The Mechanism for Activation of GTP Hydrolysis on the Ribosome

Rebecca M. Voorhees, T. Martin Schmeing, Ann C. Kelley, Ramakrishnan

Protein synthesis requires several guanosine triphosphatase (GTPase) factors, including elongation factor Tu (EF-Tu), which delivers aminoacyl–transfer RNAs (tRNAs) to the ribosome. To understand how the ribosome triggers GTP hydrolysis in translational GTPases, we have determined the crystal structure of EF-Tu and aminoacyl-tRNA bound to the ribosome with a GTP analog, to 3.2 Ångstrom resolution. EF-Tu is in its active conformation, the switch I loop is ordered, and the catalytic histidine is coordinating the nucleophilic water in position for in line attack on the γ-phosphate of GTP. This activated conformation is due to a critical and conserved interaction of the histidine in EF-Tu with the GTP γ-phosphate. This interaction stabilizes the γ-phosphate for hydrolysis to lead to GTP hydrolysis. The structure suggests a universal mechanism for GTPase activation and hydrolysis in translational GTPases on the ribosome.

In all stages of protein synthesis the ribosome requires exogenous protein factors, including several guanosine 5′-triphosphate (GTP)–hydrolyzing enzymes known as GTPases. These proteins are essential and highly conserved and include elongation factor Tu (EF-Tu), which delivers aminoacyl-tRNA to the ribosome as part of a ternary complex (TC) with GTP, as well as elongation factor G and initiation factor 2 (I). The architecture of the GTP-binding domain is similar in all translational GTPases from bacteria to higher eukaryotes, and hydrolysis is accompanied by conformational changes in the conserved switch I and II regions (2). Furthermore, translational GTPases bind to the same region of the ribosome in all species (3, 4). The conserved binding site and structural similarities suggest that there is a common mechanism by which the ribosome activates GTP hydrolysis in these factors. However, despite more than 40 years of research, this mechanism has remained elusive.

The catalysis of GTP hydrolysis in translational GTPases requires an invariant histidine (His56) in EF-Tu that acts as a general base, abstracting a proton from a water molecule, for in line attack on the γ-phosphate of GTP (5–7). The intrinsic GTPase activity of translational GTPases is accompanied by conformational changes in the conserved switch I and II regions (2). Furthermore, translational GTPases bind to the same region of the ribosome in all species (3, 4). The conserved binding site and structural similarities suggest that there is a common mechanism by which the ribosome activates GTP hydrolysis in these factors. However, despite more than 40 years of research, this mechanism has remained elusive.

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is low (8) and increases markedly upon productive binding to the ribosome (9, 10). For EF-Tu, efficient GTPase activation requires the binding of an aminoacyl-tRNA to its cognate mRNA codon. Activation could be accomplished either indirectly, i.e., proper ribosome binding induces an active conformation of the G factor, and/or more directly, i.e., proper ribosome binding induces an active role in catalysis. The ribosomal components that compose the GTPase binding site and therefore may be important for hydrolysis include the sarcin-ricin loop (SRL) of the 23S ribosomal RNA (rRNA) (3, 11), the L11 protein and rRNA (12), and the protein L12 (13).

Premature GTP hydrolysis in EF-Tu is thought to be prevented by a “hydrophobic gate,” consisting of residues Val 20 of the P loop and Ile 60 of switch I, which restricts access of His 84 to the catalytic water (6). Proposals of how the hydrophobic gate is “opened” include that His 84 would simply “push” through the gate when activated (6), or that a disordering (14, 15) or radical rearrangement of switch I removes this steric block (16–18). However, these hypotheses were made on the basis of structures of EF-Tu in isolation, or studies of the posthydrolysis, kirromycin-stalled complex on the ribosome. A conclusive understanding of how GTPase activation occurs on the ribosome requires the structure of a complex of EF-Tu before GTP hydrolysis.

