Cryo-EM structure of the hibernating *Thermus thermophilus* 100S ribosome reveals a protein-mediated dimerization mechanism

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In response to cellular stresses bacteria conserve energy by dimerization of ribosomes into inactive hibernating 100S ribosome particles. Ribosome dimerization in *Thermus thermophilus* is facilitated by hibernation-promoting factor (TtHPF). In this study we demonstrate high sensitivity of Tt100S formation to the levels of TtHPF and show that a 1:1 ratio leads to optimal dimerization. We report structures of the *T. thermophilus* 100S ribosome determined by cryo-electron microscopy to average resolutions of 4.13 Å and 4.57 Å. In addition, we present a 3.28 Å high-resolution cryo-EM reconstruction of a 70S ribosome from a hibernating ribosome dimer and reveal a role for the linker region connecting the TtHPF N- and C-terminal domains in translation inhibition by preventing Shine–Dalgarno duplex formation. Our work demonstrates that species-specific differences in the dimerization interface govern the overall conformation of the 100S ribosome particle and that for *Thermus thermophilus* no ribosome-ribosome interactions are involved in the interface.
**Results**

**Formation of 100S ribosomes by TtHPF.** Purified TtHPF protein without ribosome present exists as a homodimer in solution as evident from the elution volume in size exclusion gel filtration analysis (Supplementary Fig. 2A). The early elution of TtHPF compared to standard proteins, also suggests that a TtHPF homodimer does not adopt a globular shape but rather a more extended conformation. This is in agreement with observations of other long HPF proteins\(^{23–25}\). This homodimeric TtHPF protein retains its expected biological activity to induce formation of 100S ribosomes (Supplementary Fig. 2B).

We investigated the in vitro dependency of Tt100S ribosome formation on TtHPF by analytical ultracentrifugation (AUC) on isolated Tt70S ribosome mixed with purified TtHPF in a series of molar ratios assuming one copy of TtHPF to bind one Tt70S ribosome (Fig. 1a and Supplementary Fig. 3). TtHPF-mediated formation of 100S ribosomes was found to be maximal at equimolar ratios of TtHPF to 70S ribosome (Fig. 1a, b). Even at sub-molar ratios, formation of 100S ribosome was observed along with observations of strong inhibition of 100S formation at molar excesses of TtHPF to 70S ribosome (Fig. 1b and Supplementary Fig. 3). These observations of a sharp transition in promotion or inhibition of 100S formation by TtHPF agrees with previous studies on 100S ribosome formation in both *T. thermophilus* and *S. aureus*\(^{12,14}\). However, the results presented here show a much stronger inhibitory effect of TtHPF on Tt100S ribosome formation in vitro at even moderate molar excess. This observation could be explained by a strong binding of TtHPF to the Tt70S ribosome, whereby TtHPF binding sites on
ribosomes are saturated and thus acting inhibiting to Tt100S ribosome formation (Fig. 1c). We did not observe a complete conversion of 70S to 100S dimers which lead us to believe that either TtHPF is not fully active at the temperature where the experiment was performed or perhaps a population of the 70S ribosomes are protected from dimerization in some way that we could not detect. Given the AUC results on 100S formation, an equimolar ratio of purified TtHPF and 70S ribosome was used in 100S formation for cryo-EM experiments (Supplementary Fig. 2C).
**Fig. 1** Analysis of in vitro TtHPF dependent formation of Tt100S. a Analytical ultracentrifugation sedimentation profiles show 70S ribosome as control (upper left), 70S ribosome mixed with TtHPF in 0.5 times molar ratio (lower left), 70S ribosome mixed with TtHPF in equimolar ratio (upper right) and 70S ribosome mixed with TtHPF in two times molar ratio (lower right). Formation of Tt100S ribosome is evident by the peak at a sedimentation coefficient of 100S. b Graphical representation of Tt100S ribosome formation from all AUC experiments. Formation of Tt100S ribosome by TtHPF is maximal in the case where the molar ratio of TtHPF to Tt70S is 1:1. See also Supplementary Figure 3. c Schematic illustration of TtHPF and Tt70S binding events leading to Tt100S ribosome formation. Binding of one NTD of TtHPF homodimer to Tt70S leads to a complex of Tt70S-TtHPF. In the case of sub- or equimolar ratios of TtHPF and Tt70S, binding of a vacant Tt70S ribosome to the free NTD of the Tt70S-TtHPF complex leads to Tt100S formation. However, in the case of TtHPF being present in excess molar ratios, Tt100S ribosome formation is inhibited because all Tt70S ribosomes bind a TtHPF homodimer.

