm. Exclusion of ice-like and debris-like particles resulted in totals of 79,204, 109,775, 59,350 and 47,996 30S subunit-like, 50S subunit-like, or 70S ribosome-like particles at 20, 80, 200 and 600 ms, respectively. All particles classified as 30S subunit-like were pooled together into a set of 170,864 particles, and all particles classified as 50S subunit-like or 70S ribosome-like were pooled together into a set of 144,504 particles. The reason the 50S subunit-like and 70S ribosome-like particles were pooled together is that some of the 70S ribosome-like particles have the appearance of 50S subunit-like particles in particular viewing directions, so separation of these particles must be deferred to the next step.

Cryo-EM data processing. A flow-chart of the data processing protocol is shown in Extended Data Fig. 3. Micrographs were first screened at each time point, then 468, 605, 445 and 363 micrographs were selected at 20, 80, 200 and 600 ms, respectively. The beam-induced motion of the sample captured by the images was corrected using the MotionCor2 software program. The contrast transfer function (CTF) of each micrograph was estimated using the CTFFIND4 software program. Images were recorded over a defocus range of 1–3 µm on a K2 direct detector camera (Gatan) operating in counting mode with an effective magnification of 29,000× at 1.66 Å per pixel. Images were composed of 40 frames that were exposed for a total of 12 s, corresponding to a total dose of 35 e− Å−2.

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and deacylated tRNA\(^{\text{fMet}}\) in the P/I configuration. The subclass containing the 30S ICs was further refined without binning the images. The resolution of the refined 30S IC was estimated to be 4.2 Å using a resolution-estimating protocol that avoids overfitting and uses the FSC with the FSC = 0.143 criterion\(^{28}\).

The set of 144,504 combined 50S subunit-like and 70S ribosome-like particles was subjected to 3D classification, from which we obtained two major subclasses. The first subclass encompassed 50S subunits and the second encompassed 70S ribosome-like particles. We found it necessary to subject this second subclass to a second round of 2D classification because of evidence of residual compositional heterogeneity. In this step, some 50S subunits were still found, and separated from 80,138 remaining 70S ribosome-like particles. The 50S subunits obtained from this second round of 2D classification were combined with the 50S subunits from the first round of 3D classification for a total of 50,918 50S subunits.

The whole process of two rounds of 2D classification and 3D classification was repeated three times to estimate the errors associated with classifying the set of particles into 50S subunit and 70S ribosome-like particle populations (Table 1). At this point in the analysis, each 50S subunit and 70S ribosome-like particle was traced back to the time point from which it originated to determine the 50S subunit and 70S ribosome-like particle populations at each time point. The 70S ribosome-like subclass with 80,138 particles was then subjected to a round of 3D classification from which we obtained two major subclasses. The first subclass encompassed 34,096 70S ICs and the second encompassed 46,042 70S ECs. Again, this third round of 3D classification was repeated three times in order to estimate the errors associated with classifying the set of particles into 70S IC and 70S EC populations (Table 1). The subclasses containing the 70S ICs and 70S ECs were then further refined without binning the images. The Fourier amplitudes of the refined cryo-EM maps were sharpened using the ‘postprocess’ command in RELION. The resolutions of the 70S IC and 70S EC maps were estimated to be 4.0 Å and 3.9 Å, respectively, using a resolution-estimating protocol that avoids overfitting and uses the FSC with the FSC = 0.143 criterion\(^{28}\).

The percentage of particles in the 50S subunit, 70S IC, and 70S EC particle classes at each time point was calculated by summing up the number of particles in each particle class at each time point and subsequently calculating the fraction of particles in each particle class with respect to the total number of particles at each time point. To compare the percentages of particles in the 50S subunit, 70S IC and 70S EC particle classes obtained here with the concentrations of 50S subunits, 70S ICs and 70S ECs predicted by the kinetic modelling, the percentages obtained here were used to calculate the concentration of each particle class, assuming the total concentration of particles in the kinetic modelling was limited to 0.6 μM (that is, the limiting concentration of 50S subunits used in the kinetic modelling) (Extended Data Fig. 1c).

