The role of fluctuations in tRNA selection by the ribosome

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The detailed mechanism of how the ribosome decodes protein sequence information with an abnormally high accuracy, after 40 years of study, remains elusive. A critical element in selecting correct transfer RNA (tRNA) transferring correct amino acid is "induced fit" between the ribosome and tRNA. By using single-molecule methods, the induced fit mechanism is shown to position favorably the correct tRNA after initial codon recognition. We provide evidence that this difference in positioning and thermal fluctuations constitutes the primary mechanism for the initial selection of tRNA. This work demonstrates thermal fluctuations playing a critical role in the substrate selection by an enzyme.

ribosome dynamics | ribosome selection | single-molecule FRET

Translation, protein synthesis by the ribosome, is a vital cellular process involving the two-subunit ribosomal particle, multiple RNAs, and protein cofactors (1, 2). To understand how a protein molecule with correct sequence, the ribosome has to select correct transfer RNA (tRNA) as dictated by messenger RNA (mRNA). In the decoding site of the ribosome, the proper matching of the 3-nucleotide codon of the mRNA to the anticodon sequence of tRNA occurs at a rate of 20–30 per second with an error rate of 10^−3 (3).

The difference in base-pairing energy between codon and anticodon cannot explain the low error rate of translation (4). In the past decades, considerable progress has been made in understanding the ingenious mechanisms used by the ribosome to achieve this low error rate (2, 4–6). The overall selection process consists of initial selection of the ternary complex [aminoacyl-tRNA (aa-tRNA), GTP, and elongation factor Tu (EF-Tu)], GTP hydrolysis in EF-Tu, and then proofreading (6). The GTP hydrolysis ensures that initial selection is separated from the proofreading by an irreversible reaction. This two-step selection yields overall probability of an error to be the product of the error probabilities of the two steps, thus dramatically lowering the overall chance of error (7).

The initial selection of aa-tRNA consists of initial binding of the ternary complex on the ribosome, interaction between tRNA and mRNA (the codon recognition), and stabilization of the ternary complex onto the ribosome. GTPase activation takes place after additional stabilizing contacts are formed between the ribosome and the ternary complex (6, 8). In the initial selection of aa-tRNA, the ternary complex binding induces structural changes in the ribosome ("induced-fit") (5, 9) in which the 30S subunit of the ribosome changes its structure and forms additional stabilizing contacts with the incoming tRNA after the codon recognition (9). In case of one base-mismatch out of the 3 bp (near-cognate), additional binding contacts are weaker, presumably because the mismatched bases form a less compact structure for the ribosome to "wrap" around as it forms additional induced fit contacts. In other words, the tighter binding of cognate tRNA relative to near-cognate tRNA mainly is attributable to shape discrimination at the decoding site of the ribosome (6, 9).

The formation of additional stabilizing contacts between the ternary complex and the 50S subunit of the ribosome (5, 10) requires additional movement beyond the positioning of the ternary complex immediately after the codon recognition. Recent kinetic studies have shown that the rate at which these stabilizing contacts are formed favors cognate ternary complexes over near-cognate ternary complexes (6). Cryo-electron microscopic structures of these stabilizing contacts of the ternary complex with the 50S subunit of the ribosome, particularly with the sarcin–ricin loop (SRL) and the GTPase-associated center (GAC) are shown in supporting information (SI) Fig. 5 (5, 10–12).

Here we present single-molecule fluorescence resonance energy transfer (FRET) studies (13, 14) of the initial selection of aa-tRNA by the ribosome that show how the induced fit plays a dual role in initial selection. In addition to causing more stable binding of the cognate ternary complex, the formation of the additional contacts with the 30S portion of the ribosome positions the ternary complex so that thermal fluctuations are much more likely to form the additional stabilizing contacts that mark the end of initial selection. Thus, large and rare thermal fluctuations are observed to play a critical role in the high substrate selectivity of this enzymatic reaction.

This work followed the single-molecule FRET approach in our earlier work (8, 15). A total internal reflection (TIR) microscope/camera system with single-photon counting sensitivity was used. The ribosome preassembled with biotinylated mRNA and Cy5-labeled peptidyl-tRNA (P-site tRNA) (16, 17) was attached on a quartz surface through streptavidin–biotin interaction. Either cognate or near-cognate ternary complex with Cy5-labeled aa-tRNA was introduced in our stopped-flow, single-molecule apparatus (Fig. 1A). The Cy3-labeled tRNA was excited with a 532-nm laser beam, and the FRET signal was observed as the ternary complex was introduced to the immobilized ribosome complex. A critical improvement in our experiment relative to previous work was that we now were able to record a single-molecule image every 25 ms, a factor of 4 faster than our previous capability. With this higher time resolution, it is possible to observe directly that aa-tRNA is selected by and accommodated into the ribosome based on the codon–anticodon interaction.


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Abbreviations: aa-tRNA, aminoacyl-tRNA; EF-Tu, elongation factor Tu; SRL, sarcin–ricin loop; GAC, GTPase-associated center; GDPNP, 5′-guanylyl-imidodiphosphate.