Cryo-EM structure determination of Tt100S ribosome. For the first cryo-EM data set, in vitro formed and purified Tt100S ribosomes were spotted on unsupported cryo-grids and imaged in the vitreous ice state. Automatic particle picking did not perform to our satisfaction, thus care was taken by manually inspecting all picked positions to avoid ribosome dimers lying very close as well as to include non-picked ribosome dimer particles. During 3D classification, one class showed distinct density features for two ribosomes in a dimer particle (Supplementary Fig. 4) with an internal C2 symmetry that resulted in a 3D reconstruction with the highest average resolution reported yet for a 100S ribosome of 4.57 Å (Fig. 2a and Supplementary Fig. 4). From the same data set, 3D classification also showed a class with a single high resolved 70S ribosome with almost no density for the other ribosome copy of the dimer (Supplementary Fig. 4). The particles in this class were used in focused refinement using a 70S ribosome mask resulting in high-resolution 3D reconstruction of a 70S ribosome with an average resolution of 3.28 Å (Fig. 2b and Supplementary Fig. 4). We refer to the two 3D reconstructions as Tt100S (ice) and 70S (ice), respectively. In the 70S (ice) reconstruction a high resolved density corresponding to TtHPF-CTD and NTD within the 100S ribosome dimer. The two 70S copies constituting the 100S particle. There was no density for the linker region. There was no density for the TtHPF-CTD in 70S (ice) reconstruction.

**Fig. 2** Cryo-EM structures of 100S (ice) and 70S (ice). a Orthogonal views of 100S (ice) with 50S subunits in green and 30S subunits in orange showing the two 70S copies constituting the 100S particle. b Orthogonal views of 70S (ice), coloring of subunits as in (a). c Views of 100S (ice) and slice-through view with both 70S ribosome copies colored in gray and the two TtHPF protein molecules colored in orange and magenta showing location of TtHPF-NTD and CTD within the 100S ribosome dimer. d View of 70S (ice) with TtHPF-NTD colored in orange. Close-up views on 30S subunit show location of TtHPF-NTD and the linker region. There was no density for the TtHPF-CTD in 70S (ice) reconstruction.

**TtHPF-NTD interactions with 30S subunit.** TtHPF-NTD binds to the 30S subunit at a position between the head and body as previously observed in 100S ribosomes from other species.22–25 as
Fig. 3 Structure of TtHPF-NTD and its interactions with the 30S subunit. a Structure of TtHPF-NTD shown in orange cartoon. The corresponding cryo-EM density of 70S (ice), shown as semi-transparent gray surface, shows clear density for linker region. Secondary structure elements are labeled. b Close-up view on the high quality density map of the TtHPF-NTD (upper) and linker (lower) with model inside. Side chains are clearly resolved in the high-resolution density. c Close-up view on electrostatic interactions between TtHPF-NTD and ribosome centered around Arg86 and Arg93 interacting with phosphate backbone on nucleotides C1382 and G1383 in h44. d Example of stacking interaction between TtHPF-NTD residue Arg103 and nucleotide G676 of h23 in 16S rRNA. e Structure of TtHPF-NTD and its density (orange) superimposed with A-, P-, and E-site tRNAs (green, purple, blue) and mRNA (red) bound in 70S ribosome (PDB entry 4V6F). The binding position of TtHPF-NTD on the 30S subunit clearly overlaps with binding sites for all three tRNAs as well as mRNA. f Structure of TtHPF with the missing seven residues of the linker indicated by dashed line. The structure is superimposed with the chimeric structure of Tt70S-RMF (PDB entry 4V8G) showing the location of the E. coli RMF protein (violet) closely matching the binding position of the TtHPF linker region. g Coloring as in E now with 3′ end of 16S rRNA shown in violet. The linker region of TtHPF occupies a binding position on the 30S subunit that overlaps with the helix formed by mRNA and 3′ end of 16S RNA. Close-up view shows TtHPF linker region residues His104, Ser105, Tyr106, and Glu107 overlapping with Shine-Dalgarno duplex between mRNA and 3′ end of 16S RNA. Proline residues 109–112 overlap with mRNA binding position as well as for the E. coli short HPF11. Similar to other long HPF proteins, TtHPF-NTD adopts a β1-α1-β2-β3-β4-α2 topology with strands β1-β2 being parallel and β2-β3-β4 anti-parallel (Fig. 3a), with high sequence conservation to other long HPF proteins (Supplementary Figure 8). Hydrophobic residues in α1, β1, and β2 as well as α2-β4 form a hydrophobic core that stabilizes the fold of TtHPF-NTD (Supplementary Fig. 6A). Interactions between aromatic residues Tyr20 and the highly conserved Tyr77 (Supplementary Figure 8) further contribute to the stabilization of the TtHPF-NTD. Our high quality 70S (ice) density map also showed clear densities for side chains on TtHPF-NTD residues enabling a detailed analysis of interactions to the ribosome (Fig. 3b).