Additional 3D classification to find low-population intermediate conformations from the 70S particle dataset. We used masked 3D classification scheme on a dataset of 80,138 70S ribosome particles to search for rare conformations of 70S IC and 70S EC. In the masked 3D classification scheme, a mask was designed covering densities of IF1, IF2, P/P-configured P-site tRNA and P/E-configured P-site tRNA (Extended Data Fig. 4a–c). The dataset of 80,138 70S particles was subjected to 3D refinement to assign angular positions, and particle alignment was turned off during the masked classification scheme. Three types of class were obtained. The first type of classes encompasses 44% of the particles with density for IF2 and tRNA in the P/I position, and the second type of classes encompasses 48% with density for tRNA in the P/I position. The third type of classes encompasses approximately 8% without any density in the masked region. The 3D refinement of the first, second and third types of classes yielded cryo-EM maps of 70S IC, 70S EC and low-resolution 70S EC, respectively (Extended Data Fig. 4d).

Modelling of the 30S IC, 70S IC and 70S EC structures. We obtained near-atomic resolution models of the 30S IC and 70S IC by using the molecular dynamics flexible fitting (MDFF) method\(^{29}\) (Extended Data Fig. 9) and an atomic, cryo-EM-derived model of a 70S IC (PDB code 3JCJ) as the initial starting model. Similarly, we obtained an initial, near-atomic resolution model of the 70S EC using rigid-body fitting within the UCSF Chimera software program\(^{49}\) and atomic-resolution models of 70S ribosomes in the non-rotated inter-subunit orientation and lacking any tRNA or mRNA ligands (PDB codes 2AVY and 2AW4). This initial, near-atomic-resolution model of the 70S EC was further refined by subjecting it to the ‘jiggle fit’ algorithm within the COOT software program\(^{44}\) to obtain the final atomic coordinates.

**Data availability**

The cryo-EM reconstruction maps have been deposited in the Electron Microscopy Data Bank (EMDB) server under the accession codes EMDB-0643 (30S IC), EMDB-0662 (70S IC) and EMDB-0661 (70S EC). The structural models obtained by MDFF have been deposited in the Protein Data Bank (PDB) server under accession codes 607K (30S IC) and 609K (70S IC). The structural model obtained by rigid-body fitting has been deposited in the PDB server under accession code 609J (70S EC).

**Code availability**

A pseudocode describing the control actions of the software synchronizing time-resolved cryo-EM apparatus is available upon request.

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41. Rohou, A. & Grigorieff, N. CTFFIND4: fast and accurate defocus estimation from electron micrographs. *J. Struct. Biol.* **180**, 519–530 (2012).

42. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612 (2004).

43. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132 (2004).

44. Kaledhonkar, S., Fu, Z., White, H. & Frank, J. in *Methods and Protocols* (ed. Marsh, J. A.) 59–71 (Humana, 2018).

45. Kaledhonkar, S., Fu, Z., White, H. & Frank, J. in *Methods and Protocols* (ed. Marsh, J. A.) 59–71 (Humana, 2018).

46. Tan, Y.Z., Baldwin, P.R., Davis, J.H., Williamson, J.R., Potter, C.S., Carragher, B. & Lyumkis, D. Addressing preferred specimen orientation in single-particle cryo-electron microscopy. *Nat. Struct. Mol. Biol.* **14**, 793–796 (2017).

47. Fabre, M. & Grall, J. Jr. Structural and biochemical characterization of the GTP-S-, GDP·Pi-, and GDP-bound forms of translation initiation factor IF3 during transition from the 30 S initiation complex to the 70 S initiation complex. *J. Mol. Biol.* **373**, 551–561 (2007).

48. Guenneguès, M. et al. Mapping the Met-tRNA\(^{\text{fMet}}\) binding site of initiation factor IF2. *EMBO J.* **19**, 5233–5240 (2000).

49. Rohou, A. & Grigorieff, N. CTFFIND4: fast and accurate defocus estimation from electron micrographs. *J. Struct. Biol.* **192**, 216–221 (2015).

