



A cascade of structural rearrangements positions peptide release factor 2 for polypeptide hydrolysis on the ribosome

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Peptide release factor 2 (RF2) catalyzes the release of the nascent polypeptide chain from the bacterial ribosomal complex during translation termination and a subset of ribosome rescue pathways. Despite its critical role, the mechanisms that govern RF2 function and regulation remain elusive. Here, using single-molecule fluorescence resonance energy transfer, we characterize the conformational landscape that RF2 explores on the ribosomal complex and show that RF2 binding and dissociation from the ribosome follows a series of conformational rearrangements which depend on its ribosomal binding platform. We also show how further interactions with the ribosomal complex are necessary to properly position RF2 for polypeptide release. This work investigates not only the dynamics RF2 undergoes while in complex with the ribosome, but also identifies a potential mechanism by which the regulation of these dynamics may be disrupted, which may be exploited for future development of broad-spectrum antibiotics.

translation termination | ribosome rescue | single-molecule kinetics | RF2 | ArfA

An essential step in the translation of messenger RNA (mRNA) into the corresponding encoded protein (1, 2) is the release of the newly synthesized polypeptide from the transfer RNA (tRNA) bound to the ribosomal peptidyl-tRNA binding (P) site. Polypeptide release is critical for the proper termination of the current round of translation and the efficient recycling of the ribosome for subsequent rounds. It thus plays an important role in both canonical translation termination at standard three-nucleotide “stop” codons, and also in the rescue of ribosomes stalled on aberrant mRNAs, such as those lacking stop codons (“nonstop” transcripts) (3, 4).

During canonical termination, a specific family of proteins called class I peptide release factors (RFs) recognize stop codons in the aminoacyl-tRNA binding (A) site in a termination complex (TC), where they bind and position a specific RF domain, domain III (dIII), into the ribosomal peptidyl transfer center (PTC) to facilitate the hydrolysis and release of the polypeptide chain (5, 6). The ribosomal A site bearing the stop codon thus forms a binding platform that allows RFs to stably associate with TCs to perform their necessary function. Bacteria have two RFs, release factor 1 (RF1) and release factor 2 (RF2), while eukaryotes have a single RF, eRF1, which, though functionally similar, bears no structural similarity to the bacterial counterparts (7). Regardless of specific structural differences, the ability of RFs to recognize and stably bind to their respective ribosomal binding platforms and subsequently undergo structural rearrangements to facilitate polypeptide release are critical parts of the mechanism of translation in all domains of life (8).

Following peptide hydrolysis, class I RFs are assisted in dissociation from the ribosomal complex by a class II RF, release factor 3 (RF3). This activity is more important for RF1, which demonstrates a negligible rate of dissociation in the absence of RF3 (9–11). On the other hand, RF2 has been found to spontaneously dissociate from the ribosome in the absence of RF3 at a rate that is at least an order of magnitude faster than RF1 (9, 11–13). This ability of RF2 to spontaneously dissociate is likely related to its functional versatility. In certain bacterial species, RF2 has been co-opted for the rescue of ribosomal complexes stalled on nonstop mRNAs (14). In the absence of a stop codon, the ribosome continues incorporating amino acids until the 3′ end of the nonstop mRNA enters the P site. This forms a nonstop elongation complex (nsEC) bearing an empty, or partially empty, A site (15). The nsEC lacks the necessary ribosomal binding platform capable of accommodating any aminoacyl-tRNA or RF and stalls on the mRNA. A secondary protein factor, alternative rescue factor A (ArfA), can recognize such complexes and form a substitute ribosomal binding platform for RF2 on the nsEC, facilitating peptide release and ribosome rescue (16, 17). This function is unique to RF2, as RF1 is unable to stably bind this nsEC binding platform (14, 18). The ArfA-RF2 pathway serves as an important

Significance

Peptide release factor 2 (RF2) plays a crucial role in releasing the nascent polypeptide from the ribosome in several pathways, from the canonical termination of protein synthesis to the rescue of stalled ribosomes on aberrant messenger RNAs. This work elucidates the common mechanism by which RF2 is stabilized on the ribosomal complex and properly positioned to catalyze peptide release in these pathways. It further shows how dysregulation of the cascade of structural rearrangements that underlie this common mechanism can abrogate RF2 function and lead to rapid dissociation of RF2 from the ribosomal complex—a potential mechanism for the design of the next generation of antibiotics.

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fail-safe for nonstop ribosome rescue and becomes essential for survival under conditions where other nonstop rescue pathways, such as trans-translation, are inactive (4, 19–21). It has also been recently implicated as a necessary component in a newly discovered bacterial phage defense pathway (22). Like canonical termination, the recognition and stable binding to ArfA-bound nsECs by RF2 and their subsequent structural transitions are critical steps for the efficient ArfA-mediated rescue and recycling of stalled nsECs. Furthermore, since the pathway of nonstop rescue is divergent between the different domains of life (23), the bacterial nonstop rescue pathways serve as attractive targets for the next generation of antibiotics (24, 25).

Multiple structural and biochemical studies have been employed that indirectly probe the mechanistic details of RF2 function in both canonical termination and ArfA-mediated nonstop rescue (6, 9, 11, 13, 14, 16, 18, 26–40). These studies suggest that the individual steps of RF2-catalyzed peptide hydrolysis in these two distinct pathways are broadly similar. Both require a ribosomal binding platform for RF2 in the A site [comprising the stop codons UGA or UAA in TCs (3), and ArfA bound in the decoding center and the empty mRNA channel downstream of the A site in nsECs (17)]. Upon stably associating with this binding platform, RF2 is expected to transition from a “collapsed” conformation, where dIII is far from the PTC, to an “extended” conformation, where dIII is “docked” into the PTC (Fig. 1) (6, 26–29, 32–37, 40). This structural transition is thought to be triggered by specific RF2 interactions with the ribosome in TCs (6, 26, 27, 29, 31, 37, 40) or ArfA in nsECs (32–36) that stabilize an RF2 motif connecting domains II and III, termed the “switch loop.” An alanine to threonine mutation at position 18 in ArfA leads to a loss of RF2 function (19), which structural studies have attributed to the disruption of switch-loop interactions believed to be essential for triggering the collapsed to extended

transition in RF2 (33, 34). Importantly, there are also distinct differences between these pathways; while RF3 can assist in canonical termination as a catalyst for faster dissociation of RF2 from the ribosome, it is not implicated in the ArfA-mediated ribosome rescue pathway (41, 42).

However, since the real-time conformational dynamics of RF2 during either canonical termination or ArfA-mediated nonstop rescue have not been directly observed, mechanistic models of RF2 function remain speculative. In this work, we use total internal fluorescence microscopy (TIRFM) coupled with single-molecule fluorescence resonance energy transfer (smFRET) to follow the real-time dynamics of the binding and conformational rearrangements of RF2 in ribosomal complexes. We develop and utilize two smFRET signals to investigate these RF2 dynamics in the context of TCs, nsECs bound to wild-type (wt-) ArfA (wt-nsECs), and nsECs bound to mutant (mut-) ArfA (mut-nsECs). We demonstrate that ribosome-bound RF2 dynamically samples a wider conformational landscape than the previously defined collapsed and extended conformations. We show how these transiently sampled intermediate RF2 conformations are regulated by the ribosomal binding platform and, in mut-nsECs, form part of the mechanism through which mut-ArfA abrogates RF2 function. Finally, we elucidate a specific docked conformation of RF2 which must be stabilized by specific interactions with the ribosomal complex for peptide hydrolysis in both TCs and nsECs.

Results

Development of an smFRET Signal to Monitor RF2 Binding to the Ribosome. We first developed an smFRET signal to report on RF2 binding and dissociation from the ribosome. For the FRET donor, we used cyanine 3-labeled (Cy3-labeled) ribosomal

