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Heterogeneous Pathways and Timing of Factor Departure during Translation Initiation

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Abstract

Initiation of translation establishes the reading frame for protein synthesis and is a key point of regulation[1]. Initiation involves factor-driven assembly at a start codon of an mRNA of an elongation competent 70S ribosomal particle (in bacteria) from separated 30S and 50S subunits and initiator tRNA. Here we establish by direct single-molecule tracking the timing of initiator tRNA, initiation factor 2 (IF2), and 50S subunit joining during initiation. Our results show multiple pathways to initiation, with orders of arrival of tRNA and IF2 dependent on factor concentration and composition. IF2 accelerates 50S subunit joining, and stabilizes the assembled 70S complex. Transition to elongation is gated by the departure of IF2 after GTP hydrolysis, allowing efficient arrival of elongator tRNAs to the second codon presented in the aminoacyl-tRNA acceptor site. These experiments highlight the power of single-molecule approaches to delineate mechanism in complex multicomponent systems.

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Initiation is a key point of regulation of gene expression before the ribosome is committed to the energy-intensive process of synthesizing a full protein[1]. Protein factors guide and regulate initiation: three initiation factors (IF1, IF2, and IF3) are required for viability in bacteria, whereas a far larger complement of factors exists in higher organisms.

While the mechanism and overall kinetics of translation initiation in bacteria have been delineated during the past two decades[2], fundamental questions remain. The possible configurations that this multi-factor system can adopt challenge traditional biophysical methods. The timings of individual factor and tRNA assembly on the ribosome, their coordination with each other and subsequent factor dissociation that allow elongation are not known. Translation initiation may follow a linear mechanism, or branch through multiple parallel pathways. We apply here real-time single-molecule methods to track directly the dynamics of translation initiation in the model *E. coli* system. We determined the relative timing of initiator tRNA, IF2, and subunit binding, and showed how IF2 and GTP hydrolysis control the transition into elongation. Our data demonstrate that intermediate and late steps in initiation occur through heterogeneous pathways. The overall initiation rates and efficiency depend on the initiation pathway, whose selection is guided by initiation factors.

Single-molecule fluorescence experiments allow direct observation of dynamics in complex biological systems. To monitor single-molecule fluorescence at high (0.1–5 μM) concentrations of free dye-labeled biomolecules, optical confinement was achieved using zero-mode waveguides (ZMWs)[3] (Supplementary Figure 1). We recently demonstrated the power of this approach by tracking the real-time dynamics of tRNA transit through the ribosome during elongation[4]. After conducting control experiments to verify the functionality of our dye-labeled biomolecules (Supplementary Text 1 & Supplementary Figure 2), we broaden this method to follow tRNAs, protein factors, and ribosomal subunits directly during initiation and transition into elongation (Figure 1a).

Although recent experiments suggested that IF2 and fMet-tRNA^{fMet} bind sequentially to the small subunit in the formation of a 30S pre-initiation complex (30S PIC)[5], IF2(GTP) also forms a weak complex with the tRNA ($K_D \approx 1$ μM)[6,7], potentially allowing both to bind simultaneously. We determined whether IF2 and tRNA binding is simultaneous, sequential, or random by delivering a mixture of fMet-(Cy3)tRNA^{fMet}, Cy5-IF2, and Cy3.5-50S to immobilized Alexa488-30S at 20 nM to 1000 nM of each reagent (Methods 4). The appearance of a stable 50S signal ($t > 10$ s) was used to identify productive tRNA and IF2 binding events. The relative timing of IF2 and tRNA^{fMet} arrival to the ribosome was determined by single-molecule analysis (Figure 1b).

At low concentration (20 nM each) of IF2 and the initiator tRNA, tRNA arrives first in 65% of the initiation events, IF2 arrives first in 30% and only 5% show simultaneous arrival of both molecules (Figure 1c and Supplementary Figure 3). Addition of IF1 and IF3 shifts the arrival order, with 50% of ribosomes having IF2 arrive before tRNA^{fMet}, 40% having tRNA^{fMet} before IF2, and 10% showing simultaneous arrival. This is consistent with IF1 and IF3 destabilizing tRNA^{fMet} in 30S PIC[8] and increasing the affinity of IF2 to the 30S ribosomal subunit in the absence of initiator tRNA[9,10]. Increasing IF2 and tRNA^{fMet} concentrations to 1 μM raised the fraction simultaneous arrival to 45%, while lowering the

fraction of IF2 arriving first to 35% and the fraction of $tRNA^{fMet}$ arriving first to 10%. Thus, the order of IF2 and initiator tRNA arrival does not strictly follow a defined sequence, but is greatly affected by ligand concentrations and other initiation factors. While at lower concentrations, the ligands arrive independently, simultaneous arrival of both ligands could be a more common mechanism at near physiological concentrations.