50. Scheres, S. H. W. RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J. Struct. Biol.* **180**, 519–530 (2012).

51. Antoun, A., Pavlov, M. Y., Lovmar, M. & Ehrenberg, M. How initiation factors tune the rate of initiation of protein synthesis in bacteria. *EMBO J.* **25**, 2593–2590 (2006).

52. Grigoridou, C., Marzi, S., Pan, D., Gualerzi, C. O. & Cooperman, B. S. The translational fidelity function of IF3 during transition from the 30 S initiation complex to the 70 S initiation complex. *J. Mol. Biol.* **253**, 1–12 (2005).

53. Russo, C. J. & Passmore, L. A. Electron microscopy: ultrastable gold substrates for electron cryomicroscopy. *Science* **346**, 1377–1380 (2014).

54. Zhang, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat. Methods* **14**, 331–332 (2017).

55. Rohou, A. & Grigorieff, N. CTFIND4: fast and accurate defocus estimation from electron micrographs. *J. Struct. Biol.* **192**, 216–221 (2015).

56. Scheres, S. H. W. RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J. Struct. Biol.* **180**, 519–530 (2012).

57. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612 (2004).

58. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132 (2004).

59. Kaledhonkar, S., Fu, Z., White, H. & Frank, J. in *Methods and Protocols* (ed. Marsh, J. A.) 59–71 (Humana, 2018).

60. Tan, Y.Z., Baldwin, P.R., Davis, J.H., Williamson, J.R., Potter, C.S., Carragher, B. & Lyumkis, D. Addressing preferred specimen orientation in single-particle cryo-electron microscopy. *Nat. Struct. Mol. Biol.* **14**, 793–796 (2017).