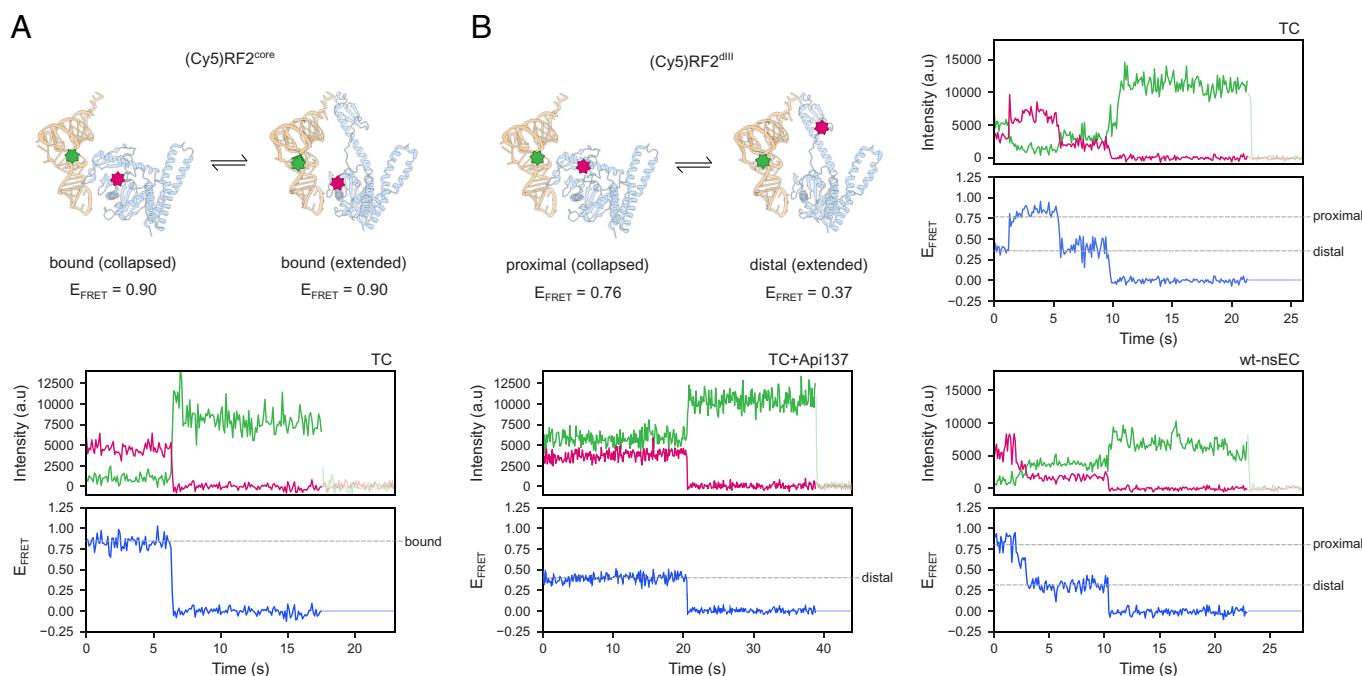


Fig. 1. smFRET signals for RF2 stable association and dynamics. (A) *Top*: structural representations of RF2-bound ribosomal complexes containing the collapsed (PDB ID: 5U9G) and extended (PDB ID: 5U9F) conformations of RF2 with the relative labeling positions for Cy3 (green) and Cy5 (red) corresponding to the (Cy5) RF2^{core} smFRET signal and the expected E_{FRET} states. *Bottom*: a representative individual E_{FRET} trajectory for the TC showing the Cy3 (green) and Cy5 (red) fluorescence intensities and the corresponding E_{FRET} (blue). (B) *Top Left*: structural representations of RF2-bound ribosomal complexes containing the collapsed and extended conformations of RF2 with the relative labeling positions for Cy3 (green) and Cy5 (red) corresponding to the (Cy5)RF2^{dIII} smFRET signal and the expected E_{FRET} states. *Top Right* and *Bottom*: representative individual E_{FRET} trajectories for the TC (*Top Right*), Api137-bound TC (*Bottom Left*), and wt-nsEC (*Bottom Right*), showing the Cy3 (green) and Cy5 (red) fluorescence intensities and the corresponding E_{FRET} (blue). For clarity, only RF2 and the P-site tRNA are displayed in all structures.

complexes labeled at a P site-bound phenylalanine-specific tRNA carrying a formylmethionine-phenylalanine dipeptide (fMet-Phe-(Cy3)tRNA^{Phe}). For the FRET acceptor, we employed cyanine 5-labeled (Cy5-labeled) RF2, where RF2 domain II (hereafter referred to as the “core” domain) is labeled at residue 184 ((Cy5)RF2^{core}). Since the core domain does not undergo significant structural rearrangements, the FRET efficiency (E_{FRET} ; defined as the Cy5 fluorescence intensity divided by the total Cy3 and Cy5 fluorescence intensities) for this signal is expected to remain the same [0.95 to 0.97, based on a FRET radius (R_0) of 55 Å for Cy3-Cy5 (43)] for the different ribosome-bound RF2 conformations previously observed in structural studies (6, 26–29, 31–37, 40) (Fig. 1A). Using this signal, we investigated RF2 binding to i) elongation complexes (ECs) with a codon for lysine (AAA) in the A site (that is not expected to stably bind RF2), ii) TCs with an A-site stop codon (UAA), and iii) nsECs containing a 3'-truncated mRNA with an empty A site, in the presence of both wt- and mut-ArfA (see *Materials and Methods* for details).

As expected, steady-state experiments of (Cy5)RF2^{core} binding to TCs and nsECs yielded one nonzero E_{FRET} state centered at ~0.9 (Fig. 1A and *SI Appendix, Fig. S1A*), according to a hidden Markov model (HMM)-based analysis (44) (see *Materials and Methods* for details). This nonzero E_{FRET} state is consistent with stable binding of RF2 to both TCs and nsECs. These experiments also yielded a zero E_{FRET} state centered at 0.0, which corresponds to either (Cy5)RF2^{core} being too far away from the P-site tRNA to yield any observable E_{FRET} (e.g., when RF2 is not bound to the ribosome) or the Cy5 acceptor fluorophore being photobleached (Fig. 1A). When performing similar steady-state experiments of (Cy5)RF2^{core} binding to ECs, we only observed the zero E_{FRET} state. Since RF2 must interact with the ribosomal complexes to probe the identity of the A site binding platform and distinguish between ECs, TCs, and nsECs, these results suggest that a transient “encounter complex” (45) is formed when RF2 first interacts with the ribosome, and that it has a lifetime much shorter than our TIRFM acquisition time of 100 ms (*Materials and Methods*). For TCs and nsECs, the presence of the ribosomal binding platforms stabilize the encounter complex and leads to more stably bound and long-lived RF2 conformations that correspond to the observed nonzero E_{FRET} state. For ECs, the lack of such a platform in the A site means RF2 cannot stably bind, and so it rapidly dissociates.

TCs Facilitate the Stable Association of RF2 More Effectively than wt-nsECs and mut-nsECs. We next employed (Cy5)RF2^{core} to estimate the rates of association (k_a s) of RF2 to TCs and wt-nsECs. Since RF2 can readily hydrolyze the polypeptide in these ribosomal complexes, thereby changing the composition of the complex during rebinding, we employed pre-steady-state experiments where we delivered (Cy5)RF2^{core} to TCs and wt-nsECs and measured the first instance where (Cy5)RF2^{core} stably binds to these complexes. For wt-nsECs, a saturating concentration of wt-ArfA (more than 50-fold excess of the (Cy5)RF2^{core} concentrations) was added along with (Cy5)RF2^{core} to the ribosomal complexes immediately prior to imaging (see *Materials and Methods* for details). By titrating the concentration of (Cy5)RF2^{core} over the course of several successive pre-steady-state experiments (*SI Appendix, Fig. S2A*), we were able to obtain the second-order k_a s of RF2 to TCs and ArfA-bound wt-nsECs (*SI Appendix, Fig. S2B*).

The k_a of (Cy5)RF2^{core} for TCs was 12.6 ± 0.7 ($\mu\text{M}\cdot\text{s}$)⁻¹. In the absence of wt-ArfA, we did not observe any stable (Cy5)RF2^{core} binding to nsECs; however, in the presence of wt-ArfA, the k_a of (Cy5)RF2^{core} for the wt-nsECs was 7.1 ± 0.7 ($\mu\text{M}\cdot\text{s}$)⁻¹ (*SI Appendix,*

Fig. S2B). Because the rate of formation of the initial encounter complex between (Cy5)RF2^{core} and the ribosome depends on the collision rate between the two, which should be approximately the same for both TCs and nsECs (45), the k_a s that we measured instead reflect the probability that the encounter complex progresses to a stable and long-lived RF2-bound ribosomal complex. The nearly twofold larger k_a of the TC over that of the wt-nsEC thus suggests that the TC binding platform facilitates productive association of RF2 more successfully than the wt-nsEC binding platform.

For mut-nsECs, the ability of RF2 to hydrolyze peptide is mostly abolished (14, 16, 19, 42). As a result, the mut-nsECs remain largely unchanged (and hydrolysis-competent) through successive rebinding of RF2. We therefore employed steady-state experiments to probe the binding of (Cy5)RF2^{core} to mut-nsECs and we estimated the corresponding k_a of (Cy5)RF2^{core} for mut-nsECs as 7.2 ± 0.3 ($\mu\text{M}\cdot\text{s}$)⁻¹ (*SI Appendix, Fig. S2B*). We highlight here that this is a steady-state estimate of this rate, and not a pre-steady-state k_a such as the ones above. Nonetheless, our results suggest that mut-ArfA does not reduce RF2 stable association with ribosomal complexes when compared to wt-ArfA, which is in agreement with previous studies (16, 19). The ability of mut-ArfA to abrogate RF2 function must therefore arise from the dysregulation of a mechanistic step that occurs after RF2 association with the mut-ArfA-bound ribosomal binding platform.

ArfA Must Accommodate into the A Site to Form a Functional Ribosomal Binding Platform for RF2. We next investigated the role of ArfA in forming a stable ribosomal binding platform for RF2 on nsECs. Previous studies have hypothesized that ArfA must adopt a specific conformation on nsECs before RF2 can stably associate (14, 17). We therefore performed steady-state smFRET experiments where we allowed wt-ArfA to accommodate into the nsEC A site before the addition of (Cy5)RF2^{core}. When a saturating concentration of wt-ArfA was added to nsECs and preincubated for 5 min prior to the addition of (Cy5)RF2^{core}, the rate of RF2 dissociation (k_d) from the resulting wt-pre-nsECs (where “pre” denotes the preincubation) was 0.26 ± 0.03 s⁻¹ (Table 1). In contrast, when a saturating concentration of wt-ArfA was added simultaneously with (Cy5)RF2^{core}, the k_d of RF2 from the resulting wt-nsEC was 0.66 ± 0.05 s⁻¹ (Table 1), demonstrating that RF2 is less stably bound to wt-nsECs than wt-pre-nsECs. This supports the hypothesis that ArfA accommodates in the A site first prior to RF2 stable association.

The k_d of RF2 from mut-nsECs was 0.47 ± 0.03 s⁻¹, a value that is lower than that from wt-nsECs but nearly double that from wt-pre-nsECs (Table 1). The similar k_d s of RF2 dissociating from mut-nsECs and wt-nsECs provides further evidence that mut-ArfA can also form a ribosomal binding platform for RF2 in mut-nsEC.

In the case of TCs, we observed an RF2 k_d of 0.16 ± 0.02 s⁻¹, a rate that is lower than the RF2 k_d for any nsEC (Table 1). This indicates that the TC forms a ribosomal binding platform that is much more effective at binding RF2 than the platform formed by wt-ArfA, even for the wt-pre-nsEC, at least under our experimental conditions.

The k_d of RF2 from TCs that we observe is nearly identical to that observed in another smFRET study for RF2 dissociation from a TC posthydrolysis in the absence of RF3 (12), but fivefold lower than that reported in a third smFRET study which employed a different RF2 construct (13). This discrepancy is likely due to differences in experimental design between these studies. Thus, although our results show that RF2 can dissociate from TCs in the absence of RF3, the relatively smaller k_d obtained from this and previous studies (9, 11, 12) leads us to suggest that RF2 binds

Table 1. Dissociation rates and conformational rearrange rates for ribosome-bound RF2

	(Cy5)RF2 ^{core}		(Cy5)RF2 ^{dIII}		
	k_d (s ⁻¹)	$k_{p \rightarrow d}$ (s ⁻¹)	$k_{d \rightarrow p}$ (s ⁻¹)	k_d^{prox} (s ⁻¹)	k_d^{dist} (s ⁻¹)
TC	0.16 ± 0.02	0.50 ± 0.02	0.35 ± 0.01	0.13 ± 0.02	0.41 ± 0.01
wt-nsEC	0.66 ± 0.05	0.35 ± 0.02	0.32 ± 0.01	0.08 ± 0.02	0.30 ± 0.02
wt-pre-nsEC	0.26 ± 0.03	0.32 ± 0.02	0.25 ± 0.01	0.08 ± 0.02	0.18 ± 0.01
mut-nsEC	0.47 ± 0.03	0.40 ± 0.02	0.33 ± 0.02	0.00 ± 0.01	1.11 ± 0.03
Api137-bound TC	—	—	—	—	0.24 ± 0.01*

The results of the HMM-based analysis for the dissociation rate (k_d) of (Cy5)RF2^{core} for different ribosomal complexes, and for the rates of the proximal-to-distal transition ($k_{p \rightarrow d}$), the distal-to-proximal transition ($k_{d \rightarrow p}$), the dissociation from the proximal state (k_d^{prox}), and the dissociation from the extended state (k_d^{dist}) of (Cy5)RF2^{dIII} for different ribosomal complexes. All dissociation rates except for k_d^{dist} of (Cy5)RF2^{dIII} from the Api137-bound TC (indicated by *) are corrected for photobleaching. Errors represent the SD of the posteriors inferred from the HMM-based analysis.

stably enough to TCs such that RF3-accelerated RF2 dissociation plays an important biological role even under normal growth conditions (*Discussion*).

Development of an smFRET Signal to Follow RF2 Conformational Dynamics. We next investigated the conformational dynamics of the ribosome-bound RF2 using a separate smFRET signal. This signal used a different RF2 construct as a FRET acceptor. Here, RF2 was labeled with Cy5 at residue 271 within dIII, the domain that extends into the PTC to carry out peptide hydrolysis ((Cy5) RF2^{dIII}) (Fig. 1*B*). We expected this smFRET signal to report on RF2 structural rearrangements that resulted in the repositioning of dIII, such as the ones captured in previous structural studies (6, 26–29, 31–37, 40) (Fig. 1*B*).

We observed two nonzero E_{FRET} states for (Cy5)RF2^{dIII} for both TCs and nsECs (Fig. 1*B* and *SI Appendix, Fig. S1B*): a low- E_{FRET} state of ~0.37 for both TCs and nsECs, where RF2 dIII is distal to the Cy3-labeled tRNA, and a high- E_{FRET} state of 0.76 for TCs and 0.70 for nsECs, where RF2 dIII is proximal to the Cy3-labeled tRNA (*SI Appendix, Table S1*). Since our smFRET signal reports only on the distance between the FRET donor and acceptor, we have adopted the labels “distal” or “proximal” (relative to the Cy3 label on the P-site tRNA, Fig. 1*B*) for our E_{FRET} states to avoid assigning structural interpretations to these states, particularly in terms of RF2 conformation, until a more detailed analysis could be carried out.

To assign these observed distal and proximal E_{FRET} states, we used an antimicrobial peptide, Api137, which binds the nascent polypeptide exit tunnel of a TC post-peptide-hydrolysis and traps RFs in an extended conformation (46, 47). In our experiments, TCs in the presence of Api137 primarily adopted a single nonzero low- E_{FRET} state centered at 0.39 (Fig. 1*B*), which we therefore assigned to the extended conformation in the Api137-bound TC. We reasoned that this RF2 conformation also yielded the similar distal low- E_{FRET} state (centered at 0.37) in the non-Api137 bound TCs, wt-nsECs, and wt-pre-nsECs. A low- E_{FRET} state was also observed in the mut-nsECs. However, this was centered at 0.43, highlighting some heterogeneity in the distal state for this complex compared to that observed for TCs and wt-nsECs (Fig. 2*A–C*). One likely source of such heterogeneity is the existence of multiple distal conformations that can yield similar E_{FRET} states in our smFRET experiments, which would require further characterization (see below).

The proximal E_{FRET} state from the other complexes was virtually absent for the Api137-bound TC (Fig. 2*D*). Since this complex is expected to not adopt a long-lived collapsed RF2 conformation, we could therefore assign the proximal state to this collapsed conformation. The slight differences in E_{FRET} for this state in a wt-nsEC (0.70) compared to that in a TC (0.76) or a mut-nsEC

(0.75) suggest subtle variations in the positioning of RF2 dIII between ribosomal complexes. However, similar to the distal E_{FRET} state, further characterization of the proximal E_{FRET} state is required to determine whether it corresponds to a single or multiple RF2 conformations (see below).

Since Api137 traps RFs in the distal state for time intervals much greater than our experimental time window (46, 47), all instances of a transition from this state to the zero E_{FRET} state in the Api137-bound TC were likely due to photobleaching of (Cy5) RF2^{dIII}. The HMM-derived rate of transition from this E_{FRET} state to the zero E_{FRET} state thus provided a conservative estimate of the rate of Cy5 photobleaching in our experimental conditions. All experimental k_d s reported in this study were therefore corrected for photobleaching using this estimated photobleaching rate (see *Materials and Methods* for details).

RF2 Adopts at Least Two P-Site tRNA Distal Conformations When Bound to Ribosomal Complexes. The differences in the E_{FRET} states for different ribosomal complexes suggests that the ribosome-bound RF2 explores a wider ensemble of conformations than what previous studies have reported. In fact, we observed RF2 structural rearrangements in all four ribosomal complexes that we studied (Fig. 2). This was in striking contrast to a previous structural study of mut-ArfA which had suggested that the mutation in ArfA inhibits RF2 structural transitions and traps RF2 in a collapsed conformation (33). If RF2 was trapped in a collapsed conformation in mut-nsECs, we would only observe the proximal state in our experiments. Furthermore, our HMM-derived rates for transitions between the distal and proximal states were very similar for all complexes (Table 1). Thus, the rates of RF2 structural rearrangements were, on average, unaffected by the identity of the specific ribosomal complex. This intrinsically dynamic behavior of the ribosome-bound RF2 appears to agree with previous studies hypothesizing that RF2 dynamics may be regulated by changes in the local ribosomal environment that are triggered by RF2 binding (18, 48). However, we have previously shown that HMMs do not distinguish between the distinct kinetic phases that arise from conformational transitions between states that have the same E_{FRET} values but that occur at different rates; instead, HMMs only report the average of these rates (44).

To investigate the existence of any distinct kinetic phases, we instead quantified the dwell time distributions of the distal state in the E_{FRET} trajectories (i.e., the times spent in the distal state before transitioning to the proximal state, Fig. 3*A*). Since the presence of distinct kinetic phases would manifest as non-single-exponential decays, we fit the resulting survival curves of the dwell time distributions to both single- and double-exponential decays (see *Materials and Methods* for details). To distinguish between the single- or double exponential fits, we used a cutoff value of 0.95 for the goodness-of-fit

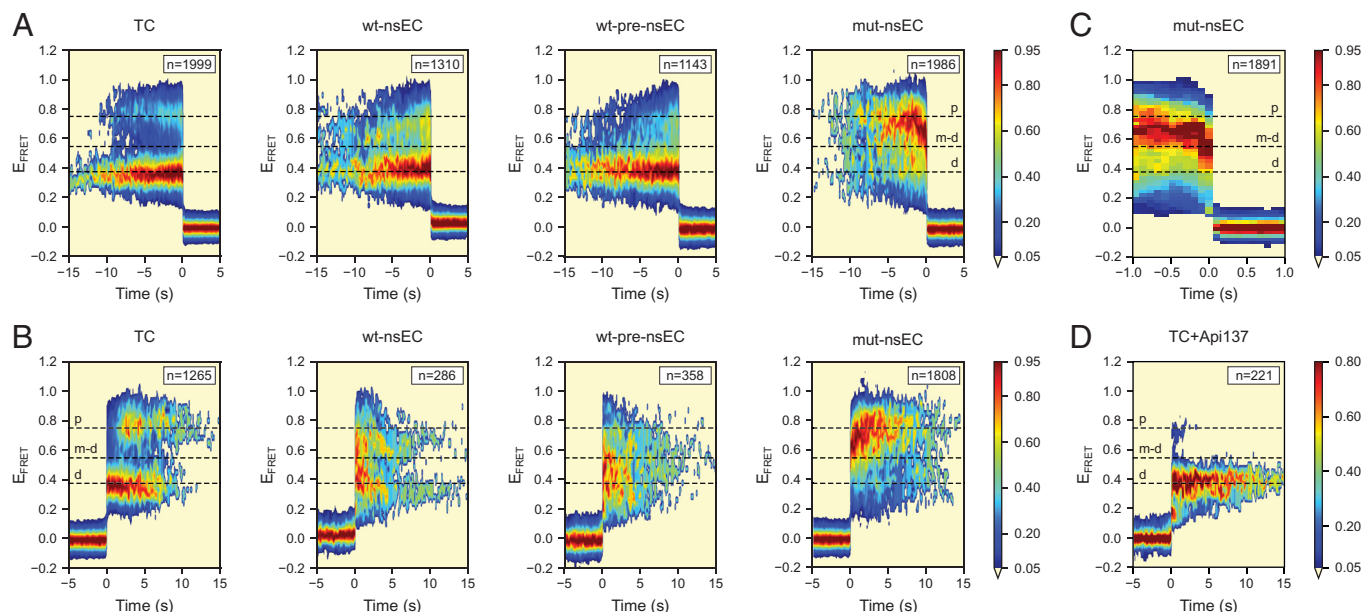


Fig. 2. 2D E_{FRET} vs time histograms of RF2 dissociation and stable association. 2D histograms postsynchronized to (A) the transition from any nonzero E_{FRET} state to the zero E_{FRET} state, following the dissociation of ribosome-bound (Cy5)RF2^{dIII} from TCs, wt-nsECs, wt-pre-nsECs, and mut-nsECs, (B) the transition from the zero E_{FRET} state to any nonzero E_{FRET} state, following the stable association of (Cy5)RF2^{dIII} to TCs, wt-nsECs, wt-pre-nsECs, and mut-nsECs, (C) the transition from any nonzero E_{FRET} state to the zero E_{FRET} state for mut-nsECs which highlights the transient mid-distal state adopted immediately prior to RF2 dissociation, and (D) the transition from the zero E_{FRET} state to any nonzero E_{FRET} state, following the stable association of (Cy5)RF2^{dIII} to Api-137-bound TCs, highlighting the excursion to the proximal E_{FRET} state in these complexes. Dashed lines show the E_{FRET} states corresponding to proximal (p), distal (d), and mid-distal (m-d) conformations of (Cy5)RF2^{dIII}.

parameter, R^2 , for supporting the single-exponential decay. Using this criterion, the observed kinetics of the TC-bound RF2 could be adequately explained by a single exponential decay ($R^2 = 0.97$) (SI Appendix, Fig. S3A), but they needed at least a double-exponential decay to be properly modeled for nsECs (with R^2 values of 0.94, 0.92, and 0.83 for the single-exponential fit for wt-nsECs, wt-pre-nsECs, and mut-nsECs, respectively) (SI Appendix, Fig. S3A). Thus, our data show that the distal state encompasses at least two distinct RF2 conformations for nsECs with indistinguishable E_{FRET} values, one of which likely corresponds to the extended conformation captured in structural studies. In the case of TCs, we note that it is possible that corresponding RF2 conformations exist that transition with similar rates, thereby giving rise to an apparent single-exponential decay.

Strikingly, when we constructed postsynchronized, two-dimensional (2D) E_{FRET} histograms of this distal state (where the transitions either to this state from the zero E_{FRET} state or the reverse is aligned to the zero time-point) (SI Appendix, Fig. S4), we see the clear appearance of one such additional state for the mut-nsEC. This transiently sampled E_{FRET} state appears to be centered around 0.55, distinct from the distal states we previously observed. We attribute this state to a conformation of RF2 where dIII is more distal to the P-site tRNA than the collapsed conformation observed in structural studies. Hereafter, we refer to this E_{FRET} state as the mid-distal state. We reason that the transient nature of the corresponding RF2 conformation and the proximity of its E_{FRET} value to the low- E_{FRET} distal state prevented its identification as a separate state by our HMM-based analysis (44), which instead assigned it as a part of the distal state.

In contrast to the distal states, the corresponding observed kinetics for the proximal state for all complexes could be well explained by a single-exponential decay (with R^2 greater than 0.95), suggesting the proximal state represents a single RF2 conformation (Fig. 3B and SI Appendix, Fig. S5B). We reason that

this proximal state must then arise from the collapsed RF2 conformation observed in structural studies. Unlike the distal states above, we can therefore use the terms proximal and collapsed interchangeably to refer to the same E_{FRET} state in our experiments. However, we have chosen, for consistency, to use proximal state for discussing our subsequent results.

RF2 Dissociates from Ribosomal Complexes Via Previously Undetected Intermediate States. Having revealed the presence of at least two distal RF2 conformations, and in particular, their emergence when RF2 transitions to the zero E_{FRET} state, we next sought to investigate their mechanistic significance to RF2 function. We quantified the preference of RF2 to dissociate from either a distal or proximal state using steady-state smFRET experiments with (Cy5)RF2^{dIII} (Fig. 1B and SI Appendix, Fig. S1B), which yielded the RF2 dissociation rate from these states (k_d^{dist} and k_d^{prox} , respectively) (Table 1). For all complexes that we investigated, k_d^{dist} was significantly greater than k_d^{prox} , suggesting that RF2 preferentially dissociates from the ribosome in a distal state. Since the rates of exchange between the two states were at least an order-of-magnitude lower than our acquisition rate (Table 1), we could discount the possibility that the ribosome-bound RF2 transiently samples the proximal state immediately prior to dissociation for a period that does not show up in our experiments (49). This preference for RF2 to dissociate from the distal state was most dramatically pronounced in mut-nsECs, where k_d^{prox} was negligible and k_d^{dist} was $1.11 \pm 0.03 \text{ s}^{-1}$, which was more than twofold higher than that of TCs ($0.41 \pm 0.01 \text{ s}^{-1}$) and four- to sixfold higher than that for wt-nsECs ($0.30 \pm 0.02 \text{ s}^{-1}$ for wt-nsECs and $0.18 \pm 0.01 \text{ s}^{-1}$ for wt-pre-nsECs).

By synchronizing every transition from a nonzero E_{FRET} state to the zero state and visualizing those transitions using 2D E_{FRET}

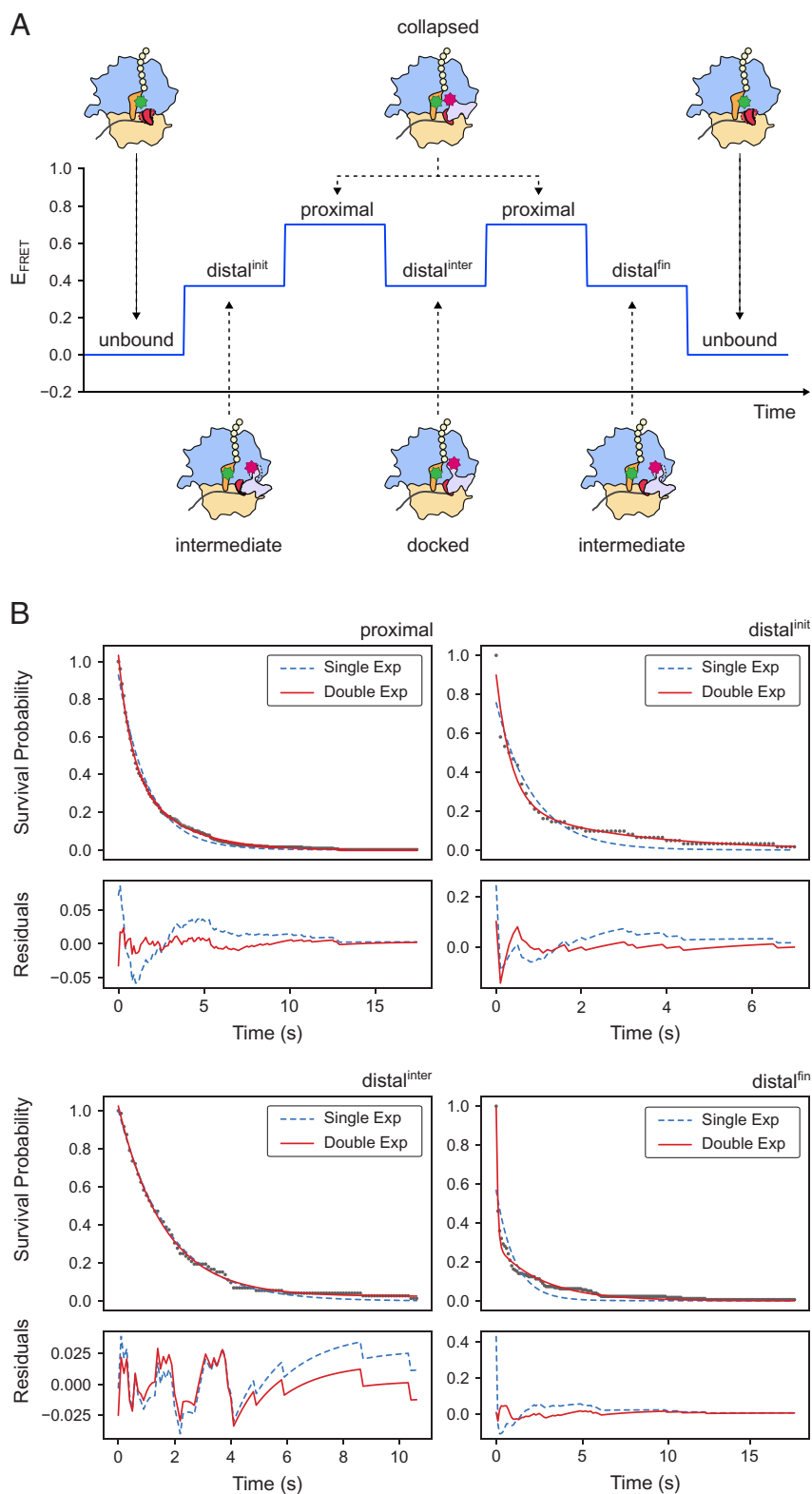


Fig. 3. Dwell-time analysis of ribosome-bound (Cy5)RF2^{distal}. (A) A hypothetical E_{FRET} trajectory of (Cy5)RF2^{distal}, showing the different observed E_{FRET} states and the corresponding RF2 conformations. (B) Survival plots of the dwell time distributions (black) for wt-pre-nsECs along with fits of single-exponential (blue) and double-exponential (red) decays (above) and the corresponding residuals (below) for proximal, distal^{init}, distal^{inter}, and distal^{fin} states.

histograms (*Materials and Methods*), we observed that RF2 preferentially dissociates from the mid-distal state in mut-nsECs (Fig. 2A). The mid-distal state exhibits a very short lifetime of <300 ms, which corresponds to a $k_d > 3.3 \text{ s}^{-1}$ (Fig. 2C). For nsECs, we saw an increased density of E_{FRET} values between the proximal

and distal states, suggesting the presence of one or multiple transient mid-distal states (Fig. 2A and *SI Appendix, Fig. S4A*). For these complexes, this mid-distal state is even more transiently sampled and, thus, even less likely to be identified by our HMM-based analysis. (44) We surmise that it is this density that

is simply significantly increased in mut-nsECs, leading to the observation of a distinct observed E_{FRET} .

To more confidently detect these intermediate conformations in the dissociation pathway of RF2 from TCs and wt-nsECs, we employed the dwell-time based analysis described above. We specifically selected only those dwells in a distal E_{FRET} state that are preceded by the proximal state and followed by the zero E_{FRET} state. We expect these dwells to report on the final behavior of RF2 immediately before dissociation (distal^{fin}) (Fig. 3A). For these distal^{fin} dwells, all complexes required at least a double-exponential fit (Fig. 3B and *SI Appendix, Fig. S5 and Table S2*), suggesting the presence of heterogeneous distal conformations with closely spaced E_{FRET} values. We hypothesize that these comprise an ensemble of transient intermediates that RF2 samples during dissociation. For nsECs, this ensemble nature is supported by the broad distribution of E_{FRET} values observed immediately prior to RF2 dissociation from these complexes (Fig. 2A). This broad distribution is not observed to the same degree for TCs, and thus the primary evidence for this is the double exponential decay obtained from the dwell-time analysis. Further studies are therefore required to characterize specific details about these intermediates in nsECs and TCs (*Discussion*).

The Binding Intermediates of RF2 Are Regulated by the Ribosomal Binding Platform. Having found that RF2 dissociates from the ribosomal complex through an ensemble of different intermediates, we next investigated whether RF2 association to ribosomal complexes also occurs through a similar ensemble. Like above, we visualized the RF2 binding pathway using postsynchronized 2D E_{FRET} histograms where every transition from the zero state to any nonzero E_{FRET} states was aligned (Fig. 2B). Density corresponding to mid-distal state is present in the RF2 binding pathway for both mut-nsECs and wt-nsECs, but less so for wt-pre-nsECs. For TCs, on the other hand, this density is virtually absent; instead, we see that the distal state is immediately sampled upon stable RF2 association before the transition to the proximal state. This immediate sampling of the distal state upon stable RF2 association is also observed in the postsynchronized 2D E_{FRET} histogram for the Api137-bound TC complex (Fig. 2D). For these complexes, sampling of the distal state is then followed by a transient excursion to the proximal state and a subsequent final excursion to a long-lived distal state, which represents the Api137-trapped extended state of RF2 after peptide hydrolysis. Taken together, this demonstrates that the pathway of RF2 association proceeds via a distal state for TCs and an ensemble of distal and mid-distal intermediate states for nsECs, which then transition to a proximal (collapsed) conformation before presumably transitioning to one or more hydrolysis competent distal conformations (see below, Fig. 3A).

Evidence for this binding pathway is also present in the dwell-time distributions of the distal states immediately following RF2 stable binding (distal^{init}). These distal^{init} dwells are preceded by the zero E_{FRET} state and followed by the proximal state, and thus report on the initial behavior of the ribosome-bound RF2 (Fig. 3A). The distal^{init} dwell-time distribution was well fit by a single exponential for TCs ($R^2 = 0.96$), but not for nsECs (R^2 values of 0.84, 0.90, and 0.89 for wt-nsEC, wt-pre-nsEC, and mut-nsEC respectively), which, similar to the distal^{fin} dwell-time distributions, required at least a double-exponential fit (Fig. 3B and *SI Appendix, Fig. S5 and Table S2*). This suggests that RF2 also samples the ensemble of transient intermediates when binding to nsECs.

Altogether, our data suggest that the ribosomal binding platform regulates the ensemble of intermediate states sampled by

RF2 during stable association. Additionally, while mut-Arfa can form a ribosomal binding platform, the specific nature of this binding platform makes RF2 more prone to populating the mid-distal state, which subsequently leads to its dissociation from the mut-nsEC. These ensembles of distal and mid-distal states thus form critical intermediates that are sampled during stable association and dissociation of RF2, and the degree to which they are populated differs between nsECs and TCs, highlighting the greater efficiency of the TC binding platform relative to that in nsECs (*Discussion*).

The Hydrolysis-Competent Docked State of RF2 Is Distinct from the Distal States that RF2 Samples During Association and Dissociation. While our results suggest that RF2 traverses through distal intermediates during binding to and dissociation from the ribosomal complex, it was not immediately clear how these intermediates are related to the extended conformation observed in structural studies. We found that the distribution of distal^{inter} dwells (distal states preceded or followed by the proximal state) could be reasonably explained as a single-exponential for all ribosomal complexes except mut-nsEC (Fig. 3B and *SI Appendix, Fig. S5 and Table S2*). Since these dwells exclusively report on the behavior of ribosome-bound RF2 distal conformations adopted after the proximal state (Fig. 3A), and are thus distinct from the above ensemble of binding intermediates, we propose that this one distal RF2 conformation corresponds to a docked conformation in which the RF2 dIII is extended into the PTC and competent for peptide hydrolysis. We hereafter refer to this as the docked conformation.

For mut-nsECs, the distal^{inter} dwell-time distribution was not well explained by a single-exponential decay ($R^2 = 0.94$) (*SI Appendix, Fig. S5 and Table S2*); however, we did not observe many dwells for this state ($n = 29$) relative to the other ribosomal complexes. This is most likely because excursions to docked conformation are rare in mut-nsECs, which would explain the inability to capture this conformation in structural studies (33). The distribution from the limited distal^{inter} dwells is double exponential. We interpret this to mean that the few instances of the mut-nsECs distal^{inter} state that we do observe correspond to the ensemble of intermediate conformations encountered during binding and dissociation, and not a singular docked conformation as observed for the other complexes. However, we note, given the low amount of data points obtained, that these dwells could represent rare excursions to the docked conformation. Regardless of the interpretation, our data show that the docked conformation is greatly destabilized in mut-nsECs. We hypothesize that the disruption of the ability of RF2 to properly dock into the PTC leads to the increased occupancy of the mid-distal conformation (and other binding intermediates) in mut-nsECs, which subsequently results in the rapid dissociation of RF2.

Discussion

The smFRET experiments we presented here probe the pathway of RF2 stable association and conformational dynamics leading up to RF2-catalyzed peptide hydrolysis for two distinct biological processes: canonical translation and Arfa-mediated nonstop rescue. Our results lead to a mechanistic model where RF2 associates with the ribosome through binding intermediates that all lead to a collapsed conformation, and then to a hydrolysis-competent docked conformation (Fig. 4A and B). We show that the initial transitions between the binding intermediates and the collapsed conformation are regulated by the ribosomal binding platform for RF2. These transitions occur in all ribosomal complexes investigated in this

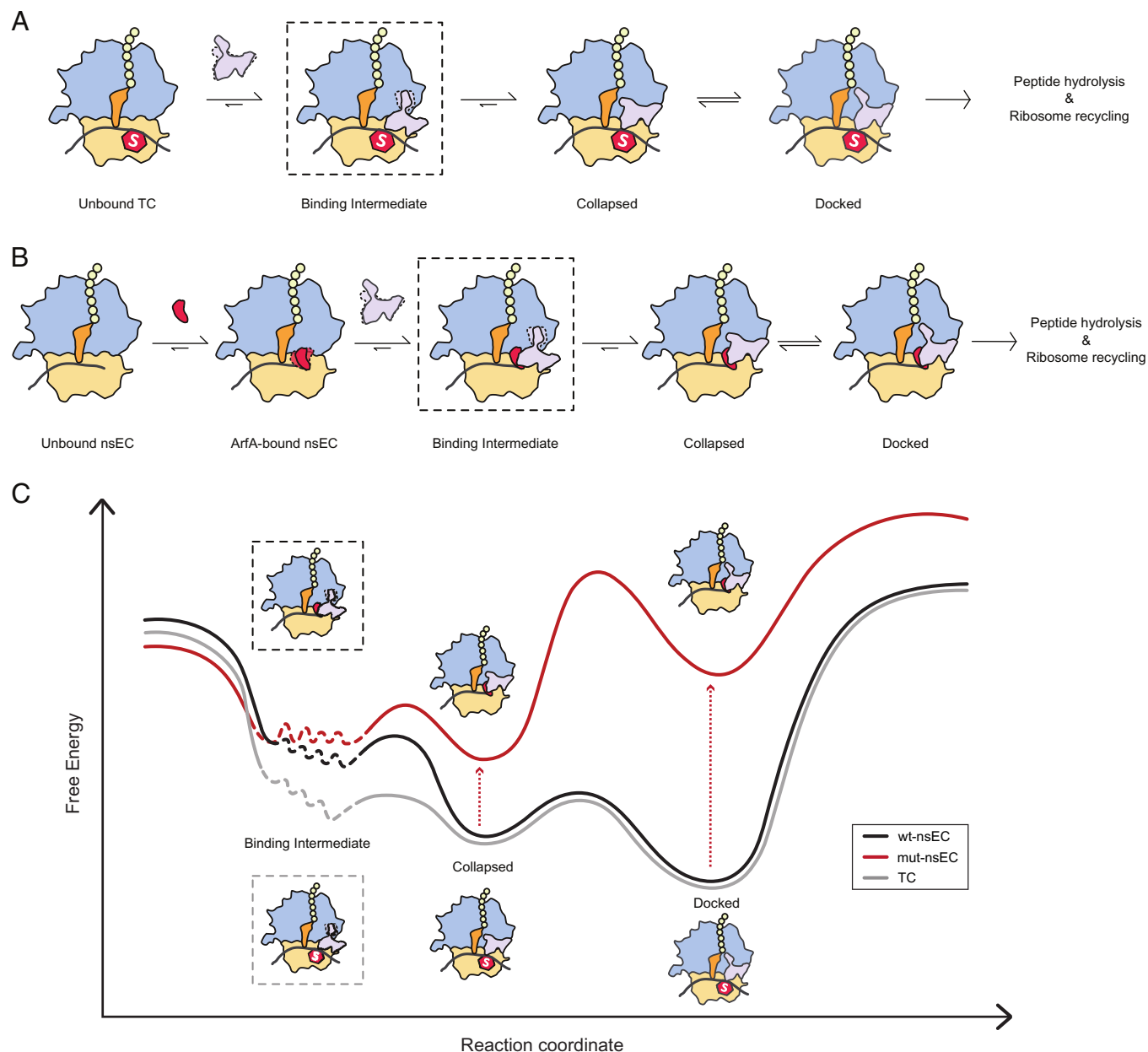


Fig. 4. Proposed model for the mechanism of RF2 function. (A) RF2 associates with the TC in an ensemble of binding intermediates, subsequently transitions to the collapsed conformation, and then docks into the PTC, allowing it to hydrolyze the newly synthesized polypeptide. (B) ArfA stably associates with the nsEC, followed by RF2 associating in an ensemble of binding intermediates, subsequently transitioning to the collapsed conformation, and then docking into the PTC, allowing it to hydrolyze the aberrant, truncated polypeptide. (C) A schematic of the RF2 conformational free energy landscapes on wt-nsECs (black), mut-nsECs (red), and TCs (gray) along with the corresponding ribosome-bound RF2 conformations, showing the cascade of structural transitions that allow RF2 to achieve the docked conformation in wt-nsECs and TCs, and the perturbation caused by mut-ArfA to the landscape that reverses the cascade and abrogates RF2 function in mut-nsECs. The dashed lines for the binding intermediates represent our lack of specific details for these conformations.

study, even in the presence of mut-ArfA. The docked state that appears following the second transition is likely stabilized by further interactions within the ribosomal complex, including those with the RF2 switch loop, as suggested in previous studies (26, 27, 29, 32–37, 50). It is the disruption of these interactions in mut-nsECs that abrogates the ability of RF2 to remain stably docked into the PTCs in this complex.

We hypothesize that upon initial formation of an RF2-ribosome encounter complex, RF2 cascades through multiple intermediate conformations, and that these transiently populated RF2 conformations allows RF2 to efficiently undergo stochastic transitions to the more thermodynamically stable collapsed and docked conformations observed in structural studies (Fig. 4C). mut-ArfA disrupts this cascade by stabilizing the intermediate distal

conformations relative to the collapsed conformation (Fig. 2), and thus inhibits transitions into the docked conformation for mut-nsECs (Fig. 3 and *SI Appendix, Fig. S5*) and abrogates RF2 function (Fig. 4C). On the other hand, the cascade of transitions through the intermediate RF2 conformations is efficient for wt-nsECs, and even more so for wt-pre-nsECs where ArfA is fully accommodated to form the ribosomal binding platform for RF2 (Fig. 2B). Because the cascade is the most efficient when the ribosomal binding platform is already preaccommodated, the intermediate conformations will be even more transiently sampled and only a single E_{RET} state will be observed before the transition to the collapsed state, as is the case for TCs (Figs. 2 and 4C). These differences in efficiency of the cascade of transitions may explain why, under steady-state X-ray crystallographic and cryogenic

electron microscopy (cryo-EM) conditions, ribosome-bound RF2 has been observed in both collapsed and docked conformations in nsECs (33), but only in the final docked conformation in TCs (6, 26, 27, 29–31, 37). Only time-resolved cryo-EM or use of the termination inhibitor blasticidin in traditional cryo-EM has captured TC-bound RF2 in the collapsed conformation. (28, 50)

While our results suggest that RF2 traverses an ensemble of intermediates during dissociation, it is not clear at this point how these intermediates relate to the ones we observe during RF2 binding—and how these intermediates differ between TCs and nsECs. A key difference between the two processes is that, in our experiments, while RF2 primarily binds a ribosomal complex where the polypeptide is not hydrolyzed, dissociation occurs primarily from complexes where the polypeptide has been hydrolyzed, except in the case of mut-nsEC. In particular, the heterogeneity observed by dwell-time analysis during RF2 dissociation from TCs, which is not observed for RF2 stable association with TCs, suggests that these two processes may have some differences. This may arise from the conformation of the ribosomal complex itself. The ribosome is locked in a single conformation (in terms of the relative rotation between the two ribosomal subunits) due to the presence of the polypeptide on the P-site tRNA during stable association of RFs (1, 51–53). Post hydrolysis, the ribosome must achieve a “rotated” conformation for RF dissociation—the main mechanism through which RF3 facilitates RF dissociation from TCs is thought to be through stabilization of this conformation in the presence of GTP (10, 29, 53–59). However, it has been demonstrated that this state can be achieved in RF2-bound ribosomal complexes, in the absence of RF3, often enough to be captured by cryoEM (29). Biochemical studies report RF2 dissociation rates from TCs on the order of 10^{-2} s^{-1} in the absence of RF3 (9, 11), which is the same order as the rate of the slow phase identified in our distal^{fin} dwell time analysis for TCs (*SI Appendix, Table S2*). This suggests that the slow phase represents a rate-limiting step in RF2 dissociation from TCs in the absence of RF3. We hypothesize that this slow phase may correspond to the rotation of the ribosome in the presence of RF2 posthydrolysis, following which RF2 dissociates rapidly.

RF2 is also implicated in an entirely separate translation pathway, that of post-peptidyl transfer quality control. In this pathway, RF2 and RF3 stably bind to an EC containing a misincorporated tRNA and release the aberrant peptide (60). It is unknown how this is achieved, since RF2 cannot stably bind to an EC under normal conditions. However, if RF3 stabilizes intermediate RF2 conformations that exist both in the association and dissociation pathway of RF2 from the ribosome (similar to those we observed in this study), that would explain how it can both enable RF2 to stably bind an EC with a weak inherent affinity for RF2, and also facilitate RF2 dissociation from a TC where the more stable conformations in the absence of RF3 are the tightly bound collapsed and docked conformations. A prediction that arises from such a scenario is that, beyond stabilizing the rotated conformation of the ribosome, RF3 can further facilitate RF2 dissociation by engineering a reversal of the conformational cascade through selective stabilization of the binding intermediates. Of course, detailed studies of the relation between RF2 dissociation and the conformational dynamics of the ribosome itself, and of the effect of RF3 on this relationship, is required to distinguish between these mechanistic models and to shed further information on the contribution of the intermediates we observe for RF2 binding and dissociation.

Altogether, the work we have presented here highlights the remarkable plasticity of RF2 in catalyzing peptide release from the ribosomal complex in different contexts, an essential step for

the proper termination of translation in both canonical and rescue pathways. Equally remarkable is the fact that RF2 undergoes conformational transitions in nsECs that are similar to the ones it undergoes in TCs. This finding highlights the ability of ArfA to functionally replicate interactions that RF2 makes with the TC. The intermediate conformations we observe in this work are relatively short-lived, and this perhaps explains why previous structural studies, which are predicted to underestimate conformational heterogeneity (61), did not capture them. Indeed, some of the conformations we report may prove to be too short-lived to be accessed by structural studies and instead may require characterization through kinetic investigations entirely.

We anticipate that the conformational dynamics of RF2 on TCs observed in this study should be largely similar for RF1, and form a part of the general mechanism through which the nascent polypeptide is released from the ribosomal complex. Of course, differences in the interactions RF1 and RF2 make with the ribosomal complex (6, 11, 27, 29, 31, 37, 50, 62–68), in addition to the greater dependence of RF1 on RF3 (9–11), suggest that key differences in the kinetics of these conformational changes may exist between different RFs and form a part of the slightly differing biological roles they play in the cell.

Understanding these dynamics and the important conformations these RFs adopt during catalytic activity is critical not only from a mechanistic standpoint but also from one focused on human health as well. Recently, trans-translation has emerged as a potential antibiotic target, with the development of drug leads that can inhibit the process (25). For many bacteria, a suitable antibiotic cocktail must target both trans-translation and the ArfA-RF2 pathway in order to be effective. Our work elucidates a potential mechanism by which the ArfA-RF2 pathway may be disrupted. The model of RF2 dynamics that we elucidate here thus forms not only an important part in our understanding of the function of RF2 specifically, and the wider family of class I RFs in general but also highlights a future target for the next generation of broad-spectrum antibiotics.

Materials and Methods

Construction and Purification of Cy5-Labeled RF2 Constructs. The K12-derived *Escherichia coli* C600 RF2 gene (*prfB*) was cloned into a pProEx-Htb plasmid according to the method previously described for RF1 (51). To enable overexpression of RF2, the autoregulatory stop codon in *prfB* was removed by deletion of the thymine at nucleotide position 76, leading to the direct translation of aspartic acid at residue position 26 in RF2. Residue 246 was mutated from threonine to alanine to mimic the activity of a B-strain *E. coli*-derived RF2, which is necessary for peptide release activity in MRE600-derived ribosomes (69). Labeling mutants of RF2 were engineered in which the native cysteine at position 274 was mutated to serine and the serine at position 184 or the valine at position 271 was mutated to cysteine for the RF2^{core} and RF2^{dIII} constructs, respectively. For both constructs, mutation of the cysteine at position 128 led to protein aggregation, and thus this cysteine was left in its native state given that it has extremely low conjugation efficiency (~3%) (70). A pET vector containing the methyltransferase enzyme gene (*prmc*) that methylates RF2 Q252 (69, 71) was co-overexpressed with RF2 in BL21-DE3 cells. The 6xHis-tagged RF2 constructs were purified over Ni²⁺-nitrilotriacetic (Ni²⁺-NTA) agarose resin followed by subsequent removal of the 6xHis-tag with Tobacco Etch Virus (TEV) protease cleavage. RF2 activity was verified using a radioactive dipeptide release assay (see below). The Cy5-conjugation reaction was performed by combining a 10-fold molar excess of Cy5-maleimide fluorophore at room temperature for 1 h in Labeling Buffer [100 mM Tris-acetate (Tris-OAc) pH₈ = 7.0, 50 mM potassium chloride (KCl), and 1 mM tris(2-carboxyethyl)phosphine (TCEP)]. The labeled (Cy5)RF2 constructs were further purified from excess fluorophore and unlabeled RF2 using size exclusion chromatography with a HiLoad Superdex 75 column pre-equilibrated in 2x Translation Factor Buffer [20 mM Tris-chloride (Tris-Cl),

pH_{4°C} = 7.5, 100 mM KCl, and 10 mM β-mercaptoethanol (βME)] followed by hydrophobic interaction chromatography (HIC) with a TSKgel Phenyl-5PW column (Tosoh Bioscience) using RF2 HIC Buffer A [100 mM sodium hydrogen phosphate/sodium dihydrogen phosphate (Na₂HPO₄/NaH₂PO₄), pH_{RT} = 7.0, 1 M ammonium sulfate ((NH₄)₂SO₄)] and an increasing linear gradient of RF2 HIC Buffer B (100 mM Na₂HPO₄/NaH₂PO₄, pH_{RT} = 7.0) over 20 column volumes.

Construction and Purification of ArfA Constructs. The gene for ArfA and ArfA^{A18T} [lacking the 17 C-terminal residues to mimic the stem-loop cleavage by RNase III that occurs in vivo (72)] was cloned into a pProEx-Htb plasmid and expressed in BL21-DE3 cells. The 6xHis-tagged ArfA constructs were purified over Ni²⁺-NTA agarose resin followed by subsequent removal of the 6xHis-tag with TEV protease cleavage.

Labeling and Amino-Acylation of tRNA Constructs. *E. coli* tRNA^{fMet} (MP Biomedicals) was aminoacylated with methionine or [³⁵S]-methionine using methionyl tRNA synthetase and then formylated using formyltransferase as previously described (43). tRNA^{Phe} was labeled at position 47 via the naturally occurring 3-(3-amino-3-carboxypropyl)-uridine residue using a 20-fold molar excess of Cy3 N-hydroxy succinimide (NHS) ester (GE Lifesciences) in tRNA Labeling Buffer [50 mM HEPES, pH_{RT} = 8.0, 0.9 M sodium chloride (NaCl)] with 20% labeling efficiency. The Cy3-labeled tRNA^{Phe} was purified from the unlabeled fraction using HIC with a TSKgel Phenyl-5PW column (Tosoh Bioscience) using tRNA HIC Buffer A [10 mM ammonium acetate (NH₄OAc), pH_{RT} = 6.3, and 1.7 M (NH₄)₂SO₄] and an increasing linear gradient of tRNA HIC Buffer B [10 mM NH₄OAc, pH_{RT} = 6.3, 10% methanol (CH₃OH)] as previously described (43). Labeled Cy3-tRNA^{Phe} was aminoacylated using phenyl-tRNA synthetase as previously described (43).

Preparation of mRNAs. The mRNAs used in this study were derived from the T4 bacteriophage gene product 32 and were in vitro transcribed using T7 RNA polymerase from linearized double-stranded DNA templates, as previously described (43). The general mRNA sequence used is 5′-[GG]CAACCUAAAACUUA CACAGGGCCCUAAGGAUUAUUUAUG(XYZ)_n-3′, where the bracketed nucleotides are those necessary for in vitro transcription, the underlined nucleotides are for oligonucleotide hybridization (see below), the bold-underlined nucleotides are the Shine-Dalgarno sequence necessary for ribosome binding, and the italicized-underlined nucleotides represent the start codon AUG (the first codon in the wild-type sequence of T4gp32). The bolded nucleotides in parenthesis referred to as (XYZ)_n refer to the number of codons postmethionine (i.e., codons 2-n) from the wild-type sequence of T4gp32 that are included in the construct, where n = 20 for our experiments except for the truncated nsEC constructs. For TCs, codon 3 was mutated to UAA. The differences between the three mRNA constructs are shown below, starting from AUG (codon 1 in wild-type T4gp32):

EC: AUG-UUU-AAA-(4-20)

TC: AUG-UUU-UAA-(4-20)

nsEC: AUG-UUU

For surface immobilization in the smFRET studies, the mRNAs were hybridized to a complementary 3′-biotinylated DNA oligonucleotide (5′-TGTGTAAGTTTAG GTTGATTG-Biotin-3′, Integrated DNA Technologies) prior to complex assembly, as previously described (43).

Preparation of Ribosomal Complexes. Ribosomes and translation factors were purified as described previously (43). TCs, ECs, and nsECs were prepared in Polymix Buffer [50 mM Tris-OAc, pH_{RT} = 7.5, 100 mM KCl, 5 mM magnesium acetate (Mg(OAc)₂), 5 mM NH₄OAc, 0.5 mM calcium acetate (Ca(OAc)₂), 6 mM βME, 5 mM putrescine dihydrochloride, and 1 mM spermidine] as previously described (43, 51). In all cases, initiation complexes were enzymatically prepared by incubating 1.8 μM ribosome with 2.3 μM initiation factors (IF1, IF2, IF3), 2.7 μM fMet-tRNA^{fMet} (or radioactive [³⁵S]-methionyl-tRNA^{fMet} for biochemical studies), 5.4 μM mRNA in presence of 2 mM GTP at 37 °C for 20 min. The reaction mixture was then combined with an equal volume of 15 μM EF-Tu, 5.4 μM Phe-(Cy3)tRNA^{Phe} or Phe-tRNA^{Phe}, 6 μM EF-G, and incubated at 37 °C for 10 min in order to promote the formation of ribosomal complexes containing fMet-Phe dipeptides that were stalled at the codon in the third position (60). These ribosomal complexes were then further purified using sucrose density gradient ultracentrifugation (10 to 40% sucrose w/v) for 12 h at 25,000 RPM using an SW41 rotor in a Beckman Optima L70 Ultracentrifuge (Beckman) in Buffer C [50 mM Tris-HCl, pH_{RT} = 7.5, 70 mM ammonium chloride (NH₄Cl), 30 mM KCl, 7 mM

magnesium chloride (MgCl₂), 10 mM βME, 1% beta-D-glucose] for TIRFM experiments, or were buffer exchanged into Buffer C using P6 desalting spin columns (Bio-Rad) for biochemical assays. Purified ribosomal complexes were flash frozen in liquid nitrogen, and stored at −80 °C.

Radioactive Dipeptide Release Assays. Enzymatically assembled ribosomal complexes carrying a [³⁵S]-methionyl-Phe-tRNA^{Phe} in the P site were preincubated in Buffer C at room temperature for 5 min. Additionally, a mixture of RF2 and 1 mM GTP was separately preincubated in the same buffer as the ribosomal complexes at the specified temperature for 5 min. Upon initiation of a time course reaction, the ribosome-containing and RF2-containing mixtures were combined to final concentrations of 100 nM ribosomal complexes, 1 mM GTP, and varying concentrations of RF2. Small samples were taken out at specific time points and quenched with an equal volume of ice-cold 25% formic acid. The desired dipeptides were separated from other products using electrophoretic thin layer chromatography (eTLC) with pyridine-acetate (0.05% pyridine, 20% acetic acid) at 1,200 V for 30 min (60, 73, 74) (SI Appendix, Fig. S6 A and B). The amount of dipeptide was quantified (SI Appendix, Fig. S6 C-E) using ImageQuant v5.2.

smFRET Experimental and Imaging Conditions. Microfluidic flow cells were prepared using quartz microscope slides (G. Finkenbeiner) and borosilicate coverslips (VWR) passivated with a mixture of polyethylene glycol and dilute biotinylated polyethylene glycol, as previously described (73, 75, 76). The slide surfaces were functionalized with streptavidin and ribosomal complexes were surface-immobilized via a preannealed, biotinylated DNA oligonucleotide tether, as previously reported (51, 73). The data were collected with a wide-field, prism-based total internal reflection fluorescence (TIRF) microscope with a 100 mW diode-pumped solid-state 532 nm laser (Laser Quantum gem 532) operating at a power of 30 mW (or 15 mW for pre-steady-state experiments), measured at the prism, for Cy3 excitation (51, 73, 75). The fluorescence emissions were collected through a 60×, 1.2 NA, water-immersion objective (Nikon), and imaged through a Dual-View (Photometrics) multicolor imaging device using an iXon Ultra 888 electron-multiplying charge-coupled device (EMCCD) camera (Andor) with 2×2-pixel binning and a 100 ms acquisition time per frame. All steady-state experiments were performed at room temperature in Buffer C containing an oxygen-scavenging system composed of 50 U/mL glucose oxidase (Sigma) and 370 U/mL catalase (Sigma), a triplet-state quencher system composed of 1 mM cyclooctatetraene (Sigma) and 1 mM p-nitrobenzyl alcohol (Fluka), and a nonspecific interaction blocking system composed of 1 μM duplex DNA oligo and 1 μM BSA (75). Pre-steady-state experiments were performed by flowing in (Cy5) RF2^{core} or (Cy5)RF2^{core} and ArfA (1 μM) to tethered TCs or nsECs, respectively, at (Cy5)RF2^{core} concentrations of 2.5, 5, 10, and 20 nM (prepared by serial dilution from the highest concentration).

E_{FRET} vs. Time Trajectory Analysis. As previously described (77), corresponding fields-of-views comprising the fluorescence emissions of hundreds of Cy3 and Cy5 single emitters were aligned using an Iterative Closest Point (ICP) algorithm (78) to identify the best affine transformation between the two channels. The time-dependent fluorescence emissions from the Cy3 and Cy5 single emitters thus colocalized were fit to Gaussian distributions and extracted to yield fluorescence intensity vs time trajectories. Bleed-through from the Cy3 channel to the Cy5 channel was corrected using a bleed-through parameter (3.5% for all nsECs with (Cy5)RF2^{core}, 5% for TCs with (Cy5)RF2^{core}, 4% for all complexes with (Cy5)RF2^{dlil}). Only trajectories exhibiting anticorrelated Cy3 and Cy5 intensities (*I*_{Cy3} and *I*_{Cy5}, respectively) and photobleaching (i.e., complete loss of fluorescence emission) in a single step were selected and truncated at the time point of Cy3 photobleaching. The corresponding E_{FRET} vs. time trajectories were obtained using the equation E_{FRET} = (*I*_{Cy3}/*I*_{Cy5} + *I*_{Cy3}) (79) and were used for further analysis.

Kinetic Analysis of Pre-Steady-State E_{FRET} vs. Time Trajectories. Trajectories were analyzed to identify the first binding event of (Cy5)RF2^{core} (defined as the first appearance of *I*_{Cy5} and corresponding anticorrelated drop in *I*_{Cy3} upon addition of (Cy5)RF2^{core}), which were then used to obtain the time to (Cy5)RF2^{core} binding for that individual trajectory. Survival plots of the distributions of binding time so obtained at each concentration of (Cy5)RF2^{core} were fit to exponential decays using Python scripts, using the function $y = Ae^{-kt}$ for the single exponential decay, where *y* is the survival probability at time *t*, *k* is the rate constant, *A* is the normalization constant, and $y = Ae^{-k_1t} + Be^{-k_2t}$ for the double exponential

decay, where y is the survival probability at time t , k_1 and k_2 are the rate constants for the two phases, A and B are the corresponding populations for those phases. These yielded the apparent pseudo-first-order rates of association (k_a^{app} s) at these concentrations of (Cy5)RF2^{core}. Survival plot fits which yielded an R^2 value of 0.95 or above for a single exponential fit were taken to be well explained by single exponential decays. The rest were fit using a double exponential decay function. For TCs, the survival plots for RF2 concentrations at 2.5 and 5 nM could be well explained by single exponential decays, while for 10 and 20 nM, the survival plots required at least double exponential decays. The faster phases of the double exponential decays, in these cases, showed a dependence on (Cy5)RF2^{core} concentration and were taken to be the k_a^{app} s at these concentrations. The slower phases, which accounted for a population of ~10% of the total number of binding times, were found to be orders of magnitude slower than the k_a^{app} s and were likely the result of nonspecific binding of (Cy5)RF2^{core} at these higher concentrations. For nsECs, the survival plots for all concentrations could be well explained by single exponential decays. Subsequently, these pseudo-first-order k_a^{app} s were plotted as a function of (Cy5)RF2^{core} concentrations, and fit to a linear equation ($y = mx + c$) to obtain the second-order rate of association, k_a , for these complexes (SI Appendix, Fig. S2).

Global HMM Analysis of Steady-State E_{FRET} vs. Time Trajectories. The trajectories for all steady-state experiments for each construct were analyzed using a global variational Bayes HMM analysis, as previously described (44). Briefly, the individual E_{FRET} vs. time trajectories in the datasets for each ribosomal complex were assumed to be independent and identically distributed (i.i.d.) according to a single global HMM for that complex, which was then inferred using the vbFRET algorithm (80). For (Cy5)RF2^{core} datasets and (Cy5)RF2^{dIII} with the Api137-bound TC, a global 2-state HMM was used. For the other (Cy5)RF2^{dIII} datasets, a global 3-state HMM was used for all datasets except for mut-nsEC. In the mut-nsEC, the global 3-state model yielded two zero E_{FRET} states and a very broad nonzero E_{FRET} state encompassing both the extended and the collapsed states. This is likely due to low occupancy of the ribosome-bound nonzero E_{FRET} states of (Cy5)RF2^{dIII} on mut-nsECs, which led to the HMM picking up variations in the zero E_{FRET} state as a separate state instead. To get around this, a 4-state HMM was used, which captured two zero E_{FRET} states, the mid- E_{FRET} state, and the high E_{FRET} state. This 4-state model was collapsed into three states by combining both "zero" E_{FRET} states into a single state, i.e., combining the transitions to and from the separate zero E_{FRET} states into that for a single zero state, and the transitions between them into self-transitions of the zero state. These transition probabilities from the HMM transition matrices were used to calculate the rate between specific states using the equation

$$k_{ij} = -\frac{\ln(1 - P_{ij})}{\tau},$$

where k_{ij} is the transition rate between states i and j , P_{ij} is the corresponding transition probability, and τ is the duration of a single frame (81).

Accounting for Photobleaching Using Api137. The zero E_{FRET} state corresponds to both a ribosome lacking a bound (Cy5)RF2 and one where the Cy5 dye had photobleached; therefore, the rate of transition into the zero state, as calculated by the HMM-based analysis described above, is a sum of the rate of RF2 dissociation and that of Cy5 photobleaching. However, since Api137 can trap RFs in the extended state on the ribosome for extended periods of time that greatly exceed our time window (46, 47), all instances of a drop in E_{FRET} to zero can be attributed solely

to photobleaching for an Api137-bound complex. Thus, the rate of transition to the zero E_{FRET} state for this construct was used to estimate the photobleaching rate for all other constructs in our experimental conditions. It should, however, be noted that the rate of photobleaching for any fluorophore is influenced by several factors that can be directly related to the individual complexes themselves. For example, a higher E_{FRET} state for a donor-acceptor pair on a given complex typically corresponds to more (indirect) excitations of the acceptor fluorophore compared with a lower E_{FRET} state for the same pair on a different complex observed under the same experimental conditions. Thus, complexes exhibiting a higher E_{FRET} will likely have a higher rate of photobleaching than those with a lower E_{FRET} . Since the Api137-bound TC mostly exhibits the mid E_{FRET} state (~0.39) of (Cy5)RF2^{dIII}, this correction represents a lower bound for the global photobleaching rates, and thus the apparent dissociation rates (k_d s) calculated using this correction represent a higher bound for the real dissociation rate. It is thus reasonable to expect, for example, that the high E_{FRET} state (~0.9) for the (Cy5)RF2^{core}-bound complexes [or indeed the E_{FRET} states for the closed conformation of (Cy5)RF2^{dIII} (>0.7)] would consequently have a higher rate of photobleaching, and thus the rates of dissociation from these states reported in this work are likely overestimated.

Construction of Postsynchronized 2D Histograms. To visualize transitions from all of the nonzero E_{FRET} states to the zero E_{FRET} state for each dataset, the idealized trajectories from the global HMM analysis [using the Viterbi algorithm (80)] were split into dwells in the nonzero E_{FRET} states and dwells in the zero E_{FRET} state using an E_{FRET} threshold of 0.25. All transitions from an E_{FRET} state above 0.25 to one below were collected for the entire dataset and postsynchronized to time zero, which was then used to construct a 2D E_{FRET} vs. time histogram. Only a single dwell in the nonzero E_{FRET} states (i.e., $E_{FRET} > 0.25$) immediately preceding the transition and a single dwell in the zero E_{FRET} states (i.e., $E_{FRET} < 0.25$) immediately after the transition were considered. Each time-point (frame) in the histogram (i.e., the data points contained in a column at a given time t) was normalized to 1. Similarly, for transitions from the zero E_{FRET} state to all of the nonzero E_{FRET} states, a similar procedure was used, except only transitions from an E_{FRET} state below 0.25 to one above were considered. For the histograms showing transitions from the mid- E_{FRET} state to the zero E_{FRET} state and the reverse, only transitions between the mid- E_{FRET} state and zero E_{FRET} state (and single dwells in these states immediately before and after the transitions) were considered.

Dwell-Time Analysis of Individual E_{FRET} States. The idealized trajectories from the HMMs for each complex yielded the dwell times for individual states. For a given state, these dwells were used to construct a dwell time distribution and construct a survival plot, similar to the procedure for the pre-steady-state kinetics. Using Python scripts, these were fit to exponential decays, using the function $y = Ae^{-k_1t}$ for the single exponential decay and $y = Ae^{-k_1t} + Be^{-k_2t}$ for the double exponential decay, with the terms defined as above. A threshold of $R^2 = 0.95$ for the single exponential fit determined whether the single exponential fit was sufficient to explain the decay or whether at least a double exponential was required. For parsed dwells, the above analysis was restricted to only include dwells of a given state that originated from and resulted in specific states.

Data, Materials, and Software Availability. The raw data associated with this paper is available on Zenodo (82).

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