The clear density observed for side chains on TtHPF-NTD showed interactions predominantly with 16S rRNA through electrostatic interactions between basic residues and phosphate groups of the rRNA backbone in h30, h31, and h44, e.g., between Arg86 and Arg93 phosphates on nucleotides C1382 and G1383 in the base of h44 (Fig. 3c and Supplementary Fig. 6B–D). We also observed clear side chain density for interactions between TtHPF-
NTD and 16S rRNA through stacking interactions between Tyr31 and A773 in the loop of h24 as well as by Arg103 with G676 in the loop of h23 (Fig. 3d and Supplementary Fig. 6E). The only interaction observed between the THPF in the 70S (ice) reconstruction and a ribosomal protein is the contact formed between Tyr25 in uS11 and Pro112 of the linker between NTD and CTD (Supplementary Fig. 6F).

The binding site of THPF-NTD on the 30S subunit overlaps with binding sites for tRNAs in A-, P-, and E-sites as well as the mRNA binding groove on the small ribosomal subunit (Fig. 3e). The tight tethering of THPF-NTD to 16S rRNA helices positions the folded NTD right in the A- and P-sites on the 30S subunit, a binding position that precludes binding of tRNA in either of these two sites (Fig. 3e). The long a2 helix and the start of the THPF linker region occupy a position that would cause a sterical clash with tRNA in E-site (Fig. 3e). Previous studies also indicated overlap of the binding position of long HPF proteins on 30S subunit with A- and P-site tRNAs22-25, but here we show that the THPF-NTD inhibitory effect on translation is due to a complete sterical hindrance of tRNA binding in any of the three A-, P-, and E-sites. This also agrees with biochemical observations of reduced translation activity in in vitro assays12,14. In previous structures of long HPF proteins, the density for the LHPF-NTD and the linker region was poorly resolved allowing the linker region to only be traced to approximately residue 101 to 106 depending on the study23-25. In our 3.28 Å 70S (ice) reconstruction the electron density for both the NTD and the linker was well resolved (Figs 2d, 3a, b) and allowed us to build the THPF linker until residue 122, only leaving a gap of seven residues between the N and C-terminal domains (Fig. 3f). The THPF linker region extends toward the mRNA exit site in proximity of the 16S rRNA 3′ end (Fig. 3e), a position close to that occupied by E. coli RMF protein on the small subunit11 (Fig. 3f). The part of the linker region closest to the 3′ end of 16S rRNA is a stretch of residues from His104 to Pro112, where the four proline residues 109-112 are arranged in a distorted poly-proline-II helix (Fig. 3g). This type of secondary structure is known for its ability to interact with nucleic acids36. This binding position of the THPF linker is in the same region where E. coli RMF11 was observed to bind and will interfere with helix formation between Shine–Dalgarno (SD) and anti-Shine–Dalgarno (aSD) sequence (Fig. 3g) during translation initiation37,28, hence causing inhibition of translation.