61. Raw, A. S., Coleman, D. E., Gilman, A. G. & Sprang, S. R. Structural and biochemical characterization of the GTP-S-, GDP-P-, and GDP-bound forms of a GTPase-deficient Gly\(^{\text{36}}\) – Val mutant of Gα\(_{i1}\). *Biochemistry* **36**, 15660–15669 (1997).
**Extended Data Fig. 1 | Cryo-EM reconstructions.** a, b, 3D cryo-EM-derived Coulomb potential maps\(^\text{30}\) of the 30S IC (a) and the 30S subunit + fMet-tRNA\(^{\text{fMet}}\) complex (b) obtained from a control experiment in which the 30S IC in Tris-polymix buffer and a solution of Tris-polymix buffer lacking 50S subunits were injected into the microfluidic chip designed to give the longest reaction time (~600 ms), mixed, allowed to react, and sprayed onto an electron microscopy grid that was rapidly plunged into liquid ethane. The sizes of the resulting populations of the 30S IC and the 30S subunit + fMet-tRNA\(^{\text{fMet}}\) complex were 75% and 25%, respectively, which demonstrates that most of the 30S ICs remain intact during the mixing-spraying process. c, Plot of the concentrations of the 50S subunit, 70S IC and 70S EC as a function of time generated by using the initial 50S subunit and 30S IC concentrations analogous to those used in our mixing-spraying microfluidic chip (that is, 0.6 \(\mu\)M and 1.2 \(\mu\)M, respectively) and modelling the kinetics of subunit joining using the kinetic scheme and set of rate constants reported previously for a subunit-joining reaction performed in the presence of IF1 and IF2, but in the absence of the IF3\(^{31}\). A detailed description of the kinetic modelling can be found in the Methods. The plot predicts that the 70S IC population should peak within 50–250 ms after mixing of the 50S subunit and 30S IC, and that these 70S ICs should mature to a notable population of 70S ECs within the next several hundreds of milliseconds. Therefore, to ensure that we would capture formation of the 70S IC and its maturation to the 70S EC, we selected microfluidic chips designed to provide reaction times of approximately 20 ms, 80 ms, 200 ms and 600 ms. The free 50S subunit, 70S IC and 70S EC populations observed in our time-resolved cryo-EM experiments are shown as blue diamonds, light grey circles and dark grey triangles, respectively.
Extended Data Fig. 2 | Time-resolved cryo-EM grid preparation apparatus. a, A photograph of the mixing-spraying, time-resolved cryo-EM apparatus, labelled to show all major components. The mixing-spraying microfluidic chip is mounted inside an environmentally controlled chamber. A syringe pump, which is controlled by a laboratory-written, Visual Basic and C++ software program called Howard5e45, is used to inject the reactants from inlets 1 and 2 into the microfluidic chip. Once in the microfluidic chip, the reactants are mixed and allowed to react for the reaction time specific to the microfluidic chip being used. The electron microscopy grid is held at the end of the plunger by a pair of tweezers. The Howard5e software controls and synchronizes the syringe pump as well as the plunger that holds the tweezer-mounted electron microscopy grid45. Thus, as the sprayers discharge the reaction from the microfluidic chip onto the electron microscopy grid, the plunger is activated to plunge the grid into cryogen45. b–f, Images of cryo-EM grids prepared by the mixing-spraying, time-resolved cryo-EM apparatus, going from low to high magnification. b, Grid-view depicting droplets of different sizes deposited on the grid. c, Square-view depicting droplet distribution over the holes. d, Hole-view depicting ice distribution over holes. For image acquisition, thin ice regions were selected. e, A representative micrograph showing good particle density. f, Power spectrum of the acquisition image in e.
Extended Data Fig. 3 | Flow-chart of the work process for single-particle analysis and 3D refinement. In the first step, particles were auto-picked from the images recorded for the individual time points. Auto-picked particles were then extracted using 2× binning of the images and subjected to 2D classification to discard ice-like and/or debris-like particles and define 30S subunit-like, 50S subunit-like and 70S ribosome-like particle classes. Representative 2D classes of 30S subunit-like, 50S subunit-like and 70S ribosome-like particles are shown on the left and right of the flow chart. A detailed account of the classification scheme is provided in the Methods. In brief, following 2D classification at each time point, two particle datasets were created. The first particle dataset was composed of 170,864 30S subunit-like projections, and the second was composed of 144,504 50S subunit-like and 70S ribosome-like projections. The first particle dataset with 170,864 30S subunit-like projection classes was subjected to 3D classification, which yielded two major subclasses. The first of these contained 86,367 30S ICs and the second contained 17,686 30S subunit + fMet-tRNA\(^{FMet}\) complexes. The second particle dataset, containing the 144,504 50S subunit-like and 70S ribosome-like particles, was also subjected to a combination of 3D and 2D classification to separate compositional heterogeneity consisting of the 50S subunit ribosome and 70S ribosome. After performing a combination of 3D and 2D classifications, two particle datasets were created, the first containing 50,918 50S subunit particles and the second containing 80,138 70S ribosome-like particles. Further 3D classification was performed on the dataset containing 80,138 70S ribosome-like particles, which yielded 70S IC and 70S EC classes. Particles from 50S subunit, 70S IC and 70S EC were traced back to each time point, as tabulated at the bottom of the flow chart.
Extended Data Fig. 4 | Masked classification scheme to look for rare conformations of the 70S IC and 70S EC. a–c. The mask (grey) covering densities of IF1 (magenta), IF2 (purple), P/P-tRNA (orange) and P/E-tRNA is shown in different views. The views depict the position of the mask with respect to the 30S subunit (pale yellow) and the 50S subunit (blue). d, For the masked 3D classification scheme, this mask was applied to the dataset of refined 80,138 70S particles, which yielded mostly three types of class. The first class encompasses 44% of the particles with density for IF2 (purple) and tRNA in the P/I position (green), the second class encompasses 48% with density for tRNA in the P/P position (orange), and the third class encompasses approximately 8% without any density in the masked region. The 3D refinement of the first, second and third types of class yielded cryo-EM maps of 70S IC, 70S EC and low-resolution 70S EC, respectively.
Extended Data Fig. 5 | Selection of noise particles and angular coverage of 70S IC with addition of noisy particles. a, Noise particles selected from the gain-corrected micrograph. The noise particles that did not exhibit any ribosome particle-like features were selected from the background (green circles). b, The angular coverage of the 70S IC with respect to the view depicted in the centre panel, as a function of the level of added noise.
Extended Data Fig. 6 | Fourier shell curves and cryo-EM reconstructions for the 30S IC, 70S IC and 70S EC. a–c, Fourier shell curves (FSC) for the 30S IC (a), 70S IC (b) and 70S EC (c). The resolutions of these structures were estimated using a resolution-estimating protocol that avoids overfitting and uses the FSC and 0.143 criterion\textsuperscript{28}. d–f, Cryo-EM reconstructions of 30S IC (d), 70S IC (e) and 70S EC (f). g–i, Angular orientation coverage of 30S IC (g), 70S IC (h), and 70S EC (i), presented corresponding to the views depicted in d, e and f, respectively. j–l, Directional FSC plots\textsuperscript{46} of the cryo-EM reconstructions of the 30S IC (j), 70S IC (k) and 70S EC (l).
Extended Data Fig. 7 | IF2–70S ribosome interactions in the 70S IC.  