Here, we report the crystal structure of EF-Tu bound to the 70S ribosome along with Trp-diRNA(TMP), and the antibiotic paromomycin, stalled in the activated conformation by the GTP analog β-γ-methyleneadenosine 5′-triphosphate (GDPCP), at 3.2 Å resolution. The structure reveals the GTPase-competent form of EF-Tu and suggests a universal mechanism for GTP hydrolysis on the ribosome.

The overall conformations of the ribosome, EF-Tu, and the aminoacyl-tRNA are as previously reported (15) (Fig. 1A), and all the conformational changes predicted to communicate codon recognition to EF-Tu are observed, except that the switch I loop is ordered in the GTPase center (Fig. 1B).

As well as providing insight into the mechanism of GTP hydrolysis of all translational GTPases, this structure represents a key, missing state in the decoding pathway. High-resolution crystal structures have previously been determined for the isolated TC (19) and the posthydrolysis state stalled on the ribosome by kirromycin (15). In conjunction with these two structures, this structure of the activated GTP state bound to the ribosome provides insight into the chemical details of the complete hydrolysis pathway of EF-Tu.

Before ribosome binding, the TC contains an unbound tRNA, and switch I, switch II, and the P loop are ordered around the GTP molecule in the active site. However, the catalytic His 84 (switch II) is rotated away from GDP in an inactive con-
formation (19). Ribosome binding and codon recognition induce the bent tRNA in the A/T state (where the anticodon interacts with the mRNA in the A site and the acceptor end is bound to EF-Tu) (20), as well as conformational changes in the tRNA, 3OS ribosomal subunit, and EF-Tu essential for triggering GTP hydrolysis (15). Productive binding of the TC to the ribosome results in a shift of the G domain of EF-Tu by up to ~7 Å, to avoid a steric clash between switch I and the SRL of the 23S rRNA. Ribosome binding also causes a distortion in the 3′ end of the aminoacyl-tRNA between residues 72 and 75 as reported (15), which releases contacts between the tRNA backbone and the switch I loop (fig. S1). However, in contrast to our previous prediction, this alone does not disorder switch I, which is largely unchanged from its conformation in the isolated TC (Fig. 2A) (19).

Although switch I remains ordered in the active site, the catalytic histidine is in its activated conformation (Fig. 2). We see no evidence that large rearrangements are required for catalysis (fig. S1) (15–18). Instead, the “open” state is reached by a 1.2 Å shift in the side chain of Ile84, an ~80° rotation of the side chain of Val83, and a small movement of the backbone of residues 19 to 22 away from the GTP analog (Fig. 2). The conformation of switch I is similar to that modeled for the eukaryotic ortholog of EF-G bound to β-γ-imidodiphosphosphate (GDPCP) on the ribosome (21).

These small changes in the active site would subtly increase the space between switch I and the P loop, possibly facilitating GTP hydrolysis. However, our analysis suggests that even the “closed” hydrophobic gate (19) could accommodate the activated conformation of His84 and would not preclude binding of a water in its catalytic position, though a water is not observed. Instead, to prevent GTP hydrolysis before codon recognition, the activated conformation of His84 must be unfavorable in solution. Indeed, the adjacent residues, Gly83 and Pro82, are more than 98% conserved, and mutational data suggest that the flexibility of these residues may be important (19, 22).

GTPase activation by the ribosome therefore does not involve an opening of the hydrophobic gate, but rather requires the specific positioning of His84 into the active site. In this activated structure of EF-Tu, the phosphate of residue A2662 of the SRL orders His84 in its catalytic conformation, which then acts as a general base to allow the nucleophilic water molecule to attack the γ-phosphate of GTP (Figs. 2 and 3). When positioning His84, the phosphate of A2662 could also conceivably participate in a charge-relay system that facilitates the deprotonation of the water by His84, playing a role analogous to that of the catalytic aspartic acid in serine proteases (23). However, the existence and contribution of such a mechanism would need to be assessed by biochemical experiments. The activation does not require movements within the SRL, as its overall conformation is similar to that in previous 70S ribosome structures (13, 24). This critical role of the sarcin-ricin loop answers the long-standing question of why the SRL is important for binding and GTPase activation by the ribosome. The toxin o-sarcin acts by cleaving the 23S rRNA between residues G2661 and A2662 (11), exactly the phosphate group activating His84.

It is therefore clear why cleaving this bond, or introducing mutations at nearby sites in the SRL (25), leads to defects in His84 positioning and GTP hydrolysis (11, 26). o-Sarcin is active against both bacterial and eukaryotic ribosomes and causes similar defects in the function of their respective translational GTPases (11, 26). We therefore predict that the positioning of the catalytic histidine into the active site by A2662 of the SRL is the universal mechanism for GTPase activation on the ribosome. EF-G has additional interactions between its domain 3 and A2660 of the SRL, which explains why depurination of A2660 by the toxin ricin affects EF-G, but not EF-Tu (26).

Ribosome association alone is not sufficient for A2662 to activate His84. Rather, all of the conformational changes that occur upon cognate codon recognition in the ribosome (codon-anticodon monitoring, domain closure), the tRNA (A/T state, 3′ end distortion), and EF-Tu (β-loop interaction with 3OS shoulder, G domain shift) (15) are essential for properly positioning the GTPase center of EF-Tu for activation by the SRL. The energy required to induce these movements is balanced against the energy derived from binding of a cognate tRNA, including that from interactions of the 16S rRNA residues A1492, A1493, and G530 with the minor groove of the codon-anticodon helix (27). Even subtle changes in the position of the G domain relative to the SRL would cause defects in GTPase activation by preventing A2662 from properly placing His84 into the active site.

The activation of GTP hydrolysis in EF-Tu by the SRL is similar to the regulation of cellular GTPases by their partner proteins. Unlike the classic cases of Rho and Ras, in which their GTPase activator proteins (GAPs) directly donate catalytic residues (28, 29), other G proteins are activated by “regulator of G-protein signaling” (RGS) proteins, which instead stabilize the active conformation of the GTPase (30). By analogy, the SRL acts as an RGS-type GAP for translational GTPases.

All components required for GTPase activation should be present in this catalytically active structure. Notably, we see no evidence that ribosomal protein L12, predicted to be important for hydrolysis, is interacting with EF-Tu, though such an interaction would be compatible with crystal packing. Indeed, it is sterically impossible for L12 or any other protein to interact with GTP, consistent with data suggesting that L12 does not function directly in catalysis (13, 31, 32). Furthermore, deletion of the eukaryotic orthologs of L12 from yeast ribosomes is not lethal (33), indicating that L12 may not be critical for achieving GTP hydrolysis.

Following hydrolysis, inorganic phosphate (P_i) is released from EF-Tu. The γ-phosphate of GTP makes several contacts with switch I through residue Thr61 (Fig. 3 and fig. S3), and release of P_i could destabilize and perhaps even require movement of switch I. This and the loss of interactions with the 3′ end of the tRNA backbone (fig. S1) lead to the previously observed disordering of switch I (15, 18). Furthermore, comparison of the kirromycin- and the GDPCP-stalled complexes shows a rotation of ~4° of the G domain relative to domains 2 and 3 (fig. S4). There is a corresponding movement in the acceptor stem of the tRNA, in order to maintain interactions with switch II (15), which could explain the subtle
Evolution of Yeast Noncoding RNAs Reveals an Alternative Mechanism for Widespread Intron Loss

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The evolutionary forces responsible for intron loss are unresolved. Whereas research has focused on protein-coding genes, here we analyze noncoding small nucleolar RNA (snoRNA) genes in which introns, rather than exons, are typically retained. Intron loss is therefore therefore likely to be biased toward mechanisms of intron gain and loss, which speak to questions about both the origins of introns and the marked differences in the intron-exon patterns observed across eu- karyotes (5)—for example, whether spliceosomal introns arose within eu karyotes (“introns late”), within an ancestor of both prokaryotes and eukaryotes (“introns early”), or even before the emergence of protein-coding genes (“introns first”) (6). The last two hypotheses depend on the feasibility of comprehensive intron loss within both the prokaryotic and archaean lineages, whose modern representatives lack spliceosomal introns. Within eu karyotes, the hemiaascomycetous yeasts show substantial intron loss, with modern species like Saccharomyces cerevisiae and Candida albicans devoid of introns in >90% of their genes (7). A postulated mechanism for this loss is reverse transcription of spliced RNA, followed by homologous DNA recombination that replaces the intron-containing genomic sequence with the intronless copy (8). Previous studies of intron loss have focused on protein-coding genes, however, and are therefore likely to be biased toward mecha-

In eukaryotes, protein-coding genes are frequently interrupted by introns, which must be precisely removed from RNA transcripts by the nuclear spliceosome (7). Over evolutionary time scales, the presence of introns is dynamic, with intron gain and loss rates varying substan-
tially across eukaryotic lineages (2–4). The mechanisms of intron gain and loss speak to questions about both the origins of introns and the markedly different intron-exon patterns observed across eu-
karyotes (5)—for example, whether spliceosomal introns arose within eu karyotes (“introns late”), within an ancestor of both prokaryotes and eukaryotes (“introns early”), or even before the emergence of protein-coding genes (“introns first”) (6). The last two hypotheses depend on the feasibility of comprehensive intron loss within both the prokaryotic and archaean lineages, whose modern representatives lack spliceosomal introns. Within eu karyotes, the hemiaascomycetous yeasts show substantial intron loss, with modern species like Saccharomyces cerevisiae and Candida albicans devoid of introns in >90% of their genes (7). A postulated mechanism for this loss is reverse transcription of spliced RNA, followed by homologous DNA recombination that replaces the intron-containing genomic sequence with the intronless copy (8). Previous studies of intron loss have focused on protein-coding genes, however, and are therefore likely to be biased toward mecha-

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Supporting Online Material for

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This PDF file includes:

Materials and Methods
Figs. S1 to S4
Table S1
References
Materials and Methods

Ribosomes from *Thermus thermophilus* harboring a C-terminal truncation of protein L9(1) were purified as previously described(2) from cells grown at the Bioexpression and Fermentation Facility at the University of Georgia. mRNA with the sequence 5′GGCAAGGAGGUA AAAAUGUUCUGGAAA was purchased from Dharmcon (Thermo Scientific). Trp-tRNA_Trp was prepared as described (3).

Complexes of trp-tRNA_{Trp}-GDPCP-70S ribosome were prepared and purified by Ni-NTA affinity purification as described (3) with the exception that the antibiotic paromomycin was included in the reaction mixture to increase sample yield. The trp-tRNA_{Trp} used in this study contained a G24A mutation, which increased the reproducibility of crystal growth. On cognate mRNA, it is known that this tRNA behaves identically to wild type trp-tRNA_{Trp}(4) and crystal structures of G24A and tRNA_{Trp} and bound to the ribosome on the cognate codon are extremely similar (3).

Crystals were grown as described(5) by vapor diffusion in sitting drop trays by addition of 3 μL reservoir (100 mM MES pH 6.3, 60-100 mM KCl, 50 mM sucrose, 1% glycerol, and 5.3% (w/v) PEG20K) to 3 μL of the 70S-TC sample, and cryo-protected stepwise to a final solution of 100 mM MES pH 6.3, 100 mM KCl, 50 mM sucrose, 1.1% glycerol, 6.0% (w/v) PEG20K, 15 mM MgOAc2, and 30% (w/v) PEG400 before being frozen by plunging into liquid nitrogen.

Data was collected at beamline ID 14-4 of the European Synchrotron Light Source(6), and integrated and scaled using XDS(7). These crystals are in space group P21, with unit cell dimensions a=197.6 b=274.9 c=282.5, β=91.8. This crystal form is related to our previous crystals (5) (3), which were also in P21, but the length of the a axis is doubled in the previous form. The two forms are related by a small translation of the ribosome, which converts the non-crystallographic symmetry in the larger unit cell into crystallographic symmetry, leading to only one ribosome molecule in this smaller asymmetric unit.
The structure was determined by molecular replacement in CNS(8), using a ligand-free ribosome as the search model. Iterative rounds of model building and refinement were carried out in coot(9) and CNS(8) as previously described(5). To avoid contamination of R_{free}, the R_{free} set was inherited from our previous data with the conversion h_{new}=l_{old}/2, k_{new}=-k_{old}, l_{new}=h_{old} to account for the change in unit cell. All figures were made in Pymol(10).
fig s1. Binding the ribosome induces a distortion in the 3' end of the aminoacyl-tRNA (green—activated structure, grey—isolated TC) that disrupts interactions between the Switch I loop of EF-Tu (red—activated, grey—TC) and the tRNA backbone.
fig s2. In the activated structure of EF-Tu bound to the ribosome, the Switch I loop (red) is in a well ordered and located in the GTPase center. We see no evidence that a disordered or radically remodeled Switch I (pink)(11) is required for GTPase activation and hydrolysis on the ribosome.
fig s3. Several interactions between EF-Tu and the GTP stabilize the $\beta$ and $\gamma$ phosphates that are important for GTP hydrolysis. Additionally, several interactions between the Switch I residue Thr61 and the $\gamma$ phosphate explain how release of Pi results in the disordering of Switch I.
**fig s4.** A small rotation in the G-domain of EF-Tu relative to domains 2 and 3 is observed between the GDPCP (red) and kirromycin (pink) stabilized structures bound to the ribosome.
Table s1. Summary of crystallographic data and refinement

<table>
<thead>
<tr>
<th>Data collection</th>
<th>70S-TC-GDPCP (merged from 3 crystals)</th>
</tr>
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<tbody>
<tr>
<td>Space Group</td>
<td>P2₁</td>
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<tr>
<td>Cell dimensions</td>
<td></td>
</tr>
<tr>
<td>( a, b, c ) (Å)</td>
<td>( a=197.6 \ b=274.9 \ c=282.5 )</td>
</tr>
<tr>
<td>( \alpha, \beta, \gamma ) (°)</td>
<td>( \alpha=90.0 \ \beta=91.8 \ \gamma=90.0 )</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50-3.1 (3.2-3.1) †</td>
</tr>
<tr>
<td>( R_{sym} ) (%)</td>
<td>22.8 (126.3)</td>
</tr>
<tr>
<td>( I/\sigma I )</td>
<td>6.98 (1.16) *</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.8(89.6)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>5.2 (4.3)</td>
</tr>
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</table>

| Refinement                              |                                       |
| Resolution (Å)                          | 50.0-3.1†                              |
| No. unique reflections                  | 537024                                |
| \( R_{work}/R_{free} \)                 | 23.1/26.8                             |
| No. atoms                               |                                       |
| RNA                                     | 102,537                               |
| Protein                                 | 50,602                                |
| \( B \)-factors                         |                                       |
| RNA                                     | 83                                    |
| Protein                                 | 87                                    |
| R.m.s deviations                        |                                       |
| Bond lengths (Å)                        | 0.007                                 |
| Bond angles (°)                         | 1.2                                   |

* \( I/\sigma I = 1.88 \) at 3.2 Å resolution (using a bin from 3.4-3.2 Å resolution)
† \( R_{work}/R_{free} = 33.2/35.4 \) for data from 3.2-3.1 Å resolution
References