**THPF-CTD bridges interactions in 100S ribosome interface.**

The 100S (ice) reconstruction showed a density located in the dimerization interface of the ribosome copies (Fig. 2c) that we attributed to the THPF-CTD homodimer. Given only a medium resolution in this part of the reconstruction (Supplementary Fig. 4B) we collected a second cryo-EM data set this time with isolated 100S ribosome spotted on cryo-grids with a continuous amorphous carbon support. Processing of single particles essentially followed the steps of the first data set processing (Supplementary Figure 5) with 3D classification showing one class of 100S particles that aligned with C2 symmetry resulting in a final 3D reconstruction with an average resolution of 4.13 Å surpassing that of previous 100S ribosome reconstructions including our 100S (ice) reconstruction (Fig. 4a and Supplementary Figure 5). This 3D reconstruction we refer to as 100S (amc). As the conformation of THPF was found to be identical in the three different reconstructions (Supplementary Figure 5C), we initially thought of combining 100S particles from the two data sets (ice and amc) aiming for a higher resolved reconstruction allowing a closer analysis of the 100S dimerization interface around the THPF-CTD homodimer. However, although the overall conformation of the 100S (ice) and 100S (amc) was identical, there was a slight difference when looking specifically at the dimerization interface where the 100S (amc) has two additional sites of interaction between the small subunit head domains centered at ribosomal proteins uS7 and uS9 (Supplementary Figure 7A). In addition, we observed a different conformation of H69 of 23S rRNA between 100S (ice) and 100S (amc) reconstructions separate. The density around the THPF-CTD homodimer region in the 100S (amc) was slightly better resolved compared to the 100S (ice) reconstruction (Supplementary Figure 5B), thus THPF-CTD was modeled in the 100S (amc) density (Figs 4a, b). The homodimer of THPF-CTD adopts a conformation similar to that observed for BiHP23, SaHPF24 and LHPF22 with each copy of the THPF-CTD forming a central three-stranded beta-sheet flanked by an alpha helix (Fig. 4a, small insert). This is in line with the high degree of sequence conservation in the CTD (Supplementary Figure 8). The central beta-sheets are extended by one strand by interacting with the other copy of THPF-CTD in the region connecting to the linker region (Fig. 4b). The interactions between the two beta-sheets of the two THPF-CTD copies are dominated by hydrophobic interactions, e.g., Ile169 on one copy interacts with Ile169 on the other copy together capping the CTD dimerization interface lined by Val160 and Val171 on both copies (Fig. 4b). Further stabilization of the CTD dimerization interface comes from intricate stacking interactions of aromatic residues Phe158 and Tyr173 tethering the two THPF-CTD together (Fig. 4b). This very tight interaction between the THPF-CTD copies resembles that observed in SaHPF-CTD24.

Interestingly, in our T100S ribosome we do not observe the network of interactions in the dimerization interface observed for 100S ribosomes from other species22-25. As speculated from analysis of 16S rRNA secondary structure diagrams (Supplementary Figure 1), the inter-ribosome interaction between 16S rRNA h26 of one ribosome copy and uS2 on the other ribosome copy is not present in our T100S ribosome reconstructions from 100S (amc) nor 100S (ice) (Fig. 4c and Supplementary Figure 7C). The h26 does indeed forms a helix protruding from the small subunit, however the length of h26 is much too short to make it all the way to uS2 protein on the other ribosome to interact (Fig. 4c). Thus, no additional stabilization of the ribosome dimerization interface can be attributed to the h26-uS2 interaction.

We also do not observe an interaction similar to that between the SaHPF-CTD homodimer and 16S rRNA h40 in our T100S ribosome (Fig. 4d and Supplementary Figure 7D). As the length of h40 is similar to that in other species (Supplementary Figure 1H), where it makes contact with the THPF-CTD homodimer, the conformation of the T100S ribosome must be slightly different from that of 100S ribosomes from other species given there is no interaction. Thus contrary to what has been observed for other species of 100S ribosome dimers, the ribosome dimerization interface for the *Thermus thermophilus* 100S ribosome is centered on the THPF-CTD homodimer and only comprises interactions between ribosomal protein uS2 and THPF-CTD on the same ribosome copy with the opposite THPF-CTD and protein uS2. These results provide evidence that formation of *Thermus thermophilus* 100S ribosome dimers by long HPF proteins is attributed to the LHPF protein alone and not inter-ribosome interactions.

**Discussion**

Formation of 100S ribosomes is a ubiquitous bacterial response to cellular stresses and unfavorable growth conditions12. In such circumstances, bacterial cells rely on second messenger signaling molecules, e.g., (p)ppGpp, to tune the process of translation to...
conserved energy. Though the mechanism of ribosome dimerization is different between gamma proteobacteria and non-gamma proteobacteria, the regulation of the proteins involved in ribosome dimerization seems conserved. In *E. coli*, the protein responsible for ribosome dimerization, RMF, is transcriptionally upregulated in response to secondary messengers (pppGpp) and cAMP during nutrient starvation. Similarly, in organisms employing LHPF proteins for ribosome dimerization their regulation is also dependent on (pppGpp). The stringent response is also conserved in *T. thermophilus*, thus making it likely that TtHPF is regulated by (pppGpp) in response to cellular stresses.

In this study we presented the cryo-EM reconstruction of *Tt*100S ribosome along with biophysical characterization of 100S ribosomes as a function of TtHPF molar ratio. The results obtained from AUC analysis showed increasing formation of 100S ribosome in response to increasing molar ratios of TtHPF with a maximum conversion of 70S ribosome to 100S ribosome dimers at equimolar ratios of TtHPF (Fig. 1). At excess molar ratios of TtHPF to ribosome, we observed a strong inhibition of 100S ribosome formation (Supplementary Fig. 3) in agreement with previous experiment, but with our results showing a much higher sensitivity of Tt100S formation to TtHPF amounts. Thus, similar binding events likely govern formation of 100S ribosome dimers in *T. thermophilus* and *S. aureus*. Despite the clear formation of Tt100S ribosomes indicated by the 100S peaks in the sedimentation profiles, we also observed a 70S sedimentation peak that we naturally attribute to 70S ribosomes. In the experiment with *T7*70S ribosome and TtHPF mixed in 1:1 molar ratio, approximately only half of the *T7*70S ribosomes are converted to Tt100S ribosomes. Whether this is a reflection of our purified TtHPF protein not being fully active is difficult to assess. It could also be that the temperature of 20°C during the AUC experiment is inhibiting for 100S ribosome formation given the thermophilic nature of the source organism. However, no other experiments of 100S formation from other species has ever reported complete conversion—often the conversion rate has been quite low. Perhaps the incomplete conversion to 100S reflects yet undiscovered properties of the mechanism of dimerization or that a certain populace within the ribosome pool are protected from dimerization. This would prevent a complete shutdown of translation and ascertain that some 70S ribosomes are still available for protein production during stress. The decrease in Tt100S ribosome formation upon excess molar ratios of TtHPF we interpret as all possible binding sites on single ribosomes filling up with TtHPF homodimers effectively leaving no vacant Tt70S ribosomes to form 100S dimers.

In the crystal structure of the chimeric complex of *T. thermophilus* 70S ribosome with *E. coli* HPF and RMF reported by Polikanov et al., a model for the 100S ribosome was proposed based on an *E. coli* 100S reconstruction. It was suggested that the 30s subunits in the 100S ribosome would form two points of...
contact centered at ribosomal proteins uS9, uS10, and h39 of 16S rRNA and around ribosomal proteins uS2, uS3, uS4, and uS5\(^5\). However, our structures clearly show this not to be the case. In our Tt100S ribosome reconstructions, the ribosomal proteins proposed to interact are located far from each other on the respective ribosomes within the dimer (Fig. 5a).

So far structures of LHPF-mediated 100S ribosomes from *B. subtilis* and *S. aureus* determined by cryo-EM show a high degree of conformational homogeneity with the ribosomes constituting the 100S ribosome dimers superposing very well (Fig. 5b)\(^22-25\). A common feature for the Bs100S and Sa100S ribosomes is the LHPF homodimer protein that facilitates ribosome dimerization in all these structures. The LHPF-CTD interacts with uS2 and h40 and additional stabilization of the 100S ribosome dimerization interface comes from inter-ribosome interactions by h26 and uS2 (Fig. 5b and Supplementary Fig. 1). As shown in our structure of the Tt100S ribosome, the dimerization interface is not stabilized by the h26-uS2 interaction between the ribosome copies in the 100S dimer (Fig. 4e) nor by interactions between THF-CTD and h40 (Fig. 4f). This leaves only THF-CTD interaction with uS2 to stabilize the dimerization interface. Earlier speculations on Tt100S ribosomes adopting a tilted conformation to bring h26 into interaction distance with uS2\(^24\) are thus not correct according to our 100S structure. We observe a slightly altered staggered conformation of the *T. thermophilus* ribosome, both the Tt100S (ice) and Tt100S (amc), compared with other 100S ribosome structures (Fig. 5c and Supplementary Figure 7E). When superposing one 70S copy of our Tt100S ribosome to fit with a 70S copy of Bs100S and Sa100S, the superposed 70S copy fits very well. However, the other 70S copy of our Tt100S ribosome does not superpose well with the other ribosome copy on the Bs100S and Sa100S ribosomes indicating a different conformation for the Tt100S ribosome. Based on the results we have presented here for the Tt100S ribosome structure, the Tt100S ribosome dimerization follows that of other organisms relying on LHPF proteins for 100S ribosome formation\(^12,22-25\), where 100S ribosome dimerization is a result of binding of a homodimeric LHPF protein that brings the two ribosomes into a staggered conformation. The differences that we do see, e.g., the h26-uS2 interaction, between our 100S and 100S ribosomes from other species having long HPFs are overall not very large but might reflect species-specific differentiations of the LHPF-ribosome dimerization interface to modulate or regulate stabilization according to species-specific needs.

As expected since *T. thermophilus* has a LHPF protein, the Tt100S ribosome is markedly different in its staggered conformation compared with the structure of *E. coli* 100S ribosome (Fig. 5d) where RMF and a short HPF are required\(^7\). The binding of these two proteins induces the formation of Ec100S ribosomes in which the 30S subunits interact in a back-to-back fashion
for the TTHPF linker region connecting the NTD and CTD. The linker region extends to a region, which would clash with formation of the SD-aS helix during translation initiation (Fig. 3g) providing yet another inhibitory mechanism for TTHPF on translation. Alternatively, the presence of the linker region in the same area as RMF is coincidental and the role of the linker region may be in synchronization of the dimerization by the CTD and the blocking of mRNA and tRNA binding by the NTD.

Although the precise role for sequestering ribosomes as translational inactive 100S ribosome dimers is not known, various reasons can be envisioned. Structural studies of the bacterial RNA polymerase (RNAP) in complex with the bacterial small ribosomal subunit have shown that RNAP interacts through its β′-subunit with ribosomal protein uS2 and h40 in a conformation where the mRNA exit tunnel of RNAP is positioned near the 3′ end of 16S rRNA35. If this RNAP-30S subunit structure represents an intermediate during translation initiation, then this step would be inhibited by 100S ribosome conformation as uS2 and h40 are buried within the 100S ribosome dimerization interface and thereby inaccessible35 (Fig. 6d). In a different study showing how RNAP binds to the complete 70S ribosome, the RNAP mRNA exit tunnel faces the mRNA entry site on the ribosome between uS3, uS4, and uS5. In this conformation the ω-subunit of RNAP interacts with ribosomal protein uS236. The complex of RNAP-70S ribosome referred to as “the expressome” was shown to form only during transcription elongation36. As in the former RNAP-30S subunit case with uS2 being embedded within the 100S ribosome dimerization interface, interactions between RNAP and 70S ribosome would be sterically hindered causing a decrease in translation activity. When overlaying the structure of the expressome with our Tt100S ribosome we see that it is not the RNAP ω-subunit that is sterically prevented from binding to uS2, but rather the RNAP subunit a that sterically clashes with the 100S ribosome at a position close to the L1 stalk (Fig. 6b). However, in either case, the arrangement of the 100S ribosome precludes the formation of the expressome.

The conformation of the 100S ribosome might also reflect the cellular need to disassemble and recycle the ribosomes to be able to use them in translation again. Studies have reported a universally conserved GTPase named HflX that binds the large ribosomal subunit37. HflX was shown to dissociate 70S ribosome into small and large subunits in a GTP-dependent manner, with the HflX protein staying attached to the large subunit after dissociation of 70S ribosome38. In S. aureus, HflX was also shown to prevent 100S ribosome formation also dependent on GTP, even in the presence of SaHPF39. A cryo-EM structure of the E. coli 50S subunit with HflX bound, located the protein in the ribosomal A-site causing structural rearrangements within the 50S subunit likely to be responsible for the 70S ribosome dissociating capabilities of HflX38. Superposing our cryo-EM structure of TTHPF bound to the ribosome with the E. coli 50S-HflX structure shows no steric overlap between TTHPF and HflX (Fig. 6). Thus, it may be possible for HflX to bind a 100S ribosome and disassemble it.

**Methods**

**Cloning and expression of TTHPF.** Genomic DNA from *Thermus thermophilus* cells was extracted using Trizol (Sigma). The TTHPF gene (UniProt Q5SIS0) was PCR amplified using forward primer also encoding a TEV-protease cleavage site, 5′-GAGGAGAAGCCCGGTTCATCA-3′, and reverse primer 5′-GAGGAGAAGCCCGGTTCATCA-3′. The PCR product was cloned in pET46-Ek/LIC plasmid (Merck) and verified by sequencing. Protein was expressed in *Escherichia coli* BL21 (DE3) cells by auto-inducing ZYP-5052 growth medium supplemented with 300 μM ampicillin. Cells were grown at 37 °C until reaching OD₆₀₀ of 0.6 followed by overnight incubation at 18 °C. Harvested cells were resuspended in lysis buffer (50 mM Heps/KOH pH 7.5, 300 mM KCl, 5 mM MgCl₂, 20 mM
imidazole, 10% v/v glycerol, 1 mM DTT) supplemented with protease-inhibitor tablet (Sigma) and 5 U/mL DNasel (ThermoFisher).

**Purification of T. thermophiles ribosome.** Resuspended cells were lysed by sonication. Lysate was cleared by centrifugation at 30,000 × g and 20 °C for 45 min and supernatant loaded onto a 5 mL HisTrap column (GE Healthcare) equilibrated in lysis buffer. All purification steps were done at room temperature. The HisTrap column was washed in 20 column volumes (CV) buffer W (50 mM Mepes/Hepes/KOH pH 7.5, 1 M KC1, 50 mM imidazole, 10% v/v glycerol, 1 mM DTT) and 2 CV lysis buffer. Bound protein was eluted in buffer E (50 mM Mepes/Hepes/KOH pH 7.5, 200 mM KC1, 300 mM imidazole, 10% v/v glycerol, 1 mM DTT) with a 20 CV linear gradient from 50 mM to 300 mM imidazole. Fractions containing TTHPF protein were pooled and 1:100 w/w TEV-protease (made in-house) added. The solution was dialyzed overnight against 2 L buffer D (20 mM Hepes/Hepes/KOH pH 7.5, 100 mM KC1, 10% v/v glycerol, 1 mM DTT). The dialyzed protein solution was diluted with one volume buffer G (50 mM Hepes/KOH pH 8.0, 10% v/v glycerol, 1 mM DTT) and loaded onto a 9 mL Source15Q column (GE Healthcare) equilibrated in buffer G (same as buffer G plus 50 mM KC1). The column was washed for 4CV with buffer G and bound protein eluted with a 20 CV linear gradient to buffer Q1000 (same as buffer S plus 150 mM KC1). Peak fractions containing TTHPF were concentrated to ∼5 mL volume by centrifugation in VivaSpin concentrators with a 5000 Da cutoff. The protein solution was loaded on a HILoad Superdex 75 column (GE Healthcare) equilibrated in buffer F (20 mM Mepes/Hepes/KOH pH 7.5, 100 mM KC1, 10% v/v glycerol, 1 mM DTT). Peak fractions were concentrated to 8 mg/mL. The dialyzed protein solution was adjusted to 100 nM (0.5 mg/mL) 100S concentration. Quantifoil R2/1.3 300 mesh grids were glow discharged for 40 s at 3 mA before use and flash frozen in liquid ethane cooled by liquid nitrogen using a FEI Vitrobot MarkIV.

Similar 100S ribosome sample preparation procedure was used prior to sample application onto Quantifoil R2/2 300 mesh grids with amorphous carbon coating. Sample concentration was adjusted to 13 nM (0.06 mg/mL) with the same Vitrobot settings as described above.

**Cryo-EM data collection and processing.** Data was collected automatically using SerialEM45 on a spherical aberration (Cs) corrected FEI Titan Krios transmission electron microscope at liquid nitrogen temperature operating at an accelerating voltage of 300 kV equipped with a K2 Summit camera (Gatan) at a nominal magnification of 59,000× resulting in a 0.143 Å/2 frames per acquisition. All image processing steps with movie frame alignment and determination of CTF parameters as well as single particle processing and refinement was done within cisTEM46. Particles from the unsupported ice data set were picked with a difference–free automatic picking with manual inspection of all picked positions. Particles were extracted with a 700-pixel box and all 93,133 particles 2D classified. Particles in class averages showing clear structural features for both ribosomes within the dimer were used further in 3D classification and refinement steps. Particles in 3D class IV (see Supplementary Figure 3) were aligned and C2 symmetry was used for the final refinement resulting in a reconstruction at 4.57 Å (0.143 FSC) average resolution. This reconstruction is referred to as 100S (ice) particles. From 3D class II were refined using a 70S mask resulting in a 70S ribosome at 3.28 Å (0.143 FSC) average resolution (referred to as 70S (ice)). A similar processing procedure was used for data collected from amorphous carbon grids. After manual inspection of automatically picked particle positions, 55979 particles were used in 2D classification. Following 3D classification and refinement, particles in 3D class II were aligned and C2 symmetry was imposed (see Supplementary Figure 4) in the final refinement resulting in a reconstruction at 4.13 Å (0.143 FSC) average resolution (referred to as 100S (amc)). For all three final reconstructions local calculations were resolved using ResMap58.

**Model refinement and validation.** For initial model fitting and building, the 70S ribosome EM density map was sharpened automatically using phenix.auto_sharpen. The crystal structure of the T. thermophiles 70S ribosome (PDB entry 4Y9B) was edited removing rRNAs, tRNA and antibiotic compound and used as the initial model. The small and large subunit were fitted separately into corresponding density using rigid body fitting in UCSF Chimera58. To improve model fit to density, each subunit was further fitted by molecular dynamics flexible fitting with manual inspection and building of the fitted models using Coot50. TTHPF-NDT residues 2–122 were built in the density map of the 70S (ice) ribosome guided by a crystallographic model of TTHPF-NDT (PDB entry 2TWF, unpublished) and de novo building for the linker region. The fitted model of T70S ribosome with TTHPF-NDT was further used to generate models of T100S ribosomes in 100S (ice) and 100S (amc) EM densities using Naminderator. For TTHPF-CTD building, the SciHFL-CTD (PDB entry 6FXC) was docked into the density and used as a template for model building of residues 129–185. Models of 70S (ice), 100S (ice), and 100S (amc) were all refined using real space refinement59. Finally, refined models were all validated with MolProbity60 with all statistics listed in Supplementary Table 1. Figures were prepared using UCSF Chimera and PyMol (Schrodinger). Ribosomal proteins are all named based on the new system61.

**Data availability**

Atomic models have been deposited with the Protein Databank with the following accession codes: 70S (ice) PDB accession code 6GZQ, 100S (amc) PDB accession code 6GZX and 100S (amc) PDB accession code 6GZX. EM density maps have been deposited within the EMDB with accession codes for 70S (ice) EMDB-0101, 100S (ice) EMDB-0104, and 100S (amc) EMDB-0105. Other data are available from the corresponding author upon request.
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**Additional information**

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