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trace is the Cy5 fluorescence intensity. FRET efficiency is defined as the green time trace shows the Cy3 fluorescence intensity, and the orange GTP analogue, GDPNP. Materials and methods are explained elsewhere (8). (Experimental setup to observe the initial selection of aa-tRNA by the ribosome (8).

...particles) ribosome complexes are superimposed after synchronizing to the first individual FRET trajectories for the cognate (56 particles) and near-cognate (72 near-cognate delivery of GDPNP stalled ternary complexes (see SI Text). The histogram of the mid-FRET state shows double exponential decay (Fig. 2). These results suggest that the mid-FRET state previously defined as the GTPase-activated state in fact is composed of two different states: a short-lived state (state 3) and an intermediate state between state 2 and state 3. Under this scenario, state 3 is connected directly to 3. It is still possible to get transitions to/from state 3 that are not affected by adopting a different kinetic scheme.

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...charge-coupled device (CCD).

...to quantify the initial selection efficiency (8, 18). The absolute FRET efficiencies differ from those reported previously because of a difference in quantum yield in the Cy5 spectral region of the newly used charge-coupled device (CCD).

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To analyze the detailed kinetics, it is important to properly set the boundary FRET levels among background (0 FRET), the cognate-recognition (low-FRET), and the stabilized/GTPase-activated (mid-FRET) states. We initially chose the boundary values to be 1 standard deviation from the mean low- and mid-FRET efficiencies. We then varied the values to achieve the least sensitivity to change of transition rates between the low- and mid-FRET states (see SI Text). This algorithm minimizes the number of false transitions attributable to technical noise of the experimental apparatus. Once the threshold is determined, the lifetime histogram of each state is determined.

The histogram of the mid-FRET state shows double exponential decay (Fig. 2). These results suggest that the mid-FRET state previously defined as the GTPase-activated state in fact is composed of two different states: a short-lived state (state 3) in Fig. 3 and a stably bound state (state 3) (8). The detection of the 3 state suggests that the ribosome complex fluctuates reversibly between the codon-recognition and the GTPase-activated states rather than making a single, discrete transition. The suggested kinetic scheme in Fig. 3 is an analytical solution of what we observed. As an alternative scheme, state 3 may be an intermediate state between state 2 and state 3. Under this scenario, 3 is connected directly to 3. It is still possible to get lifetimes that can be fitted with double exponentials under certain conditions (e.g., lifetime of state 3 is negligibly short compared with that of state 3, and the rate forward from state 3 is negligibly low compared with the rate backward). Nonetheless, the existence of fluctuations to/from state 3 is not affected by adopting a different kinetic scheme.

Here, we explicitly assume that the formation of a long-lived mid-FRET state is required for GTPase activation and hydrolysis (5, 20). Even though GTPase activation and hydrolysis are very fast compared with the initial selection process, the fact that cognate tRNA is much more likely to enter into the stably bound mid-FRET state before GTP hydrolysis but has been shown not to affect earlier steps of aa-tRNA selection (19, 20). In the presence of GDPNP, the majority of cognate ternary complex stalls on the ribosome in a stable, GTPase-activated configuration (8, 19, 20).

Both cognate and near-cognate ribosome complexes reach the mid-FRET state (FRET efficiency 0.4, required for GTPase activation) from the low-FRET state (FRET efficiency 0.3, the cognate-recognition state) as shown Fig. 1B (8). To synchronize the behavior of individual ribosome complexes after the codon recognition, individual FRET time traces are superimposed at the first observation point of FRET efficiency 0.27. From these “post-synchronized” FRET time traces (8), histograms are compiled (Fig. 1C) to show the majority of cognate ribosome complex progress quickly (<50 ms) from the codon-recognition state to the GTPase-activated state. Near-cognate ribosome complexes, on the other hand, tend to dissociate in <100 ms from the cognate-recognition state.

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times per every one successful advancement. Our kinetic analysis also reveals that cognate ribosome complexes not only make fewer unsuccessful trials, but they also try more often. On average, at 15 mM [Mg2+]2, cognate ribosome complexes try 27 times per second, whereas near-cognate ribosome complexes try only 8 times per second. As a result of such differences, the initial selection efficiency is calculated to 6.7 and 2.5 × 102 for 15 mM and 5 mM [Mg2+]2 cases, respectively (Fig. 4). In the case of 5 mM [Mg2+]2, the near-cognate rates we report here have large uncertainties because of our limited data set (see SI Text).

Discussion

Based on these findings, we propose that the codon–anticodon interaction and the domain closure, i.e., the formation of additional ribosome-tRNA-mRNA contacts at the decoding site of the 30S subunit (9, 21), cause the cognate ternary complex to be positioned slightly closer to the P-site tRNA. We observe differences in the average FRET efficiencies of the codon-recognition state for cognate and near-cognate ribosome complexes (Fig. 1C). Cognate ribosome complexes are found to yield significantly higher FRET efficiency than near-cognate complexes for the 15 mM [Mg2+]2 data. We also performed experiments in which the progression of the aa-tRNA to the position where GTPase activation of EF-Tu also would occur under normal conditions was blocked by adding the antibiotic tetracycline (22). These experiments were performed at 15 mM [Mg2+]2. Tetracycline inhibits the tRNA selection process by hindering the stable codon–anticodon interaction and thereby blocking further incorporation of aa-tRNA from the initial codon recognition. Therefore, the position of the aa-tRNA monitored in these experiments would represent the dynamics of the codon-recognition state. In the data shown in Fig. 2B, we filtered out a subpopulation of the ribosome complexes that show normal delivery of aa-tRNA to the full accommodation. Only the first 5–10 s after the ternary complex was introduced was used to build histograms. Within this time window, the percentage of complexes that showed full accommodation was below 10%. The full accommodation percentage increased to 55% over 8 min after the ternary complex delivery. As discussed in the legend of Fig. 2B, cognate ribosome complexes in the codon-recognition state have a longer lifetime and a higher FRET efficiency than do near cognates, further supporting the view that cognate ternary complexes are positioned closer than near-cognate complexes are to the stably docked mid-FRET state.

GTP hydrolysis requires the formation of ternary complex contacts with SRL and GAC as shown in SI Fig. 5 (5, 10). It is the movement of the tRNA to this mid-FRET state that provides the allosteric linkage between action at the decoding site and GTPase activation of EF-Tu at the SRL/GAC (5, 19).
Because of (i) the tens of milliseconds needed for the ternary complex to form stabilizing contacts \( k_1 \) in Fig. 4) and (ii) the inferred distance change in going from the low- to mid-FRET states, we conclude that a large and rare thermal fluctuation is required to pivot the tRNA into a position where the contacts between SRL/GAC and the ternary complex can be formed. We now argue that ribosome uses these rare fluctuations as an effective selection mechanism. Assume, for simplicity, that the thermal distribution of distances between the SRL/GAC and the binding sites on the ternary complex is described by a Gaussian distribution \( \exp[-x^2/(2\sigma^2)] \), where \( x \) is the deviation from the equilibrium position and when \( x \) is comparable or less than \( \sigma \). For \( x \gg 2-3 \sigma \), it is plausible that the tail of the distribution of positions might be better described by \( \exp[-x/\lambda] \), analogous with thermal activation out of a bound state \( \exp[-\Delta E/kT] \). The rate of forming stabilizing contacts would be proportional to \( \nu \exp[-x/\lambda] \), where \( \nu \) describes a “characteristic frequency” of the thermal fluctuations, most likely in the nano- to microsecond range.

If \( x_c \) is the critical distance that the near-cognate ternary complex has to move to form contacts with SRL/GAC, our data indicate that the cognate requires a less rare fluctuation \( x_c < -\lambda/2 \). Thus, the ternary complex is more likely to form stabilizing contacts with the cognate tRNA complex by the ratio \( \exp[-x/(x_c - \lambda/2)]/\lambda/\nu \exp[-x/\lambda] = \exp[\lambda/(x_c - \lambda/2)] \), provided the attempt frequencies \( \nu \) of cognate and near-cognate tRNA fluctuations are similar. Note that the rate of forming the stabilizing contacts is made exponentially important in the distance \( \lambda \) because docking must be preceded by a rare thermal fluctuation. The long distance (\( >7 \) nm) between the decoding site and the SRL/GAC (5, 11, 12, 20, 23) suggests that the tRNA may act as a long lever arm to increase \( \lambda \) by magnifying a slight difference in the pivot angle between cognate and near-cognate tRNA at the decoding site.

The simple model presented above does not take into account the fact that the ternary complex can form the bona fide GTPase-activated state with the SRL/GAC or a transient pseudo-GTPase-activated state. However, because states 3 and 3' are both mid-FRET states, the formation of either state requires a large thermal fluctuation. The shorter lifetime (\( <100 \) ms) components of the mid-FRET state plotted in Fig. 2A may represent unsuccessful long-distance fluctuations of the ternary complex to stably dock at the SRL/GAC. Instead, the ternary complex may form only one of the two identified contacts. (SI Fig. 5 shows the structure of the two contacts between ternary complex and SRL/GAC.)

This hypothesis was tested by our recent results from thioestreptated-ribosome-activated state with SRL/GAC or a transient pseudo-GTPase-activated state. However, because states 3 and 3' are both mid-FRET states, the formation of either state requires a large thermal fluctuation. The shorter lifetime (\( <100 \) ms) components of the mid-FRET state plotted in Fig. 2A may represent unsuccessful long-distance fluctuations of the ternary complex to stably dock at the SRL/GAC. Instead, the ternary complex may form only one of the two identified contacts. (SI Fig. 5 shows the structure of the two contacts between ternary complex and SRL/GAC.)

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