A proton wire to couple aminoacyl-tRNA accommodation and peptide-bond formation on the ribosome

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During peptide-bond formation on the ribosome, the α-amine of an aminoacyl-tRNA attacks the ester carbonyl carbon of a peptidyl-tRNA to yield a peptide lengthened by one amino acid. Although the ribosome’s contribution to catalysis is predominantly entropic, the lack of high-resolution structural data for the complete active site in complex with full-length ligands has made it difficult to assess how the ribosome might influence the pathway of the reaction. Here, we present crystal structures of preattack and postcatalysis complexes of the Thermus thermophilus 70S ribosome at ~2.6-Å resolution. These structures reveal a network of hydrogen bonds along which proton transfer could take place to ensure the concerted, rate-limiting formation of a tetrahedral intermediate. We propose that, unlike earlier models, the ribosome and the A-site tRNA facilitate the deprotonation of the nucleophile through the activation of a water molecule.

The ribosome is a large ribonucleoprotein complex responsible for synthesizing proteins in all living organisms. In bacteria, ribosomes are composed of large (50S) and small (30S) subunits comprising approximately two-thirds rRNA and one-third protein. The active site where peptide-bond formation takes place on the ribosome—also known as the peptidyl transferase center (PTC)—is located within the 50S subunit and consists mainly of RNA, with the N-terminal tail of the nearest ribosomal protein, L27, approaching within only 8–10 Å of the reaction center¹. Thus, it has been concluded that the ribosome is a ribozyme, with RNA being the major contributor to catalysis².

Peptide-bond formation is an aminolysis reaction in which the α-amine of an aminoacyl-tRNA attacks the ester carbonyl carbon of a peptidyl-tRNA, resulting in the transfer of the peptidyl moiety to the aminoacyl-tRNA bound in the aminoacyl (A) site. For each amino acid that is added to the nascent polypeptide chain, at least three protons must move within the PTC. Because primary amines in solution at near-neutral pH exist in the protonated ammonium form, it is necessary to first deprotonate the α-NH₂⁺ group of the A-site tRNA in order to obtain the nucleophilic α-NH₂ group that will initiate the reaction. In addition, a proton must be removed from this amine either during or after nucleophilic attack. Finally, a proton must be provided to the 3’-oxygen of the terminal peptidyl (P)-site tRNA ribose in order to ensure the departure of the leaving group. As a facilitator of peptide-bond formation, the ribosome could provide a path for the fast and efficient movement of protons around the PTC (i.e., through the ordering of water molecules and/or the positioning of rRNA and tRNA residues), could directly promote proton movement through acid-base catalysis or could do both. To understand how polypeptide chains are synthesized in vivo, the role of the ribosome in facilitating proton transfer must therefore be understood.

Models seeking to explain the movement of protons during peptide-bond formation are typically evaluated on the basis of their agreement with high-resolution structures of the Haloarcula marismortui (Hma) 50S subunit in complex with minimal substrate, transition-state or product analogs³–⁵. Although these structures have greatly advanced the understanding of ribosomal protein synthesis, they nevertheless have restricted the interpretation of the available experimental and computational data to the context of the isolated large ribosomal subunit. In order to extend this structural framework to the full ribosome, we determined the structures of preattack and postcatalysis complexes of T. thermophilus (Tth) 70S ribosomes containing full-size tRNA ligands at resolutions of 2.6 Å and 2.55 Å, respectively (Fig. 1, Table 1 and Supplementary Fig. 1) as well as those of two prereaction complexes featuring a short A-site substrate analog and a full-length P-site tRNA (Supplementary Fig. 2).

Although these are not the first structures of the 70S ribosome to contain full-length aminoacylated tRNA ligands¹, the 0.7- to 0.9-Å increase in resolution that we have achieved over similar structures allows a complete description of the ribosomal PTC with bound tRNA substrates or products, ions and water molecules (Figs. 1 and 2). Notably, these structures now allow us to perform a much more comprehensive structure-based assessment of the possible mechanisms for ribosome-catalyzed peptide-bond formation.

RESULTS

The structure of the PTC remains rigid throughout catalysis

In the Tth 70S-ribosome preattack complex, unreacted, nonhydrolyzable fMet-NH–tRNA²Met occupies the P site, while nonhydrolyzable

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Phe-NH-tRNA\textsubscript{Phe} is bound to the A site (Fig. 1a,b). A comparison of the PTC seen in this structure with that of the lower-resolution structure of a \textit{Tth} 70S-ribosome preattack complex\textsuperscript{1} and the structures of an \textit{Hma} 50S-subunit preattack complex with the A- and P-site substrate mimics cytidine-cytidine-hydroxypuromycin (CChPmn) and cytidine-cytidine-adenosine-phenylalanine-caproic acid-biotin (CCPcb)\textsuperscript{1} indicates that the 23S rRNA adopts similar conformations in all cases (Supplementary Fig. 3a,c). Nearly identical 23S rRNA conformations are also present in our structures of the \textit{Tth} 70S ribosome in complex with fMet-NH-tRNA\textsubscript{Met} in the P site and the substrate analogs cytidine-puromycin (CPmn) or cytidine-cytidine-puromycin (CCPmn) in the A site (Supplementary Fig. 3e). This is in contrast with earlier studies showing that the closely related analog cytidine-hydroxypuromycin (ChPmn) bound to \textit{Hma} 50S fails to trigger the induced state, which is characterized by a rearrangement of residues U2506, G2583, U2584 and U2585 that results from proper binding of the substrate to the A site (Supplementary Fig. 3f and ref. 4). Thus, all of the preattack structures presented here correspond to the induced state, with a fully accommodated A-site nucleophile poised to react with the P-site substrate.

In the \textit{Tth} 70S-ribosome postcatalysis complex, deacylated tRNA\textsubscript{Met} occupies the P site, and dipeptidyl-tRNA obtained \textit{in situ} by reaction of Phe-NH-tRNA\textsubscript{Phe} with fMet-tRNA\textsubscript{Met} is found in the A site (Fig. 1d,e), thus providing direct evidence that the ribosomes crystallized in our study are catalytically active under the buffer conditions used for complex formation. Strikingly, we observed major differences between our structure of the \textit{Tth} 70S-ribosome postcatalysis complex and that of an \textit{Hma} 50S-subunit complex\textsuperscript{2} with a product analog in the A site (Supplementary Fig. 3b). Whereas the PTC in the latter structure is in a conformation that resembles the uninduced state, our structure is very similar to the fully induced preattack state. Moreover, the base A2602 in the \textit{Hma} 50S-subunit product structure is rotated by \textdegree;180 relative to its orientation in our structure. Because the rotated conformation of this base would result in a steric clash with the full-length P-site tRNA, we conclude that our \textit{Tth} 70S-ribosome postcatalysis structure is the correct structure of the PTC after peptide-bond formation. The conformation of the 23S rRNA seen here is also very similar to that previously reported in the lower-resolution structure of a \textit{Tth} 70S-ribosome complex\textsuperscript{1} mimicking the post–peptidyl transfer state (Supplementary Fig. 3d). However, this earlier structure featured an aminoacyl-tRNA instead of a dipeptidyl-tRNA in the A site, thus making it unclear whether it represented the true postcatalysis state. From the nearly identical conformations of the 23S rRNA in the preattack and postcatalysis structures presented here...
Figure 2  Coordination of water molecules in the active site. (a,c) Coordination of water molecule W1 by the 2′-OH of 23S rRNA residue A2451, the phosphate oxygen of the A-site tRNA residue A76, the N-terminal amino group of ribosomal protein L27 and the N6 atom of 23S rRNA residue A2602 in both the preattack (a) and postcatalysis (c) complex structures. (b,d) Coordination of water molecules W1, W2 and W3 in the preattack (b) and postcatalysis (d) complex structures. In b, W2 is coordinated by the ester carbonyl oxygen of the peptidyl-tRNA, the N1 atom of 23S rRNA residue A2602 and the 2′-OH of U2584, and W3 is hydrogen-bonded to the 2′-OH, 3′-N and N3 atoms of the P-site tRNA residue A76 and the 2′-OH of C2063. In d, contacts between W2 and the peptide are lost, whereas the hydrogen bond between W3 and the P-site A76 ribose shifts toward the 3′-OH of the deacylated tRNA.

and in earlier structures of the 50S subunit in complex with transition-state analogs, we thus conclude that the PTC remains relatively rigid throughout catalysis.

### Three ordered water molecules are trapped within the PTC

Owing to the higher resolution of the current structure of the Tth 70S ribosome in complex with tRNAs as compared with previous structures, peaks of positive density corresponding to three water molecules (W1–3) are visible in unbiased Fo−Fm maps of the preattack and postcatalysis Tth 70S-ribosome complexes (Fig. 1c,f). W1 lies within a cavity formed by residues A2602 and A2451 of the 23S rRNA, the 3′ end of the A-site tRNA and the N terminus of protein L27 (Fig. 2a,c). Although density for this water was absent from the Hma 50S-subunit preattack structure, it could nevertheless be modeled as a water molecule in all of our structures on the basis of the following criteria: (i) comparison with the structures of the Hma 50S subunit in complex with the transition-state mimics DAA3 or DCA3, both of which exhibited density in a similar location that could not be replaced by soaking the crystals with high concentrations of Mn2+, K+ or Rb+ (ref. 3); (ii) tetrahedral coordination and distances from its ligands; and (iii) strength of the electron density.

Water molecules W2 and W3, which occupy pockets buried deep within the PTC (Fig. 2b,d), were previously observed in structures of Hma 50S subunits in complex with substrates or transition-state analogs3–4 and were proposed to stabilize the oxybase in the transition state and take part in proton shuttling, respectively. However, the structures presented here exhibit all three water molecules at once, showing how the waters interact with both the ribosome and its tRNA substrates. Notably, no other solvent molecules were present within the PTC, including within the narrow cavity occupied by W3. This was not the case in many of the earlier structures of Hma 50S-subunit complexes3–4, in which two water molecules were often modeled at this location, and the active site was generally accessible to the bulk solvent. In our preattack structure, the unbiased difference density observed for W3 is elongated and could best be modeled as a single water molecule populating two alternate locations with relative occupancies of 0.7 and 0.3 (Fig. 1c). We hypothesize that the latter position corresponds to a minor subpopulation in which the P-site tRNA is deacylated, thereby also explaining the relatively weak density observed for the fMet residue. For the purposes of our analysis, we will therefore consider the position of W3 modeled with an occupancy of 0.7.

### A proton wire connects the attacking amine to W1

In all of the structures presented here, a short network of hydrogen bonds analogous to a proton wire6 connects W1 to the attacking amine via the 2′-OH of the P-site tRNA A76 and the 2′-OH of A2451 (Fig. 3a). The geometry of this network is well suited for efficient proton transfer, with D−H−A distances of <3.1 Å in the Tth 70S-ribosome preattack complex and the angle ω between three successive non-hydrogen atoms ranging from 112° to 122° and thus approximating the ideal value of 109.5° for a tetravalent atom (Supplementary Fig. 4a). This is in contrast with the hydrogen-bond networks connecting the attacking amine to the 3′-O of the peptidyl-tRNA via the 2′-OH of the P-site tRNA A76 and the 2′-OH of A2451 (Fig. 3a). The angles between the α-amine, the 2′-OH of the P-site tRNA A76 and W3, and between the 2′-OH of the P-site tRNA A76, W3 and the 3′-O of the P-site tRNA A76, respectively (Supplementary Fig. 4b) or directly (ω = 61°) (Supplementary Fig. 4c). Moreover, W1 in the Tth 70S-ribosome preattack complexes forms a strong hydrogen bond (2.4 Å) with the 5′-phosphate oxygen of A76 of the A-site tRNA as well as additional hydrogen bonds with the N6 amino group of A2602 and the N terminus of L27. As a result, this water molecule appears to be tightly coordinated from all sides and is not likely to be exchanged with bulk solvent during catalysis.

The N terminus of L27 also interacts with two hydrogen-bond acceptors: the N7 imino group of the P-site tRNA A76 and the 3′-O of A2451 (Fig. 3b). The nature of these interactions is such that proton exchange between the L27 terminus and the bulk solvent would require some backbone rearrangements to take place within the
of L27, computational simulations suggest that its protonation state of the N terminus here that are key to revealing the interactions with W1. yet it is the resolution and quality of the electron density observed currently structures of the Tth 70S ribosome in complex with tRNAs, yet it is important that the structures that helped formulate these models were obtained with isolated 50S subunits and lacked full-length tRNAs, thus precluding the ordering of protein L27 (or rather, as discussed below, its functional equivalent in archaea) that is needed to render the PTC inaccessible to the bulk solvent. What's more, the water referred to here as W1 was seen only in complexes with transition-state analogs that lacked the 2'-OH of the P-site tRNA A76, thus making it difficult at the time to incorporate this molecule into the proposed reaction models.

The question that the current work addresses is the following: given the structures of the expanded PTC presented here, can we propose alternate models for peptide-bond formation that the earlier Hma 50S-ribosome structures did not allow us to foresee? In doing so, we mean not to supersede or disprove the existing models but rather to expand the conceptual framework needed to probe the mechanistic aspects of peptide-bond formation by biochemical, kinetic and/or computational means. Like the earlier proton-shuttle models, our proposed mechanism does not seek to explain the initial deprotonation of the A-site α-NH₃⁺ to give a nucleophilic amine because this event may well take place before or during the induced fit triggered by the binding of the A-site substrate. Instead, we focus our attention on the circumstances surrounding the deprotonation of the attacking amine and the formation of a tetrahedral intermediate.

The Tth 70S preattack structure presented in this work is the outcome of crystallographic experiments performed on a thermodynamically equilibrated sample containing unreactive substrate analogs. Consequently, the state visualized here may differ to some extent from the catalytically active state, which presumably occurs immediately after accommodation of the aminoacyl-tRNA into the A site. This problem is by no means unique to this particular system, yet it is important to stress that the state populated here may not be the kinetically relevant one, although the structures of the Hma 50S ribosome in complex with transition-state analogs mirror our findings. With this in mind, we assume that the Tth 70S preattack structure represents a reasonable approximation of the reactive state, which is suitable enough to allow mechanistic hypotheses to be formulated. We make similar assumptions concerning the Tth 70S postcatalysis structure.

Superposition of the preattack and postcatalysis Tth 70S-ribosome structures obtained here onto structures of the Hma 50S subunit in complex with various transition-state analogs suggests at least one alternative model for proton transfer that differs substantially from the six- or eight-membered concerted proton shuttles (Fig. 4 and Supplementary Video 1). Assuming that the α-amine of L27 is deprotonated, the presence of this basic group and of the negatively charged 5'-phosphate oxygen of the A-site A76 in the immediate vicinity of terminal residues of L27, and this would result in the breaking of several hydrogen bonds. With the assistance of a nearby hydrated magnesium ion that closes the cavity containing W1, the N terminus of L27 could therefore restrict the flow of protons between W1 and the bulk solvent. The conformation of the N-terminal segment of L27 in our current structures is in excellent agreement with that seen in earlier structures of the Tth 70S ribosome in complex with tRNAs, yet it is the resolution and quality of the electron density observed here that are key to revealing the interactions with W1.

Even though the resolution of our structures does not allow us to draw conclusions regarding the protonation state of the N terminus of L27, computational simulations suggest that its α-amine is the ionizable ribosomal group with a pKₐ of ~7.5–8.0 that was observed in the reaction with the A-site substrate puromycin. The protonation state predicted by these studies for the reaction with tRNA substrates is more problematic, however, given that they rely on a model of the PTC in which a water molecule that could in principle correspond to W1 makes a different set of interactions with components of the active site. For the purposes of the discussion below, we will assume that the α-amine of L27 undergoes a downward pKₐ shift and is thus deprotonated in the physiological pH range.

DISCUSSION

Currently, two major models have been proposed to account for the movement of protons around the active site during peptide-bond formation. Referred to as the six- and eight-membered proton shuttles, both of these models rely on the 2'-OH of the P-site tRNA A76 ribose to transfer a proton from the attacking amine to the leaving group, with the eight-membered shuttle further incorporating a water molecule corresponding to W3 from this study into the proton relay. Importantly, the structures that helped formulate these models were obtained with isolated 50S subunits and lacked full-length tRNAs, thus precluding the ordering of protein L27 (or rather, as discussed below, its functional equivalent in archaea) that is needed to render the PTC inaccessible to the bulk solvent. What's more, the water referred to here as W1 was seen only in complexes with transition-state analogs that lacked the 2'-OH of the P-site tRNA A76, thus making it difficult at the time to incorporate this molecule into the proposed reaction models.

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Figure 4 A possible alternate pathway for peptide-bond formation. (a) Concerted attack by the α-amine of the aminoacyl-tRNA onto the ester carbonyl carbon of the peptidyl-tRNA and deprotonation of the nucleophile, in a single rate-limiting step. (b) Formation of a tetrahedral intermediate, with positive and negative charges becoming separated in space and delocalized over the pockets containing W1 and W2, respectively. Gold, water molecules. (c) A partial reversal of the proton-transfer event, resulting in intermediate breakdown to yield peptidyl-tRNA in the A site and deacylated tRNA in the P site. N term, N terminus.

W1 could facilitate the formation of a partial negative charge on the water oxygen (Fig. 4a). Shortening of the O–H–O– distance between W1 and the 5′-phosphate along the reaction coordinate to form the low-barrier hydrogen bond suggested by our structures could, in turn, cause an upward shift of the pKa of W1 from its value in solution of −1.74. As the nucleophilic attack proceeds, the extremely high pKa of the α-amine would decrease substantially, ultimately triggering a concerted proton transfer from the attacking nucleophile to W1 via the proton wire formed by the 2′-OH of the P-site A76 ribose and the 2′-OH of A2451. This would result in the negatively charged tetrahedral intermediate (T−) described by recent kinetic isotope effect (KIE) experiments11, which might additionally receive a proton from the adjoining water molecule W2 to yield a neutral intermediate (T0)12 (Fig. 4b). Positive and negative charges would then be unable to propagate further, owing to a lack of neighboring groups suitable for proton transfer, and would be transiently retained within the closed pockets containing W1 and W2, respectively.

In our model, a proton wire that consists of components provided by the ribosome and both tRNAs is formed only after the aminoacyl-tRNA is fully accommodated into the A site; this ensures that the nucleophilic attack and deprotonation of the α-amine are coordinated in a single rate-limiting step. Although we propose that the 5′-phosphate oxygen of the A-site A76 aided by the α-amine of the L27 N terminus could assist in the deprotonation of the nucleophile, further biochemical and kinetic studies will need to be carried out to establish whether this is true for all or perhaps only certain incoming amino acids. Studies on the effect of L27 on peptide-bond formation performed to date have been rather limited, with the deletion of the first three residues of L27 resulting in just a modest decrease in the rate of peptide-bond formation as measured by the standard puromycin assay13.

Table 1 Data collection and refinement statistics

| Data collection | 70S–tRNA preattack | 70S–tRNA postcatalysis | 70S–CPmn preattack | 70S–CCPmn preattack |
|-----------------|--------------------|-----------------------|--------------------|---------------------|
| Space group     | P21_21             | P21_21                | P21_21             | P21_21             |
| Cell dimensions |                    |                       |                    |                    |
| a, b, c (Å)     | 209.45, 448.85, 619.02 | 209.32, 450.06, 622.23 | 209.25, 448.46, 618.08 | 207.56, 444.23, 613.03 |
| Resolution (Å)  | 224–2.60 (2.67–2.60)a | 256–2.55 (2.62–2.55)b | 363–2.90 (2.98–2.90)c | 222–2.80 (2.62–2.80)d |
| Rmerge          | 23.2 (225.0)       | 13.1 (120.9)          | 18.5 (124.4)       | 18.4 (128.6)       |
| t / σt          | 8.58 (0.97)        | 8.91 (1.02)           | 7.13 (1.08)        | 6.04 (1.02)        |
| Completeness (%)| 99.9 (99.8)        | 95.9 (85.6)           | 96.9 (98.8)        | 98.0 (97.8)        |
| Redundancy      | 3.90 (8.99)        | 3.90 (3.09)           | 3.62 (3.48)        | 3.79 (3.66)        |

| Refinement      |                |                       |                    |                    |
| Resolution (Å)  | 2.60            | 2.55                  | 2.90               | 2.80               |
| No. reflections | 1,760,252       | 1,803,654             | 1,229,259          | 1,345,662          |
| Rwork / Rfree  | 22.3/26.4       | 23.2/27.9             | 23.1/28.6          | 23.3/28.0          |
| No. atoms       | Protein 91,253  | 91,181                | 91,171             | 91,175             |
|                 | Ligand/ion 202,093 | 202,139              | 196,240            | 196,499            |
|                 | Water 3,022     | 3,062                 | 2,071              | 2,367              |
| B factors       | Protein 63.6     | 63.9                  | 64.4               | 63.4               |
|                 | Ligand/ion 59.5  | 60.5                  | 59.9               | 57.5               |
|                 | Water 44.4       | 45.5                  | 40.1               | 41.0               |
| r.m.s. deviations | Bond lengths (Å) | 0.005                 | 0.005              | 0.005              |
|                 | Bond angles (°)  | 0.982                 | 0.989              | 0.965              |

Values in parentheses are for highest-resolution shell. aTwo crystals were used to obtain the structure. b2/σt = 2 at 2.80 Å resolution. cA single crystal was used to obtain the structure. d2/σt = 2 at 3.05 Å resolution. eA single crystal was used to obtain the structure. f2/σt = 2 at 2.97 Å resolution.
From a structural perspective, our model could be favored over the six- or eight-membered proton shuttles proposed earlier. Indeed, the efficiency of proton transfer through an sp²-hybridized atom X is impaired by substantial deviations of the ω angle from the ideal value of 109.5° (ref. 14). Although the proton wire described in this study is characterized by ω angles approximating this value, the hydrogen-bond networks at the heart of the eight- and six-membered proton shuttles have a much less favorable geometry (Supplementary Fig. 4). In addition, the alternate positions observed for W3 in the preattack structure render this water molecule much less likely than W1 to be a part of the proton relay involved in deprotonating the attacking amine. Although the proton wire linking the attacking amine to W1 in the preattack Tth 70S-ribosome structure appears to be the most geometrically favorable path along which a proton could be abstracted from the nucleophile, quantitative quantum-mechanical calculations of the type performed for the six- and eight-membered proton shuttles⁷ could be performed to evaluate this prediction.

The concerted formation of a tetrahedral intermediate implied by our model is consistent with the lack of positive-charge buildup on the attacking amine in the transition state implied by the near-zero Bronsted coefficient⁵ and with KIE data, thus suggesting that the ribosome alters the pathway of the uncatalyzed reaction by means of a prearranged proton-transfer network.¹¹ The proposed pathway for proton transfer is in agreement with the rate-limiting formation of at least three hydrogen bonds in the transition state (i.e., from the attacking amine to the P-site A76 2′-OH to the A2451 2′-OH to W1), as suggested by kinetic solvent isotope effect (KSIE) studies performed with puromycin as the A-site substrate. Moreover, the trajectory for the departing proton is supported by biochemical data showing that deletion of the 2′-OH of the A76 ribose of the P-site tRNA or substitution of the 2′-OH of A2451 with groups including -H or -OCH₃ decrease the rate of peptide-bond formation by at least 100-fold¹⁶–¹⁸ or 10- to 50-fold¹⁹,²₀, respectively. It is worth noting that the controversy surrounding the role of the 2′-OH of the P-site tRNA A76 ribose during peptide-bond formation stems from the structural consequences of mutations at this position being unknown. The values quoted above may thus be largely underestimated, given that the high-resolution structures that could reveal compensatory rearrangements within the PTC²¹ and/or functional complementation by ordered solvent molecules¹⁵ have not been observed.

Finally, the effect of base substitutions at position 2602 of the 23S rRNA on the rate of peptide-bond formation in vivo are easily reconciled with our proposed model.²² Indeed, when the minimal A-site substrate puromycin is used together with ribosomes mutated at position A2602, the rate constant for the reaction is 30–320 times slower than with wild-type ribosomes, whereas the rate remains unchanged when the reaction is performed with a full-length aminoacyl-tRNA. Modeled base substitutions at this position in the Tth 70S-ribosome preattack structure suggest that hydrogen-bonding between residue 2602 and water molecules W1 and W2 is preserved in all cases, in agreement with the rate constants observed for full-length substrates. Moreover, the mild reduction in rate that is measured upon complete deletion of A2602 could be explained by W1 remaining tightly bound via three hydrogen bonds to the mutant ribosome preattack complex. Even though A2602 may seem dispensable at first, the effect of cumulative mutations within the W1-bonding pocket on the rate of peptide-bond formation must be measured in order to assess its full contribution to the reaction. A possible involvement of A2602 in coordinating the reaction should also be considered, given that this residue acts as a bridge between the two proposed catalytic waters, W1 and W2.

A key question raised by our model concerns the possible involvement of the ribosome in catalyzing the conversion of the tetrahedral intermediate into products. Increasing the rate of breakdown of the intermediate may indeed be necessary to drive the reaction forward by preventing the intermediate from reverting back to substrates. Under such circumstances, the ester bond of the peptidyl-tRNA would become exposed to hydrolysis, which in turn could result in the premature termination of protein synthesis. On the basis of the structures obtained here, an attractive possibility is that the positive charge contained within the W1 pocket would be used to break down the intermediate via the same proton wire that was used to create it. Indeed, the fast, concerted transfer of a proton from W1 to W3 to form a hydronium ion would increase the ability of this buried solvent molecule to catalyze the hydrolysis of the intermediate into products. A role for the N-terminal amino group of L27 in delaying the loss of a proton from W1 to the bulk solvent long enough to allow protonation of W3 could explain why shortening this highly defined tail by three or more residues causes growth defects and decreases the overall yield of protein synthesis.¹³ The conserved length of the L27 N-terminal extension ensures that the entire proton wire and water molecule W2 form a shielded system that can exchange a proton with the bulk solvent through only the 2′-OH of the P-site A76 ribose. According to our model, deletion of the N terminus of L27 could alter both the rates of intermediate formation and the conversion of the intermediate into products, even though the effects on the overall reaction rate could be modest depending on the nature of the incoming amino acid in the A site.

The lack of pH sensitivity for peptide-bond formation with either full-length tRNAs or with the short A-site–substrate analogs CPmn or CCPmn may also be an indication that the PTC is closed and that protons involved in the reaction are not exchanged with the bulk solvent. This is in agreement with the three preattack structures presented here, which exhibit nearly identical PTC conformations (Supplementary Fig. 3e) and a shielded W1 cavity. In contrast, the existence of an ionizable group with a pKₐ of ~7.5 in the puromycin reaction is consistent with the N terminus of L27 acting as a general base or acid once the PTC cavity is open to the solvent and the A76 phosphate oxygen of the A-site tRNA is no longer available to initiate the proton transfer. Our model does not exclude the possibility that the N-terminal amine of L27 also becomes protonated during the reaction with the full-length substrates. Such a protonation event would remain undetected in analyzing the pH profile of the reaction because the breakdown of the intermediate is likely to occur faster than the exchange of a proton with the bulk solvent. In light of our proposed mechanism, we therefore suggest that mutations that render the pocket containing W1 accessible to the bulk solvent, such as the deletion of residue A2602 (ref. 24) or alterations to the N terminus of L27 (ref. 13), will need to be reevaluated in terms of their effect on the pH sensitivity of the reaction.

No homolog of L27 exists in archaea or in the cytoplasmic ribosomes of eukaryotes. In archaea L27 is replaced by protein L10e, which has an internal loop that is disordered in the Hma 50S-subunit structure but may nevertheless extend toward the PTC in a 70S ribosome with bound full-length tRNA substrates. Indeed, a cryo-EM model of the wheat-germ ribosome in complex with a P-site tRNA has shown that the eukaryotic L10e homolog (RPL10) possesses a loop that reaches toward the PTC in a manner similar to that of the N terminus of L27 (ref. 26). Although mutations within this highly conserved loop are lethal, a high-resolution structure of the archaeal or eukaryotic ribosome in complex with aminoacylated tRNA substrates and kinetic data for the reaction by ribosomes with L27, L10e or RPL10 mutant
proteins will be necessary to establish whether L10e or RPL10 could have a role comparable to that proposed here for L27.

In summary, we have obtained markedly improved snapshots of the PTC before and after catalysis. These structures have revealed a previously unseen network of hydrogen bonds that could have a catalytic role by ensuring that nucleophilic attack and deprotonation of the α-amine are concerted in the rate-limiting step. Though by no means definitive, the model put forward here is intended to steer research on the mechanism of peptide-bond formation in directions that could not be foreseen when, nearly a decade ago, structures of the 50S ribosomal subunit in complex with small tRNA mimics provided high-resolution pictures of the PTC in different functional states. Thus, our data provide a robust, expanded structural framework on the basis of which alternate reaction mechanisms could be formulated and tested with kinetic, biochemical and computational approaches.

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 codes 4QCM, 4QCN, 4QCO and 4QCP for the T70S-ribosome preattack complex; 4QCQ, 4QCR, 4QCT and 4QCT for the T70S-ribosome postcatalysis complex; 4QCQ, 4QCR, 4QCQ and 4QCP for the T70S-ribosome preattack complex with CPmn; and 4QCY, 4QCF, 4QD0 and 4QD1 for the T70S-ribosome preattack complex with CCPmn.

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

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AUTHOR CONTRIBUTIONS

C.A.I. and Y.S.P. devised experiments and prepared modified-tRNA substrates, C.A.I. and Y.S.P. analyzed the data, and C.A.I., Y.S.P. and T.A.S. crystallized ribosomal complexes and performed crystallographic data collection and processing, C.A.I. and Y.S.P. analyzed the data, and C.A.I., Y.S.P. and T.A.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Voorhees, R.M., Wexlbaumer, A., Loakes, D., Kelley, A.C. & Ramakrishnan, V. Insights into substrate stabilization from snapshots of the peptidyl transferase center of the intact 70S ribosome. Nat. Struct. Mol. Biol. 16, 528–533 (2009).
2. Nissen, P., Hansen, J., Ban, N., Moore, P.B. & Steitz, T.A. The structural basis of ribosome activity in peptide bond synthesis. Science 289, 920–930 (2000).
3. Schmeing, T.M., Huang, K.S., Kitchen, D.E., Strobel, S.A. & Steitz, T.A. Structural insights into the roles of water and the 2′-hydroxyl of the P site tRNA in the peptidyl transferase reaction. Mol. Cell 20, 437–448 (2005).
4. Schmeing, T.M., Huang, K.S., Strobel, S.A. & Steitz, T.A. An induced-fit mechanism to promote peptide bond formation and exclude hydrolysis of peptidyl-tRNA. Nature 438, 520–524 (2005).
5. Schmeing, T.M. et al. A pre-translocational intermediate in protein synthesis observed in crystals of enzymatically active 50S subunits. Nat. Struct. Biol. 9, 225–230 (2002).
6. Nagle, J.F. & Morowitz, H.J. Molecular mechanisms for peptide transport in membranes. Proc. Natl. Acad. Sci. USA 75, 298–302 (1978).
7. Trobo, S. & Ayvaz, J. Role of ribosomal protein L27 in peptidyl transfer. Biochemistry 47, 4898–4906 (2008).
8. Katunin, V.I., Muth, G.W., Strobel, S.A., Wintermeyer, W. & Rodnina, M.V. Important contribution to catalysis of peptide bond formation by a single ionizing group within the ribosome. Mol. Cell 10, 339–346 (2002).
9. Kuhlencorvet, S., Wintermeyer, W. & Rodnina, M.V. Different substrate-dependent transition states in the active site of the ribosome. Nature 476, 351–354 (2011).
10. Wolberger, C. & Avizet, J. The transition state for peptide bond formation reveals the ribosome as a water trap. Proc. Natl. Acad. Sci. USA 107, 1888–1893 (2010).
11. Miller, D.A., Singh, V., Zhong, M. & Strobel, S.A. A two-step chemical mechanism for ribosome-catalysed peptide bond formation. Nature 476, 236–239 (2011).
12. Carrasco, N., Miller, D.A. & Strobel, S.A. Minimal transition state charge stabilization of the oxyanion during peptide bond formation by the ribosome. Biochemistry 50, 10451–10458 (2011).
13. Maguire, B.A., Beniaminov, A.D., Ramu, H., Mankin, A.S. & Zimmermann, R.A. A protein component at the heart of an RNA machine: the importance of protein L27 for the function of the bacterial ribosome. Mol. Cell 20, 427–435 (2005).
14. Hammes-Schiffer, S. & Stuchebrukhov, A.A. Theory of coupled electron and proton transfer reactions. Chem. Rev. 110, 6939–6960 (2010).
15. Kingery, D.A. et al. An uncharged amine in the transition state of the ribosomal peptidyl transfer reaction. Chem. Biol. 15, 493–500 (2008).
16. Zehet, H.S., Shaw, J.J., Strobel, S.A. & Green, R. The 2′-OH group of the peptidyl-tRNA stabilizes an active conformation of the ribosomal PTC. EMBO J. 30, 2445–2453 (2011).
17. Huang, Y. & Sprinzl, M. Peptide bond formation on the ribosome: the role of the 2′-OH group on the terminal adenosine of peptidyl-tRNA and of the length of nascent polypeptide chain. Angew. Chem. Int. Ed. Engl. 50, 7287–7289 (2011).
18. Koch, M., Huang, Y. & Sprinzl, M. Peptide-bond synthesis on the ribosome: no free vicinal hydroxy group required on the terminal ribose residue of peptidyl-tRNA. Angew. Chem. Int. Ed. Engl. 47, 7242–7245 (2008).
19. Erlacher, M.D. et al. Efficient ribosomal peptidyl transfer critically relies on the presence of the ribose 2′-OH at A2451 of 23S rRNA. J. Am. Chem. Soc. 128, 4453–4459 (2006).
20. Lang, K., Erlacher, M., Wilson, D.N., Micura, R. & Polacek, N. The role of 23S ribosomal RNA residue A2451 in peptide bond synthesis revealed by atomic mutagenesis. Chem. Biol. 15, 485–492 (2008).
21. Weinger, J.S., Parnell, K.M., Dorner, S., Green, R. & Strobel, S.A. Substrate-assisted catalysis of peptide bond formation by the ribosome. Nat. Struct. Mol. Biol. 11, 1101–1106 (2004).
22. Youngman, E.M., Brunelle, J.L., Kochanak, A.B. & Green, R. The active site of the ribosome is composed of two layers of conserved nucleotides with distinct roles in peptide bond formation and peptide release. Cell 117, 589–599 (2004).
23. Bieling, P., Beringer, M., Adio, S. & Rodnina, M.V. Peptide bond formation does not involve acid-base catalysis by ribosomal residues. Nat. Struct. Mol. Biol. 13, 423–428 (2006).
24. Polacek, N. et al. The critical role of the universally conserved A2602 in 23S ribosomal RNA in the release of the nascent peptide during translation termination. Mol. Cell 11, 103–112 (2003).
25. Ban, N., Nissen, P., Hansen, J., Moore, P.B. & Steitz, T.A. The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. Science 298, 905–920 (2000).
26. Armache, J.P. et al. Localization of eukaryote-specific ribosomal proteins in a 5.5-A cryo-EM map of the BOS eukaryotic ribosome. Proc. Natl. Acad. Sci. USA 107, 19754–19759 (2010).
27. Hofer, A., Bussiere, C. & Johnson, A.W. Mutational analysis of the ribosomal protein Rpl10 from yeast. J. Biol. Chem. 282, 32630–32639 (2007).
ONLINE METHODS

Purification of *T. thermophilus* 70S ribosomes. *Thb* 70S ribosomes were prepared as described previously. The final 70S pellets were resuspended at a concentration of approximately 500 A₂₆₀ units/mL (in a buffer containing 5 mM HEPES-KOH, pH 7.6, 50 mM KCl, 10 mM NaCl, 10 mM Mg(CH₃COO)₂, and 6 mM β-mercaptoethanol), flash frozen in liquid nitrogen and stored in small aliquots at −80 °C until used in crystallization experiments.

Preparation of mRNA, tRNAs and tRNA analogs. Synthetic mRNA with the sequence 5′-GGC AAG GAG GUA AAA AUG UUC UUA-3′ was obtained from Integrated DNA Technologies. This mRNA includes a Shine-Dalgarno sequence, an AUG start codon and a phenylalanine UUC codon followed by a stop codon. Synthetic mRNA with the sequence 5′-UUC AGG CUA UGC CAG-3′ was produced from *E. coli* (with minor changes from ref. 1) and modified at the 3′-end with 1 mM of the modified nucleotide and 125 µg/mL *E. coli* CCA-adding enzyme in a buffer containing 50 mM glycine-NaOH, pH 9.2, 30 mM KCl, 12 mM Mg(CH₃COO)₂, 100 µg/mL bovine serum albumin and 3 mM CTP (with modifications from ref. 31). To perform a similar replacement of the terminal A76 for the mRNA, CCA-adding enzyme was used. In the first step, the incorporation/hydrolysis equilibrium of the reaction catalyzed by CCA-adding enzyme was shifted towards hydrolysis by addition of 250 µM Na₂P₂O₇ in order to remove the terminal nucleotides. In the following step, the hydrolysis reaction was reversed by addition of 2 U/mL *E. coli* inorganic pyrophosphatase (NEB), and the resulting incorporation of the modified terminal residue was allowed to proceed for 1 h at 37 °C. The resulting full-length 3′-NH₂-tRNA⁺ and 3′-NH₂-tRNA⁺⁺ were enzymatically acylated with phenylalanine or methionine, respectively, to yield Phe-NH-tRNAPhe and Met-NH-tRNAMet. The latter was then formylated as described to yield Met-NH-tRNAMet⁺. All obtained aminocyl-tRNA species were further purified by HPLC on a 20-mL C4 reversed-phase column (Vydac) in a buffer containing 20 mM NH₄CH₃COO, pH 5.5, 400 mM NaCl and 10 mM MgCl₂, with a 0–40% linear gradient of methanol for elution. The fractions from the C4 column that contained the desired tRNAs were pooled, concentrated, flash frozen in liquid nitrogen in small aliquots and stored at −80 °C until further use in crystallization experiments.

The aminocyl-tRNA mimics—cytidine-puromycin (CPmn) and cytidine-cytidine-puromycin (CCPmn)—were obtained from Thermo Scientific.

Complex formation. Ribosome–mRNA–tRNA complexes were formed by programming of 5 µM *Thb* ribosomes with 10 µM mRNA and incubation at 55 °C for 10 min. This was followed by addition of 20 µM P- and A-site tRNA substrates (with minor changes from ref. 1). Each of the last two steps was allowed to reach equilibrium for 10 min at 37 °C. In the cocryocrystallization experiments with aminocyl-tRNA analogs, CPmn and CCPmn were used at final concentrations of 100 mM Tris-HCl, pH 7.6, 2.9% (w/v) PEG 20000, 50 mM KCl, 10 mM NaCl, 10 mM Mg(CH₃COO)₂ and 6 mM β-mercaptoethanol. Crystals were cryoprotected stepwise with a series of buffers with increasing MPD concentrations until reaching a final concentration of 40% (v/v) MPD, in which they were incubated overnight at 19 °C. In addition to MPD, all stabilization buffers contained 100 mM Tris-HCl, pH 7.6, 2.9% (w/v) PEG 20000, 50 mM KCl, 10 mM NaCl, 10 mM Mg(CH₃COO)₂ and 6 mM β-mercaptoethanol. During crystal cryoprotection with aminocyl-tRNA analogs, CPmn and CCPmn were added to all stabilization solutions at 50 µM or 20 µM, respectively. After stabilization, crystals were harvested and immediately flash frozen in a nitrogen cryostream at 80 K.

Data collection and processing. Diffraction data were collected at beamline X25 at the Brookhaven National Laboratory and at beamline 24ID-C at the Advanced Photon Source. A complete data set for each ribosome complex was collected at 100 K from multiple regions of the same crystal with 0.2° oscillations and 0.9789-Å wavelength. The raw data were integrated and scaled with XDS. All of the crystals belonged to the primitive orthorhombic space group P2₁2₁2₁ with approximate unit-cell dimensions of 210 × 450 × 620 Å and contained two copies of the 70S ribosome per asymmetric unit (Table 1). Each structure was solved by molecular replacement with Phaser from the CCP4 suite. The search model was generated from the previously published structure of *Thb* 70S ribosome with bound mRNA and tRNAs (PDB 2WDG and 2WDJ). The mRNA and all three tRNAs as well as 23S rRNA residues comprising the peptidyl transferase center were excluded from the starting search model. The initial molecular replacement solutions were refined by rigid-body refinement with the ribosome split into multiple domains, followed by seven cycles of positional and individual B-factor refinement with PHENIX. Noncrystallographic symmetry restraints were applied to four domains of the 30S ribosomal subunit (head, body, spur and helix 44), and three domains of the 50S subunit (body, L1-stalk and N terminus of the L9 protein).

After initial refinement, electron density corresponding to the mRNA, all three tRNAs, water molecules (W1, W2, and W3) and amino acid moieties attached to the 3′-terminal residues of the A- and P-site tRNAs became evident in the unbiased *Fo* − *Fc* difference maps. To further improve the quality of the difference maps and to aid in model building, we used an approach in which bulk solvent correction was omitted for the ribosome’s active site region. To protect specific features of the density from being included into the solvent mask (Supplementary Fig. 1a), a sphere of evenly spaced dummy atoms with zero occupancy (Supplementary Fig. 1b) was used to cover the active site region during macromolecular mask generation but was ultimately excluded from the refinement process. All dummy atoms in the sphere grid were placed at 0.7–1.2 Å intervals as suggested by PHENIX developers, and a sphere radius of 15 Å was chosen to completely cover the active site. Any dummy atom located <2 Å away from any atom of the physical model was excluded. The resulting unbiased *Fo* − *Fc* maps revealed more prominent features and had continuous electron densities for the amino acid moieties (Supplementary Fig. 1c). Owing to the absence of bulk solvent correction, the scale of these maps within the active site region was shifted in a seemingly arbitrary fashion. An equivalent contour level was obtained by visual comparison of the active site densities between the bulk solvent-corrected and uncorrected maps.

**Model building.** The mRNA, full-length E-site tRNA and residues 1–75 of A- and P-site tRNA ligands were built into the unbiased difference density maps from the initial round of refinement, and the refinement scheme described above was performed after the addition of each ligand. A high-resolution structure of unmodified tRNA⁻ from *E. coli* (PDB 3L0U) was used as a guide to build the A- and E-site tRNAs. All of the hydrogen bonds defining the tRNA secondary and tertiary structures, as well as those involved in the mRNA-tRNA and 23S-rRNA base pairs, were included as distance restraints during refinement. The 3′-terminal A76 residues of A- and P-site tRNAs with amino acids attached were omitted until the remainder of the active site of the ribosome had been built and refined. They were then placed together with water molecules into the unbiased difference density maps with previous *Thb* 70S or HSA 50S structures as guides. Initial models for the 3′-terminal A76 residues of the A- and P-site tRNAs with the amino acids or with dipeptide attached via amide bonds were created de novo. The corresponding restraints needed for positional and B-factor refinement

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were generated manually with existing restraints from the CCP4 library for AMP, phenylalanine and formylmethionine as templates. Geometric restraints for the refinement of aminoacyl-tRNA mimics, CPmn and CCPmn, were taken from the CCP4 library.

The final models for each ribosome complex were generated by multiple rounds of model building in COOT\textsuperscript{38} and subsequent refinement in PHENIX\textsuperscript{36}. The statistics of data collection and refinement for each complex are compiled in Table 1.

The 0.7- to 0.9-Å increase in resolution achieved over existing structures of similar 70S complexes\textsuperscript{1} enabled us to build a more complete and accurate model of the 70S ribosome. The markedly improved overall quality of the electron density maps allowed us to rectify some main chain–register problems and to improve the number of residues in the favored and allowed regions of the Ramachandran plot for many ribosomal proteins in our starting model.

Additionally, we found that the ligand coordinated by residues Cys9, Cys12, Cys26 and Cys31 of ribosomal protein S4 is a 4Fe-4S iron-sulfur cluster instead of a Zn\textsuperscript{2+} ion, as suggested by all previous structures of the \textit{Tth} 70S ribosome. Initial refinement with a Zn\textsuperscript{2+} ion placed in the center of the positive \(F_o - F_c\) density peak adjacent to the protein S4 cysteine residues, which were connected to the Zn\textsuperscript{2+} ion by coordination bonds, demonstrated a substantial distortion in the protein’s backbone geometry and lingering strong peaks of positive density. This suggested that the ligand coordinated by cysteines of protein S4 should be larger in size, should possess more electrons and should have the same tetrahedral coordination geometry as compared with the Zn\textsuperscript{2+} ion. The only ligand that meets all these criteria, is known to be coordinated by cysteines and has biological significance for the \textit{Tth} species is the cubic 4Fe-4S iron-sulfur cluster. Thus, all structures presented here were built and refined with a 4Fe-4S cluster coordinated by ribosomal protein S4 of the 30S subunit.

**Figures and video.** All figures showing atomic models and video were generated with PyMOL (http://www.pymol.org/).

28. Selmer, M. et al. Structure of the 70S ribosome complexed with mRNA and tRNA. Science \textbf{313}, 1935–1942 (2006).
29. Schmitt, E., Blanquet, S. & Mechulam, Y. Crystallization and preliminary X-ray analysis of \textit{Escherichia coli} methionyl-tRNA\textsubscript{Met(f)} formyltransferase complexed with formyl-methionyl-tRNA\textsubscript{Met(f)}. Acta Crystallogr. D Biol. Crystallogr. \textbf{55}, 332–334 (1999).
30. Jünmann, R. et al. \textit{In vivo} deuteration of transfer RNAs: overexpression and large-scale purification of deuterated specific tRNAs. Nucleic Acids Res. \textbf{24}, 907–913 (1996).
31. Fraser, T.H. & Rich, A. Synthesis and aminoacylation of 3′-amino-3′-deoxy transfer RNA and its activity in ribosomal protein synthesis. Proc. Natl. Acad. Sci. USA \textbf{70}, 2671–2675 (1973).
32. Korostelev, A., Trakhanov, S., Laurberg, M. & Noller, H.F. Crystal structure of a 70S ribosome-tRNA complex reveals functional interactions and rearrangements. Cell \textbf{126}, 1065–1077 (2006).
33. Polikanov, Y.S., Blaha, G.M. & Steitz, T.A. How hibernation factors RMF, HPF, and YfIA turn off protein synthesis. Science \textbf{336}, 915–918 (2012).
34. Kabsch, W. Xds. Acta Crystallogr. D Biol. Crystallogr. \textbf{66}, 125–132 (2010).
35. McCoy, A.J. et al. Phaser crystallographic software. J. Appl. Crystallogr. \textbf{40}, 658–674 (2007).
36. Adams, P.D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. \textbf{66}, 213–221 (2010).
37. Byrnes, R.T., Konevega, A.L., Rodnina, M.V. & Antson, A.A. The crystal structure of unmodified tRNA\textsubscript{Phe} from \textit{Escherichia coli}. Nucleic Acids Res. \textbf{38}, 4154–4162 (2010).
38. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. \textbf{60}, 2126–2132 (2004).