**a**, The positions of IF2 (dark purple), 70S P/I fMet-tRNA^fMet (green), uS12 (yellow), and H69, H71, H80, H89 and H95 (SRL; all shown in blue) within the cryo-EM reconstruction of the 70S IC (transparent grey) were obtained using the MDFF method. **b**, A magnified view of the structure shown in **a**, highlighting the interactions that IF2 makes with the 70S ribosome in the 70S IC.
Extended Data Fig. 8 | Portion of the Coulomb potential map corresponding to the guanosine nucleotide obtained from the 4 Å resolution, cryo-EM reconstruction of the 70S IC. a, b. Rigid-body fitting was used to position either GTP (a) or GDP-P_i (b) into the Coulomb potential map. The Coulomb potential map is shown as a blue mesh. The initial position of P_i relative to GDP for the rigid-body fitting was taken from the structure of the GDP-P_i-form of the G protein G_{i1} (PDB code 1AS2)47.
Extended Data Fig. 9 | Views of the major components of the 30S IC and 70S IC after structural modelling of the 30S IC and 70S IC using the MDFF method. a, fMet-tRNAfMet (orange) in its 30S P/I configuration in the 30S IC. b, IF1 (magenta) in the 30S IC. c, IF2 (light purple) in the 30S IC. d, fMet-tRNAfMet (green) in its 70S P/I configuration in the 70S IC. e, IF2 (dark purple) in the 70S IC. For each component, the reconstructed Coulomb potential map is represented by the mesh and the structural model is represented by secondary structure cartoons.
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☐ □ Clearly defined error bars
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Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection: LEGINON

Data analysis: MotionCor2, CTFIND4, RELION 2.0, Microsoft Excel, UCSF Chimera software v1.12, COOT 0.8.9, VMD, MATLAB 2015b

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The cryo-EM reconstruction maps were deposited in the EMDB server under the accession codes EMD-0643 (30S IC), EMD-0662 (70S IC), and EMD-0661 (70S EC). The structural models obtained by MDF were deposited in the PDB server under accession codes 6O7K (30S IC) and 6O9K (70S IC). The structural model obtained by rigid-body fitting was deposited in the PDB server under accession code 6O9J (70S EC).
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | The number of particle images were chosen with auto-picker algorithm in RELION 2.0, and amply sufficient for the statistics. All of these strategies has been already published and widely discussed in research conferences. |
| Data exclusions | The principle of data exclusion, via classification was performed on previously published strategy which is widely accepted |
| Replication | Replication of the data analysis is used routinely as a principle of the resolution test. In our analysis resolution tests yielded standard deviation in the range of 0.7-1.5. |
| Randomization | This is part of the Relion software approach, and has been discussed at length in published article Scheres, S.H.W. (2012) J. Struct. Biol. |
| Blinding | Particle selection and classification decisions that were done independently by two team members were in overall agreement |

Reporting for specific materials, systems and methods

### Materials & experimental systems

| n/a | Involved in the study |
| --- | --- |
| ☒ | Unique biological materials |
| ☒ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☒ | Palaeontology |
| ☒ | Animals and other organisms |
| ☒ | Human research participants |

### Methods

| n/a | Involved in the study |
| --- | --- |
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |