Crystal structure of release factor RF3 trapped in the GTP state on a rotated conformation of the ribosome

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ABSTRACT

The class II release factor RF3 is a GTPase related to elongation factor EF-G, which catalyzes release of class I release factors RF1 and RF2 from the ribosome after termination of protein synthesis. The 3.3 Å crystal structure of the RF3-GDPNP-ribosome complex provides a high-resolution description of interactions and structural rearrangements that occur when binding of this translational GTPase induces large-scale rotational movements in the ribosome. RF3 induces a 7° rotation of the body and 14° rotation of the head of the 30S ribosomal subunit, and itself undergoes inter- and intradomain conformational rearrangements. We suggest that ordering of critical elements of switch loop I and the P loop, which help to form the GTPase catalytic site, are caused by interactions between the G domain of RF3 and the sarcin–ricin loop of 23S rRNA. The rotational movements in the ribosome induced by RF3, and its distinctly different binding orientation to the sarcin–ricin loop of 23S rRNA, raise interesting implications for the mechanism of action of EF-G in translocation.

Keywords: translation; GTPase; termination; translocation; switch loop; domain rearrangement

INTRODUCTION

Advances in elucidation of ribosome structure at the all-atom scale have provided a rich description of the molecular architecture of these very large molecular complexes (Schuwirth et al. 2005; Korostelev et al. 2006; Selmer et al. 2006). Despite this wealth of structural information, our knowledge falls short of an understanding of the mechanism of protein synthesis at the molecular level. This is because the structural complexity of the ribosome is compounded by its conformational dynamics, which are essential to its function (Korostelev et al. 2008b; Munro et al. 2009; Dunkle and Cate 2010; Frank and Gonzalez 2010). The importance of ribosome dynamics is highlighted by observations from several different approaches, including cryo-electron microscopy (cryo-EM) (Frank and Agrawal 2000; Fischer et al. 2010; Ratje et al. 2010), X-ray crystallography (Ogle et al. 2001; Schuwirth et al. 2005; Korostelev and Noller 2007; Zhang et al. 2009) and Förster resonance energy transfer (FRET) (Ermolenko et al. 2007a; Cornish et al. 2008, 2009; Fei et al. 2008; Marshall et al. 2008; Munro et al. 2010). Among the different modes of ribosome movement,

one of the most well known is the large-scale rotation between the 30S and 50S subunits, first observed in cryo-EM reconstructions of ribosomes bound with elongation factor EF-G (Frank and Agrawal 2000). Counter-clockwise rotation has been correlated with movement of tRNAs into hybrid states (Moazed and Noller 1989; Spiegel et al. 2007; Agirrezabala et al. 2008; Julian et al. 2008; Dunkle et al. 2011) and has been proposed to be the basis of a ratcheting mechanism responsible for the coupled translocation of mRNA and tRNA during protein synthesis (Frank and Agrawal 2000; Valle et al. 2003; Frank et al. 2007). Beside intersubunit movement, there is good evidence for rearrangement of many subfeatures of the large and small ribosomal subunits, including the head of the 30S subunit, the L1 stalk, the L11 stalk, and even separate domains of the tRNAs during ribosome function. The challenge is to determine precisely how and when these movements occur, and to understand how they contribute to the mechanisms of translation.

Counter-clockwise intersubunit rotation is induced in response to binding of EF-G (Frank and Agrawal 2000), ribosome recycling factor (RRF) (Gao et al. 2005; Dunkle et al. 2011), or the class II release factor RF3 (Klaholz et al. 2004; Ermolenko et al. 2007a; Gao et al. 2007), a GTPase structurally related to EF-G that is responsible for promoting dissociation of the class I release factors RF1 and RF2 from the ribosome following termination (Goldstein and Caskey 1970; Freistoffer et al. 1997; Klaholz 2011). Although these con-

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.031187.111>.

formational changes in the ribosome are stabilized by interactions with translation factors, they appear to be inherent dynamic properties of the ribosome. For example, both intersubunit rotation and movement of the L1 stalk occur spontaneously in the absence of factors or GTP, and thus can be driven by thermal energy alone (Cornish et al. 2008, 2009; Fei et al. 2008). Elongation factor EF-Tu is not known to cause rotation (Allen et al. 2005; Schmeing et al. 2009), yet the conserved G domains of all of the GTPase factors bind to the sarcin–ricin loop (SRL) of 23S rRNA and share other common ribosomal binding surfaces. How interactions with these translation factors induce large-scale conformational changes in the ribosome, how these dynamic events enable basic ribosomal functions, and the role of GTP hydrolysis in the diverse functions of these factors are all poorly understood.

Here, we present the 3.3 Å crystal structure of the 70S ribosome bound with RF3 and the nonhydrolyzable GTP analog GDPNP. Binding of the factor induces a 7° rotation of the 30S subunit relative to the 50S subunit and a 14° rotation of the head of the 30S subunit relative to its body. These resulting large-scale structural rearrangements are predicted to distort or disrupt interactions that are responsible for binding of the class I release factors, explaining catalysis of their release by RF3. RF3 itself undergoes major inter- and intradomain conformational rearrangements between its free GDP state and its ribosome-bound GDPNP state. In our structure, the functionally important switch loop I is well ordered, allowing the first description of the interactions of the active site of the RF3 GTPase with GDPNP. Interestingly, the orientation of the G domain of RF3 relative to the 50S ribosomal subunit is distinctly different from that observed for EF-G·GDP, EF-Tu·GDP, or EF-Tu·GDPNP (Gao et al. 2009; Schmeing et al. 2009; Voorhees et al. 2010), suggesting the possibility of the existence of more than one binding mode for the G domains of the GTPase translation factors during different stages of their interactions with the ribosome. Finally, the observed ribosomal conformational rearrangements result in opening of the constriction between the head and platform of the 30S subunit that obstructs movement of the tRNA anticodon stem–loop (ASL), between the P and E sites (Schuwirth et al. 2005), and causes displacement of features of 16S rRNA that contact the anticodon stem of P-site tRNA by a distance closely matching that required for movement of the ASL from the P site to the E site. This is an observation that may have implications for the molecular mechanics of translocation.

RESULTS

Structural rearrangements of the ribosome

Complexes of *Escherichia coli* 70S ribosomes bound with *E. coli* RF3 were formed in the presence of GDPNP, mRNA, and tRNA as described in the Materials and Methods section. Under the solution conditions used for crystallization, tRNA

dissociated from the complex (Supplemental Fig. S1), but mRNA remained bound. The complex crystallized in space group P2₁2₁2₁ with cell dimensions 257.6 × 312.9 × 328.6 Å containing a single 70S ribosome per asymmetric unit, a previously unobserved ribosome crystal form. The 3.3 Å electron density map reveals well-resolved features of the rRNA, r-proteins, and RF3-GDPNP (Fig. 1; Supplemental Fig. S2), although parts of the 30S subunit head and the L1 stalk are less well-ordered. There is clear density for the pentameric complex of proteins L10(L12)₄, in which the structures of L10 and the N-terminal domains of the four L12 molecules can be fitted (Supplemental Fig. S3A). The orientation of the L10(L12)₄ complex is rotated by ~60° around a hinge point between the globular N-terminal domain of L10 and its long, C-terminal α-helix, relative to that seen in the 70S ribosome-EF-G complex (Gao et al. 2009). This was the only previous 70S ribosome crystal structure in which the L10(L12)₄ pentamer was observed (Supplemental Fig. S3B,C).

RF3 induces large-scale rotational movements of the head and body of the 30S subunit, and other smaller conformational changes in both ribosomal subunits compared with the classical-state ribosome (we refer to the classical-state RF2 termination complex for comparison unless otherwise noted) (Fig. 2). The body of the 30S subunit is rotated 7° counter-clockwise relative to the 50S subunit, as viewed from the 30S solvent face (Fig. 2A), around an axis of rotation perpendicular to the subunit interface centered

FIGURE 1. Example of electron density maps. (A) Electron density for rRNA. Stereo view of 3.3 Å resolution 2F_o–F_c electron density contoured at 2.5 σ, showing contact between 16S rRNA (cyan, left), and 23S rRNA (gray, right), around intersubunit bridge B3. (B) Electron density map for RF3. Stereo representation of unbiased 3.3 Å resolution F_o–F_c electron density for release factor RF3, contoured at 2.4 σ.

FIGURE 2. Conformational changes in the ribosome in the RF3-GDPNP complex. (A) 7° counter-clockwise rotation of the 30S subunit (*foreground*) in the RF3 complex, as viewed from the solvent surface of the 30S subunit, relative to the classical-state RF2 complex (Korostelev et al. 2008a). The 50S subunit (gray) is in the background. (B) Stereo view showing 14° counter-clockwise rotation of the head domain of the 30S subunit, as viewed from the *top* of the subunit. The subunit interface is at the *top* of the figure. 16S rRNA and 30S proteins are colored magenta (RF3 complex) and cyan (RF2 complex).

at intersubunit bridge B3. A subtler counter rotation of the platform of the 30S subunit helps to preserve the intersubunit contacts in bridge B4 (see below). Rotation of the 30S subunit body is similar to that observed in cryo-EM reconstructions of EF-G and RF3 complexes (Frank and Agrawal 2000; Klaholz et al. 2004; Gao et al. 2007) and for the recent crystal structure of a complex containing tRNA trapped in the P/E hybrid state in the presence of RRF (Dunkle et al. 2011). In the RF3 complex, there is also a dramatic 14° counter-clockwise rotation (when viewed from above) of the head of the 30S subunit (Fig. 2B). Rotation of the head opens the previously noted constriction between the 30S P and E sites (Schuwirth et al. 2005) and results in movement of P-site elements in the head into a position juxtaposed with E-site elements of the 30S body (discussed below). Combined rotation of the 30S subunit body and head results in overall movements as great as 35 Å between equivalent positions in the classical and RF3-bound rotated states.

Intersubunit and head rotation result in rearrangement or disruption of many of the dozen bridges that connect

the 30S and 50S subunits (Fig. 3; Supplemental Table S2; Yusupov et al. 2001; Schuwirth et al. 2005). Several of the most central bridges are preserved by flexing of local structural elements, while the peripheral bridges are rearranged or completely disrupted and replaced by new bridges in the rotated structure. For example, both bridges joining the head of the 30S subunit to the top of the 50S subunit (bridges B1a and B1b) are disrupted; in their place, a new bridge, R1b, is formed between 50S protein L5 and 30S proteins S13 and S19, resulting in displacement of the L5 contact to the 30S head by a distance of >30 Å (Fig. 3C,D). Changes in bridges involving the 30S subunit body are similar, but not identical, to those reported for the RRF hybrid-state structure (Dunkle et al. 2011). The contacts forming bridge B2a, which connects helices 44 and 45 of 16S rRNA to helix 69 of 23S rRNA and contains the highest density of intersubunit interactions, are almost completely preserved between the classical and rotated states. This is accomplished by rotation of helix 69 to match the movement of the 30S subunit (Supplemental Fig. S4A). In contrast to the RRF hybrid-state structure, where there is some rearrangement of the secondary structure of helix 69, in the RF3 complex the terminal base pair (C1925–G1929) is preserved, and the conserved U1926 remains in its intercalated position (Supplemental Fig. S4B). Helix 34 of 23S rRNA, which contacts 30S protein S15 to form Bridge B4 (Culver et al. 1999), bends in its internal loop around positions 705/726, resulting in a 6 Å displacement at its apical loop to maintain interaction with S15 in the RF3-bound rotated state (Supplemental Fig. S5A). Bridge B7a, formed by tertiary contacts between A702 of 16S rRNA and G1846 and A1848 of 23S rRNA, is disrupted in the RF3 structure and replaced by bridge R7a, in which A702 is displaced by 13 Å to hydrogen bond with phosphate 1847 (Supplemental Fig. S5B).

Binding of RF3 to the ribosome

RF3 binds to the ribosome at the subunit interface, bridging the 30S and 50S subunits in a position that overlaps with those of EF-G and EF-Tu (Fig. 4A; Supplemental Fig. S6). Like the other GTPase factors, RF3 contacts the 50S subunit surface at the sarcin–ricin loop (SRL) (helix 95) of 23S rRNA. However, domain I (the G domain) of RF3 binds to the SRL of the 50S subunit in an orientation that differs markedly from that observed for EF-Tu and EF-G (Gao et al. 2009; Schmeing et al. 2009; Voorhees et al. 2010), as discussed below. In contrast to EF-Tu and EF-G, the G domain of RF3 contacts protein L6 and not the L11 stalk (Fig. 4B). RF3 is well ordered, allowing many individual side chains to be resolved in an unbiased electron density map (Fig. 1B). Binding to the ribosome results in extensive structural rearrangement of RF3 relative to its free GDP form, both in the relative orientations of its three domains and in the structure within the domains themselves. Relative to the

FIGURE 3. Rearrangement of intersubunit bridges. (A,B) Molecular surface representations of (A) 30S subunits and (B) 50S subunits showing intersubunit bridge contacts of the classical-state RF2 70S ribosome complex (*left*) (Korostelev et al. 2008a) and the rotated RF3 70S complex (*right*). Bridge contacts are colored according to whether they are maintained (blue), disrupted (green), or newly formed (red) in the rotated state. Classical-state bridges are numbered according to Yusupov et al. (2001). (C,D) Disruption and reformation of intersubunit bridges involving the head of the 30S subunit. (C) In the classical-state RF2 complex, bridge B1a is formed between protein S13 and helix 38 of 23S rRNA, and B1b between S13 and L5. (D) In the RF3 complex, a large-scale counter-clockwise rotation of the 30S head results in disruption of bridges B1a and B1b; bridge R1b is formed between proteins S13 and S19 from the 50S subunit, involving a 34 Å displacement of the intersubunit contacts.

G domain, domain II undergoes a 7° rotation and domain III is rotated by 55°, enabling contacts with the two ribosomal subunits that are responsible for stabilizing intersubunit rotation. The large-scale movement of domain III, which was already apparent from cryo-EM reconstruction (Gao et al. 2007), results in displacements of up to 35 Å (Fig. 5A,B).

Many additional differences are seen in the crystal structure of ribosome-bound RF3, most notably conformational changes within domains I and II. In domain I, residues 39–69, which contain switch loop I, a functionally

important feature that is conserved among G proteins (Wittinghofer and Vetter 2011), and disordered in all previous structures of RF3 and EF-G, become ordered in the RF3-GDPNP-ribosome complex, completing the enclosure that surrounds GDPNP within its binding site (Fig. 6A). As discussed below, ordering of the region upstream of switch loop I results in formation of a unique contact with the SRL around positions 2655/2664, which contributes to the unusual orientation of the G domain on the SRL. Residues 91–95 in the rearranged switch loop II form part of the γ -phosphate-proximal end of the GDPNP binding site and additionally create an interaction with positions 442–443, potentially stabilizing the rotation of domain III. On the 30S subunit, domains II and III of RF3 form a complementary surface that contacts protein S12 and 16S rRNA helices h5 and h15 (Fig. 4C). The universally conserved His311 in domain II contacts the surface of 16S rRNA at a site centered on the universally conserved A55–U368 tertiary base pair, which connects h5 and h15. Domain III interacts with the lower edge of protein S12 and makes salt bridges to the adjacent phosphate at position 359 in 16S rRNA.

GDPNP is held by extensive contacts with backbone and side-chain groups of the conserved P loop, switch 1, and NKXD motifs of RF3 (Fig. 6A). The triphosphate and ribose of GDPNP are buried in a narrow cavity inside domain I of RF3, while its guanine moiety is exposed to the backbone of the sarcin loop of 23S rRNA, where phosphate 2656 forms a hydrogen bond with its 2-amino group. The guanosine is further positioned in its RF3-binding pocket by H-bonds between its N1 and N2 positions with Asp145,

FIGURE 4. Binding of RF3 to the 70S ribosome. (A) Stereo view of RF3 (orange) bound at the entrance to the interface cavity of the 70S ribosome. (B) RF3 contacts viewed from inside the interface cavity. Domain I (the G domain; yellow) of RF3 interacts with the sarcin-loop (SRL) of 23S rRNA (gray) and with protein L6 (light magenta). (C) View from inside the interface cavity, showing contacts between domains II (blue) and III (magenta) of RF3 with helix 5 of 16S rRNA (cyan) and protein S12 (dark blue).

FIGURE 5. Conformational rearrangements in RF3. (A,B) Comparison of crystal structures of free RF3-GDP (A) (Gao et al. 2007) and ribosome-bound RF3-GDPNP (B). A large-scale rotational movement of domain III (magenta) and smaller movements in domains I (yellow) and II (cyan) can be seen. Disordered segments in RF3-GDP are shown as dotted lines. Rearranged segments in RF3-GDPNP containing switch loops I and II are shown in blue and green, respectively. GDP and GDPNP are shown in red. (C) Ordering of residues 39–69 and repositioning of residues 70–73 of RF3 (including the switch loop 1 residues 64–72) creates a contact between the G domain of RF1 and the SRL of 23S rRNA. Residues 39–63 are shown in dark blue, the switch loop in cyan, and the rest of RF3 in yellow.

and O6 with Asn142; packing against the Lys143 side chain and of its ribose against Val46; and an H-bond between the backbone oxygen of Val46 and the ribose 3'-OH group. The α phosphate makes H-bonds to the side chain and backbone of Thr28. The $F_o - F_c$ difference electron density for GDPNP shows a peak positioned next to the scissile β - γ phosphate linkage (Fig. 6B). This density likely corresponds to a bound magnesium ion seen in many G proteins (Wittinghofer and Vetter 2011), which coordinates with the β and γ phosphates and with the side-chain hydroxyls of Thr27 and Ser69. The β phosphate is also held by H-bonds to the backbone amide groups of Ala24, Gly25, and Thr27, and the γ phosphate by H-bonds with backbone NH groups of Asp23 and Gly91. His92, which has been proposed to act as a general base in the GTPase reaction (Voorhees et al. 2010), is oriented away from the γ -phosphate (Fig. 6C).

Binding of viomycin in the RF3 complex

The RF3-70S ribosome complex was crystallized both in the presence and absence of the antibiotic viomycin, a translocation inhibitor whose inclusion improved the diffraction properties of the crystals (Supplemental Table S1). An $F_o - F_c$

difference electron density map shows density for viomycin in the decoding site of the 30S subunit (Supplemental Fig. S7), at a location essentially identical to that recently identified by Steitz and coworkers for binding of viomycin to classical-state *Thermus thermophilus* 70S ribosomes (Stanley et al. 2010). Viomycin is bound to a site that is normally occupied by stacking of bases A1492 and A1493 at the top of helix 44. Both bases are flipped out in our structure (Supplemental Fig. S7), as in the Stanley et al.

FIGURE 6. Interactions in the GTP binding site of RF3. (A) An intricate network of H-bonds is formed between the base, ribose, and phosphate groups of GDPNP and domain I of RF3, including elements of switch I (residues 64–72), P loop (residues 21–28), NKXD motif (residues 142–145), and phosphate 2656 of the SRL of 23S rRNA. A Mg atom (green) is coordinated by Thr27, Ser91, and the β and γ phosphates of GDPNP (see Supplemental Fig. S9). (B) Difference electron density showing Mg bound to GDPNP. An $F_o - F_c$ difference map was calculated using the complete RF3-GDPNP-70S ribosome model, shown here contoured at 4.5 σ . The Mg ion (green) is coordinated with the β - and γ -phosphate oxygens of GDPNP and with the side-chain hydroxyls of Thr27 and Ser 69. (C) His92 of RF3 is positioned at a distance of 8 Å from the γ phosphate of GDPNP. The $2F_o - F_c$ electron density map is shown around His92 and GDPNP, contoured at 1.3 σ and 2.3 σ , respectively.

(2010) structure, similar to the conformations induced by binding of paromomycin (Ogle et al. 2001), which binds to a site that overlaps with that of viomycin. Although viomycin itself has been shown to stabilize intersubunit rotation in solution (Ermolenko et al. 2007b), binding of viomycin to the classical-state ribosome suggests that its effects on intersubunit rotation in solution can be overcome by forces generated during formation of the crystal lattice. It is not obvious from the location of viomycin in the 30S decoding site how it influences intersubunit rotation. In any case, the rotational state that we observe for the RF3 complex is formed independently of the presence of viomycin; identical intersubunit and head rotations are observed in a lower-resolution (3.8 Å) structure obtained from crystals grown from an otherwise identical RF3 complex formed in the absence of viomycin (Supplemental Fig. S8; Supplemental Table S1). Apart from the flipping of bases A1492 and A1493 of 16S rRNA, and rearrangement of the nearby A1913 of 23S rRNA, no major viomycin-dependent structural differences were detected (overall RMSD = 0.76 Å), within the limits of the resolution of the two structures (Supplemental Fig. S10).

DISCUSSION

Release of RF1 and RF2 by RF3

Our structure provides a structural basis for understanding how RF3 catalyzes dissociation of release factors RF1 and RF2 from the ribosome following termination of protein synthesis (Goldstein and Caskey 1970; Freistroffer et al. 1997). Termination complexes contain RF1 or RF2 bound to the ribosome in its classical, nonrotated state (Rawat et al. 2003, 2006; Korostelev et al. 2008a, 2010; Laurberg et al. 2008; Weixlbaumer et al. 2008). It has been shown that deacylation of peptidyl-tRNA must occur prior to removal of RF1/RF2 by RF3 (Zavialov et al. 2002), most likely to allow P/E hybrid-state formation of the deacylated tRNA and, therefore, intersubunit rotation. With RF3-GDPNP bound, the resulting rotated state of the ribosome creates clash between domain IV of RF2 (positions 330–340) and the head of the 30S subunit (16S rRNA positions 1208–1210), and between domain I of RF2 (positions 45–50) and the L11 stalk (23S rRNA positions 1067–8). Thus, both of the major rotational changes induced by RF3—of the 30S subunit head and body—would contribute to disruption of interactions between RF2 and the ribosome. GTP hydrolysis would then lead to release of RF3-GDP, caused by the predicted disruption of contact between domain III of RF3-GDP and the 30S subunit in the rotated state.

Although the structure of free RF3-GTP is not known, if it resembles that of ribosome-bound RF3-GDPNP, steric clash between domain III of RF3 and protein S12 would interfere with binding of RF3-GTP to the nonrotated termination complex. Ehrenberg and coworkers have proposed

that RF3-GDP first binds the termination complex, followed by ribosome-facilitated nucleotide exchange, in which GDP is replaced by GTP (Zavialov et al. 2001). Indeed, when RF3-GDP is docked on the SRL of the nonrotated RF2 termination complex by aligning its G domain with the position of the G domain of RF3-GDPNP in our structure, the steric clash predicted for RF3-GDPNP with the nonrotated ribosome is relieved; in addition, this modeling predicts formation of an interaction between domain III of RF3 and domain I of RF2 (Supplemental Fig. S9), suggesting a potential functional role for domain I of the type I release factors. However, the fact that RF3-GDP does not bind stably to the termination complex (Pel et al. 1998; Supplemental Fig. S10) is inconsistent with this scenario in its simplest form. An alternative possibility is that the termination complex exists in equilibrium between its rotated and nonrotated states, and RF3-GTP binds to and traps the complex in its rotated form. Further experiments will be needed to resolve these possibilities.

GTP hydrolysis

Based on the crystal structure of an EF-Tu-GDPCP-ribosome complex, Ramakrishnan and coworkers proposed a universal mechanism for GTPase activation of translational GTPases by the ribosome (Voorhees et al. 2010). A conserved histidine (His84 in EF-Tu) that has been implicated in catalysis of GTP hydrolysis (Cool and Parmeggiani 1991), interacts with the phosphate of SRL nucleotide A2662, thus orienting it toward a water positioned next to the γ phosphate of GDPCP. Voorhees et al. (2010) propose that this histidine plays a crucial catalytic role, acting as a general base to abstract a proton from the water for in-line attack on the γ phosphate of GTP, and predict that its positioning by A2662 of the SRL is a universal mechanism for GTPase activation on the ribosome.

Our structure of RF3-GDPNP bound to the ribosome is inconsistent with the universal mechanism proposed by Voorhees et al. (2010). The corresponding histidine residue in RF3, His92, is 8 Å from the γ -phosphate of GDPNP (Fig. 6C), ruling out its participation as a general base. The contribution of a general base to catalysis by EF-Tu and other G proteins in this way is the subject of ongoing discussion. Liljas et al. (2011) note that in numerous previously published structures of translational GTPases, this histidine is seen in many different positions, but rarely at the water molecule adjacent to the γ -phosphate, and cite studies implicating the γ -phosphate itself as a general base. In an analysis of the mechanism of the related Ras GTPase, Herschlag and coworkers (Maegley et al. 1996; Lassila et al. 2011) show that for a dissociative transition state, there would be little advantage for removal of a proton from the attacking water. Instead, they point out that the atom undergoing the largest change in charge during formation of the transition state is the β - γ bridge oxygen, and thus

stabilization of its developing negative charge would make a more substantial contribution to catalysis. Finally, EF-G-2, which carries out ribosome-stimulated GTP hydrolysis (Connell et al. 2007), contains a tyrosine rather than a histidine residue at the corresponding position of its P loop, which is incompatible with the universality of the proposed mechanism.

In contrast to the EF-Tu complex (Voorhees et al. 2010), SRL nucleotide A2662 does not contact GDPNP or RF3. The sole interaction between the SRL and GDPNP is between phosphate 2656 and the 2-amino group of the guanine of GDPNP. We suggest that, in the case of RF3, ribosome-dependent GTP hydrolysis is an indirect result of the ordering of RF3 when it binds to the ribosome. In all previous structures of RF3 and EF-G, including the structure of the EF-G-ribosome complex (Gao et al. 2009) the region of the G domain corresponding to residues 39–69 are disordered; in the RF3-ribosome structure described here, this region becomes ordered (Fig. 5C; Supplemental Fig. S11), which results in positioning of critical elements of the GTPase active site. Upstream of switch loop 1, interactions between Lys47 and Arg49 and SRL nucleotides 2664 and 2663, respectively (Fig. 5C; Supplemental Fig. S11), anchor Val46 in the active site and contribute to the conformational change in switch loop 1 that includes positioning of Ser69 to coordinate the critical Mg ion at the β - γ -phosphate linkage.

Possible implications for the mechanism of translocation

The 7° rotation of the 30S subunit body in our structure is similar to that observed for the recent crystal structure of a hybrid-state complex formed with RRF (Dunkle et al. 2011) and for cryo-EM reconstructions of EF-G- and RF3-containing complexes (Frank and Agrawal 2000; Klaholz et al. 2004; Ratje et al. 2010). The pronounced 14° rotation of the 30S head more closely resembles the conformation of the 30S subunit in cryo-EM structures of a newly identified state (“substate II”) observed in cryo-EM reconstructions of a 70S ribosome-EF-G complex (Ratje et al. 2010) and of an 80S ribosome-eEF2 complex (Spahn et al. 2004), and in a crystal structure of the vacant yeast 80S ribosome (Ben-Shem et al. 2010). In the RF3 complex, the positions of the tRNA-binding elements of the 30S subunit head, including G966, G1338, and A1339, which form the main contacts between the ribosome and the ASL of P-site tRNA (Berk et al. 2006; Korostelev et al. 2006; Selmer et al. 2006), are dramatically displaced. Specifically, G1338, which contacts the minor groove of the anticodon stem of P-site tRNA in the classical state, moves by 23 Å toward the E site from its location in the nonrotated ribosome, virtually identical to the distance traversed by the ASL during translocation from the 30S P site to the E site. In the same rotational movement, the gap between the head and platform, which in the

classical state is too narrow to allow passage of the ASL between the P and E sites (Schuwirth et al. 2005), is widened from 15 to 22 Å, sufficient to permit translocation of the ASL. Figure 7 illustrates how the positions of the tRNAs would change between the classical and rotated RF3-bound states with their ASLs following the movement of the head of the 30S subunit, as suggested by cryo-EM studies (Fischer et al. 2010; Ratje et al. 2010). Thus, a combination of 30S body and head rotations similar to those observed here is sufficient to allow for the repositioning of the tRNA ASLs that is required for translocation.

Previous crystal structures of EF-G-GDP and EF-Tu-GDP or EF-Tu-GDPCP bound to the ribosome showed a nearly identical binding orientation of their G domains to the SRL of 23S rRNA (Gao et al. 2009; Schmeing et al. 2009; Voorhees et al. 2010). However, orientation of the G domain of RF3-GDPNP differs from that of the other two factors. This is illustrated in Figure 8A, in which the structures of the respective G domains of EF-Tu, EF-G, and RF3 are aligned by a least-squares fit. Although the orientation of the SRL relative to the G domain is virtually identical for EF-Tu and EF-G, that of RF3 is rotated by $\sim 45^\circ$ from that of the other factors. Since the orientation of the SRL itself in the 50S subunit is unchanged between the different factor complexes within experimental error, this illustrates the distinctly different orientation of the G domain of RF3.

It is not known whether the other GTPase factors can bind to the SRL in the RF3 orientation; however, it seems unlikely that this orientation is unique to RF3, given the conservation and structural similarities between the G domains of EF-G and RF3 (Fig. 8B). This possibility is of some interest, because the orientation of EF-G on the 70S ribosome, as seen in all but one cryo-EM reconstructions (Frank and Agrawal 2000; Stark et al. 2000; Valle et al. 2003; Ratje et al. 2010) and in the single crystal structure of an EF-G-70S ribosome complex (Gao et al. 2009), places domain IV of EF-G in the intersubunit cavity in a position that creates

FIGURE 7. Geometry of head rotation and tRNA positioning in the 30S subunit. P-site ASL contacts in the 30S subunit head are displaced by 23 Å in the RF3 complex, relative to their position in the classical-state RF2 complex. Docking of P-site (orange) and E-site (red) tRNAs on the (A) classical-state RF2 and (B) rotated RF3 structures illustrates that the combined 30S head and body rotations seen in the RF3 structure are sufficient to translocate an ASL from the 30S P site to the E site. The views in A and B are shown in exactly the same orientation relative to the 50S subunit. In B, the initial positions of the P- and E-site tRNAs as in A are shown in transparent orange and red, respectively.

FIGURE 8. Implications of the orientation of RF3 domain I for EF-G binding. (A) Orientations of domain I of EF-Tu (Voorhees et al. 2010), EF-G (Gao et al. 2009), and RF3 (this work) relative to the SRL of 23S rRNA. The domains were aligned in three dimensions by virtue of their conserved structural elements. The conserved functional elements of the respective factors are indicated: switch 1 (blue); switch 2 (green); P loop (magenta); NKXD motif (cyan). The G nucleotides are shown in red. The orientation of the SRL in the RF3 complex is rotated by $\sim 45^\circ$ relative to those of the other factors. (B) Three-dimensional alignment of Domains I, II, and III of RF3-GDPNP (yellow) and EF-G (green) (Gao et al. 2009) show clear structural homology. (C) Observed orientation of EF-G (green) bound to the classical-state 70S ribosome-EF-G complex (Gao et al. 2009), in which an A-site tRNA has been docked in the A site; note the steric clash with A-site tRNA. (D) Orientation of EF-G in the classical-state ribosome with its domain I docked on the 50S subunit in the same orientation as that of RF3-GDPNP. Note the absence of steric clash with A-site tRNA or the ribosome.

severe clash with a tRNA bound to the A site (Fig. 8C). Since the biological role of EF-G requires that it acts on ribosomes containing tRNAs bound to both the A and P sites, it must bind to the ribosome initially in a different orientation. Alignment of the G domain of EF-G with that of RF3 results in a remarkable repositioning of EF-G, in which domain IV avoids all contact with A-site tRNA, and instead transversely spans the entrance to the interface cavity, where its tip contacts the top of the shoulder at the outer edge of the 30S subunit (Fig. 8D). This orientation of EF-G may be similar to that seen in an earlier cryo-EM reconstruction of EF-G-GTP trapped in the presence of thiostrepton (Stark et al. 2000). In our structure, residues 45–53 of RF3 form a loop that makes an additional contact

to the SRL that may stabilize its orientation. The corresponding region of EF-G is disordered in the EF-G-GDP:70S ribosome-fusidic acid complex; thus, it is possible that it rearranges during initial binding of EF-G to form a similar interaction that stabilizes an RF3-like orientation. Resolution of these and the many other unanswered questions concerning ribosome structural dynamics will depend on elucidation of the structures of additional functional complexes, and crucially, on critical tests of mechanistic models.

After completion of this work, the crystal structure of *E. coli* RF3 bound to *T. thermophilus* ribosomes was reported by Ramakrishnan and coworkers (Jin et al. 2011). Their structure differs from the one presented here in several respects. The rotation of the 30S subunit body, smaller rotation of the 30S subunit head, and presence of a P/E hybrid-state tRNA all very closely resemble those observed by Cate and coworkers for *E. coli* hybrid-state ribosomes complexed with RRF (Dunkle et al. 2011). Few details of the interactions between RF3 and the ribosome or the structural rearrangements in RF3 are reported, including structures for the previously disordered loops, most likely because of lower resolution and low occupancy of RF3-GDPNP for their complex. The greater degree of head rotation seen in our structure and in the subpopulation reported by Spahn and coworkers (Ratje et al. 2010) suggests that this conformation may represent an intermediate state that is disfavored in the presence of a bound tRNA.

MATERIALS AND METHODS

Preparation of ribosome–RF3 complex

Tight-couple 70S ribosomes were prepared from *E. coli* strain CSH142/L9ΔK (Lieberman et al. 2000), which lacks 50S ribosomal protein L9. Typically, 2 L of cells grown to mid-log at 37°C was spun down, resuspended in buffer A (20 mM Tris-Cl at pH 7.0, 100 mM NH₄Cl, 10.5 mM MgCl₂, 0.5 mM EDTA, and 5 mM βME), lysed, and pelleted through two 10-mL cushions containing 1.1 M sucrose and high-salt buffer A (20 mM Tris-Cl at pH 7.0, 500 mM NH₄Cl, 10.5 mM MgCl₂, 0.5 mM EDTA, and 5 mM βME), in a Beckman Ti60 rotor at 39,000 rpm for 20 h at 4°C. Each pellet was resuspended in 25 mL of high-salt buffer A, then pelleted in a Ti60 rotor at 55,000 rpm for 2 h at 4°C. This was repeated once. The salt-washed ribosome pellets were dissolved in a total volume of 2 mL of buffer A and layered onto six 38-mL 10%–35% sucrose gradients containing 20 mM Tris-Cl (pH 7.0), 100 mM NH₄Cl, 6 mM MgCl₂, and 5 mM βME. Gradients were centrifuged in a Beckman SW28 rotor at 19,000 rpm for 16 h at 4°C. The 70S ribosome peak was collected, and the MgCl₂ concentration was increased to 10 mM before pelleting in a Beckman Ti45 rotor at 36,000 rpm for 22 h at 4°C. The ribosome pellet was dissolved in 100 μL of buffer A, aliquoted, quick-frozen in liquid N₂, and stored at –80°C.

E. coli RF3 was amplified from MRE600 genomic DNA using primers 5'-TTTCCATCCCATATGACGTTGTCTCCTTATTTGC and 5'-CCTATACCGGATCCTTAATGCTCGCGGGTCTGGTGG, then ligated into the NdeI/BamHI sites of pET21b (Novagen).

Five additional His residues were added to the C-terminal His by site-directed mutagenesis (Kunkel 1985), to generate pRF3-C-6His. RF3-C-6His protein was expressed in strain BLR(DE3) by induction with 1 mM IPTG for 3 h. The cell pellet from 1 L was resuspended in 10 mL of 25 mM Tris-Cl (pH 7.5), 50 mM KCl, 15 mM imidazole, 15% glycerol, and 5 mM β ME; lysed through a French press at 18,000 psi; then centrifuged at 10,000 rpm for 20 min in a Beckman JA20 rotor at 4°C. The supernatant was incubated with 2 mL of Ni-NTA agarose resin (QIAGEN) for 1 h at 4°C. The resin was washed with 40 mL of the same buffer containing an additional 500 mM KCl. RF3 was eluted with 6 mL of 50 mM Tris-Cl (pH 7.5), 50 mM KCl, and 250 mM imidazole, then dialyzed into buffer Q (25 mM Tris-Cl at pH 7.5, 20 mM NH₄Cl, 5 mM β ME), and further purified by FPLC, using a 6-mL Resource-Q anion-exchange column (Amersham). RF3 was eluted with a 20–300 mM NH₄Cl gradient over 300 mL, dialyzed into buffer A, aliquoted, quick-frozen in liquid N₂, and stored at –80°C.

Typically, 400 pmol of ribosomes, 600 pmol of tRNA-Met (MP Biomedicals), and 1000 pmol of mRNA M0-27 (5'-GGCAAG GAGGUAUAAAAUGUAAAAA, IDT) were incubated for 20 min at 37°C in 20 mM Tris-Cl (pH 7.0), 100 mM NH₄Cl, 21 mM MgCl₂, and 1 mM EDTA. The MgCl₂ concentration was adjusted to 10.5 mM by adding an equal volume of buffer containing 20 mM Tris-Cl (pH 7.0) and 100 mM NH₄Cl, before adding 4000 pmol of RF3, GDPNP, and viomycin; and incubating for 10 min at 37°C. The final solution conditions were 20 mM Tris-Cl (pH 7.0), 100 mM NH₄Cl, 10.5 mM MgCl₂, 0.5 mM EDTA, 1.5 mM GDPNP, and 1.5 mM viomycin in a volume of 100 μ L.

Crystallization

Crystals were grown via vapor diffusion in 96-well plates by adding 3 μ L of reservoir solution (100 mM Tris at pH 7.0, 25–35 mM KCl, 6.1% PEG20K, 1% glycerol, 50 mM sucrose), to 1 μ L of sample, which results in a final concentration of 3.3 mM MgCl₂ in the drop after equilibrium. Viomycin-free complexes were crystallized under the same conditions, except that optimal diffraction was obtained with 6.8%–7.2% PEG20K. After 5 d at 22°C, crystals grew to dimensions of up to 300 $\times$ 200 $\times$ 50 μ m and were stabilized in a cryo-protection buffer containing 50 mM KOAc (pH 5.0), 6.5% PEG20K, 20% PEG400, 50 mM sucrose, 1% glycerol, 0.5 mM GDPNP, and 0.5 mM viomycin. Solid (NH₄)₂SO₄ was then added at saturating concentration to the well solution. After 3 d, crystals were harvested and flash-frozen in liquid nitrogen.

Crystal screening and data collection

Crystals were screened at beamline 12-2 at the Stanford Synchrotron Radiation Laboratory (SSRL) and at beamline 12-3-1 at the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory. Data were collected at beamline 12-2 at the SSRL, beamline 12-3-1 at the ALS, and at beamline 23ID-D at the Advanced Photon Source at Argonne National Laboratory using 0.4° oscillations. Diffraction data from four crystals were integrated, merged, and scaled in XDS (Kabsch 1993).

Molecular replacement and structure refinement

The crystal structure was solved by molecular replacement in Phaser (McCoy et al. 2007). A single ribosome was found per asymmetric unit. 30S and 50S subunits from a previously described

structure of the *E. coli* 70S ribosome in the classical state (Zhang et al. 2009) were used as the initial search model. Clear density was visible for RF3 bound with GDPNP (Supplemental Fig. S2), viomycin, the pentameric complex of proteins L10(L12 NTD)₄ (Supplemental Fig. S3A), and parts of the mRNA in the 3.3 Å $F_o - F_c$ electron density map. Two-rigid-body refinement was performed initially in Phenix (Adams et al. 2010), revealing an $\sim 7^\circ$ counterclockwise rotation of the 30S subunit. A further 300-rigid-body refinement was carried out as previously described (Korostelev et al. 2008a). TLS, B group, and individual coordinate refinements were carried out in Phenix, and minimization refinement in CNS (Brünger et al. 1998) was also used to improve the model. The model was finally refined to values of $R = 21.4$ and $R_{\text{free}} = 25.2$.

Parts of the rRNA model, such as the flipped-out 16S rRNA bases A1492 and A1493, were initially rebuilt manually in O (Jones et al. 1991). The mRNA model was built starting with the mRNA structure from the RF1 70S ribosome complex (Laurberg et al. 2008). The initial RF3 model was from the crystal structure of free RF3 in its GDP form (Gao et al. 2007). The RF3-GDP structure was docked into the unbiased $F_o - F_c$ electron density map and rebuilt manually in Coot (Emsley and Cowtan 2004).

The crystal structure of *T. thermophilus* protein L10 in complex containing two of the three L12 N-terminal domain dimers (Diaconu et al. 2005) mutated into the corresponding *E. coli* sequences was used as the initial model. Manual docking into the $F_o - F_c$ electron density map was followed by manual fitting of the main chain and side chains in Coot and refinement in Phenix. The viomycin model from the previously determined structure of viomycin bound to the classical-state 70S ribosome (Stanley et al. 2010) was used for docking into the $F_o - F_c$ electron density map. To confirm the positions of viomycin in the rotated ribosome, we compared $F_o - F_c$ map differences between RF3 70S ribosome complex crystals with and without viomycin.

Binding of RF3 to the RF2 termination complex

Termination complexes formed with 100 pmol of ribosomes, 200 pmol of tRNA^{Met}, 300 pmol of mRNA m0-27, and 600 pmol of RF2, in a total volume of 40 μ L of buffer A, were isolated by pelleting through 200 μ L of 37% (w/v) sucrose cushion in buffer A, in a TLA100 rotor at 100,000 rpm for 45 min at 4°C. The pellet was resuspended in 110 μ L of buffer A; 20 μ L (~ 15 pmol) of termination complex was added to 60 pmol of RF3, RF3-GDP, RF3-GTP, or RF3-GDPNP (1 mM final concentration nucleotide); and incubated for 5 min at 37°C, before pelleting again through a 200- μ L sucrose cushion in buffer A supplemented with 0.5 mM GDP, GTP, or GDPNP as required. The pellets were resuspended in 15 μ L of gel-loading buffer and run on a 10% polyacrylamide SDS gel (Supplemental Fig. S10).

DATA DEPOSITION

Coordinates and structure factors have been deposited with PDB IDs 3SFS, 3SGF, 3UOQ, and 3UOS.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

We thank J.P. Donohue for expert computational support and for help with preparation of figures; M. Soltis and the staff at beamline 12-2 at the Stanford Synchrotron Light Source, S. Classen at beamline 12.3.1 at the Advanced Light Source, and M. Becker and R. Sanishvili at beamline 23-ID at the Advanced Photon Source for expert advice and support during screening and data collection; M. Laurberg for advice on restraints during crystallographic refinement; J.H.D. Cate for communicating his results prior to publication and for critical discussion; S. Joseph and D. Herschlag for critical discussions; and members of the Noller laboratory for their support. This work was supported by grants from the NIH, the NSF, the Human Frontiers Science Program, QB3, and the Agouron Foundation.

Received October 17, 2011; accepted October 31, 2011.

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