50S subunit joining to a 30S PIC to form a 70S initiation complex (70S IC) is the second major molecular event of initiation. To track subunit joining, we used 50S subunits labeled with single dyes, which were shown to be functional in prior intersubunit FRET studies[11,12]. We delivered Cy5-50S subunits to immobilized 30S PICs (Methods 5). IF2 in the presence of GTP drives rapid, stable subunit joining[8] (Figure 2a). At 2 μM IF2(GTP), Cy5-50S subunits joined rapidly to 30S PICs with an observed $k_{on} = 1\times10^6\ M^{-1}s^{-1}$ ($\tau = 9$ s), forming complexes whose lifetime was limited by photobleaching ($\tau = 38$ s) (Figure 2b, Supplementary Figure 4 & Supplementary Text 2). In accordance with previous studies[8], omitting IF2 resulted in slow and unstable subunit joining, decreasing k_{on} to $0.3\times10^6\ M^{-1}s^{-1}$ ($\tau = 29$ s) and 50S lifetime to $\tau = 6$ s. In the presence of IF2 and non-hydrolysable GDPNP, 50S subunit arrival rate was similar to that of IF2(GTP). However, 50S subunit stability decreased to a lifetime of $\tau = 28$ s, consistent with prior intersubunit FRET results that GDPNP-bound IF2 can guide stable subunit joining without GTP hydrolysis[11,13]. Addition of the other two initiation factors, IF1 and IF3, at 1μM each to 2 μM IF2(GTP) did not appreciably change the k_{on} or the lifetime of the 50S subunit on our model mRNA.

Subunit joining accelerates GTP hydrolysis by IF2 and IF2(GDP) quickly dissociates from the ribosome[13]; elongator tRNA arrival finalizes transition into elongation. Yet the relative timings of IF2 release, 50S subunit joining, and elongator tRNA binding are not known. To monitor these events in real time, we delivered Cy5-IF2, Cy3.5-50S and Phe-(Cy2)$tRNA^{Phe}$ (as a tRNA:EF-Tu(GTP) ternary complex) to 30S PIC loaded with fMet-(Cy3)$tRNA^{fMet}$, simultaneously tracking 4 different labeled components (Methods 6). An IF2 signal was followed by rapid and stable subunit joining ($t > 10$ s) in the presence of GTP (Figure 3a). IF2 with GTP yielded stable tRNA binding ($t > 1$ s) post 50S subunit joining; only brief tRNA sampling occurs with GDPNP.

Post-synchronizing the four-color experiments with IF2(GTP) to 50S arrival revealed an overlap between the IF2 and 50S signals of $\tau = 2$ s on a 70S IC (Figure 3b, Supplementary Figure 5 Supplementary Text 3). This overlap time was independent of 50S subunit concentration, suggesting that unimolecular processes occur within this overlap (Figure 3c). During this period, IF2 rapidly hydrolyzes GTP, rearranges the 70S IC, and then dissociates from the ribosome; consistent with this interpretation, the lifetime of IF2-GDP on 70S ribosomes was $\tau = 1.2$ s (Supplementary Figure 2c). Interestingly, the arrival time of the elongator tRNA post subunit joining showed a similar lag of ~ 2 s. Increasing tRNA concentration beyond 200 nM had no significant effect on tRNA arrival time, suggesting that tRNA arrival is not a rate-limiting step (Figure 3c). The temporal correlation of IF2 dissociation and tRNA arrival during this 2 s window was not absolute; when single-molecule trajectories were post-synchronized to IF2 departure, tRNA arrival frequency increased after IF2 release, but ~ 20% of tRNA molecules arrived before IF2 departure.

Further analysis of these experiments explains how IF2 controls the transition into elongation. In the presence of IF2(GDPNP), very little elongator tRNA density was observed. The overall frequency of all elongator tRNA binding events within a 2 s window before and after IF2 departure was similar in the presence of either GTP or GDPNP (Figure 3d, Supplementary Text 4). However, the majority of tRNA arrival events were short-lived sampling in GDPNP whereas most of the tRNA events in GTP involved stable (>1 s) binding, indicating that prior to GTP hydrolysis, only short-lived elongator tRNA sampling events are allowed.

Single-molecule techniques can distinguish among heterogeneous populations of molecules. By tracking individual dye-labeled molecules, we have shown that initiation does not follow a strictly linear mechanism whereby the translational machinery is rigidly assembled in a well-defined order. While the ribosome must proceed through defined stages, such as forming the 30S PIC and 70S IC, there are numerous pathways available to it (Figure 4).

During the formation of 30S PIC, IF2 and fMet-tRNA^{fMet} must bind to the 30S subunit to establish reading frame on the mRNA and prime the 30S subunit for subunit joining. We observed all possible orders of binding occurring under different conditions. The binding pattern changes depending on concentrations of both molecules and on the presence of other initiation factors and our results suggest that simultaneous arrival of IF2 and the tRNA may dominate *in vivo*.

The last stages of successful initiation ensure stable 70S ribosome assembly and configure it to accept the first elongator tRNA. IF2(GTP) is required for stable 70S complex formation. GTP hydrolysis by IF2 occurs rapidly (30 ms) after subunit joining, but our data show a lag of 1–2 seconds before elongator tRNA arrival. During this period, IF2(GDP) is bound to the 70S ribosome. CryoEM structures show that the IF2(GDP) adopts a different conformation from the GTP form, and moves away from the GTPase activation center[14]. Our data show that IF2 occupancy post GTP hydrolysis on the 70S complex hinders elongator tRNA arrival, consistent with cryo-EM maps of IF2(GDP) on the 70S ribosome having partial steric clash with an incoming ternary complex (tRNA:EF-Tu(GTP)) in the A-site. These results suggest that IF2 release from the 70S complex controls the transition from initiation to elongation.

The single-molecule data presented here demonstrate the heterogeneous nature of translation initiation (Figure 4). As the process evolves, the pathways converge to an elongation-competent 70S complex, with initiator tRNA positioned in the P site, the correct intersubunit conformation, and IF2 clearance from the complex. Initiation factors guide the fidelity and timing of the process: IF1 and IF3 together regulate the order of IF2 and tRNA arrival and overall initiation efficiency, albeit via unclear mechanisms. IF2 guides the ribosome towards productive initiation and GTP hydrolysis governs transition into elongation. Single-molecule methods using ZMWs presented here can be broadly applicable to tracking compositional dynamics in other biological systems.

Methods

1. Dye-labeled ligands

Escherichia coli ribosomal subunits, initiation factors, elongation factors were prepared and purified as described[11,15–17]. $tRNA^{fMet}$ and $tRNA^{Phe}$ were labeled with fluorescent cyanine dyes at their elbow positions (U8 or U47), purified, and aminoacylated as previously described[15,17]. A single-cysteine mutant of initiation factor 2 (C599A and K791C) was labeled with monomaleimide-Cy5 (GE Lifesciences) according to instructions from GE Lifesciences[18]. Supplementary Figure 6 shows the location of dye-labeling on the biomolecules.

2. Experimental conditions

Ribosome initiation complexes were assembled at 0.25 μM 30S subunit concentration in a polymix buffer (50 mM Tris-acetate (pH 7.5), 100 mM potassium chloride, 5 mM ammonium acetate, 0.5 mM calcium acetate, 5 mM magnesium acetate, 0.5 mM EDTA, 5 mM putrescine-HCl and 1 mM spermidine) as described by Uemura *et al.*[4]. Nucleotide concentration is at 4 mM for GTP, GDP, and GDPNP in all experiments.

3. 30S PIC immobilization

Biotinylated messenger RNAs were used to immobilize 30S PICs with or without fMet-$tRNA^{fMet}$. Complexes were tethered to the biotin-PEG-derivatized quartz surface on the bottom of ZMW wells through tetrameric neutravidin adaptor molecule by establishing PEG-biotin:neutravidin:biotinylated-mRNA complexes. The mRNA used contains the following in order from 5′ to 3′: a 5′-UTR and Shine-Dalgarno sequence derived from gene 32 of the T4 phage, an AUG start codon, 6 repeats of phe-lys codons, an UAA stop codon, and 4 spacer phe codons (Supplementary Figure 1). Immobilized PICs were identified by initiator tRNA or 30S-subunit fluorescence and were distributed in ZMW holes according to Poisson statistics[4]. A single photobleaching step for the scoring dye confirms the single-occupancy of the ZMW. Control experiments without mRNA demonstrated the absence of non-specific surface interactions at concentrations up to 1μM of labeled tRNAs, factors, or ribosomes. Thus, fluorescent events observed here represent true interactions of translation components with immobilized 30S subunits.

4. Observing the order of arrival of IF2 and initiator tRNA

We delivered a mixture of fMet-(Cy3)$tRNA^{fMet}$, Cy5-IF2(GTP), and Cy3.5-50S to immobilized Alexa488-30S at 20 nM, 200 nM, or 1000 nM of each reagent. When present, IF1 and IF3 are at 1 μM. The appearance of a stable 50S signal was used to identify productive tRNA and IF2 binding events. The relative timing of IF2 and $tRNA^{fMet}$ arrival to the ribosome was determined by the order that their respective signals appear in each trace.

Observations for all experiments were done at 30 frames per second with ~33.3 ms exposure. Therefore, simultaneous arrival of fluorescence signals can be either genuine simultaneous arrival events or the two events happening sequentially within the exposure time. From the tRNA and IF2 arrival rates observed in control experiments, we calculated the percentage of apparent simultaneous events that can be attributed to sequential events

happening in quick succession within 1 frame of exposure. We subtracted that part from the simultaneous events we observed and represented the subtracted portion as “instrument uncertainty,” or the gray portion on the bar graph in Figure 1c.

5. Observing the role of IF2 in subunit joining

We delivered 200 nM of Cy5-50S to immobilized Cy3-30S at different magnesium concentataions (2.5~10 mM) both with and without 1 μM IF2 in either GTP or GDPNP. Where present, IF1 and IF3 are at 1μM. The wait time until the appearance of the 50S signal and its lifetime was analyzed to determine the efficiency of subunit joining under the different conditions.

6. Observing the relative timing of IF2 departure and elongator tRNA arrival

We delivered 20~1000 nM Cy5-IF2, 200~1000 nM Cy3.5-50S and 200~1000 nM Phe-(Cy2)tRNA^{Phe} in a ternary complex with EF-Tu and GTP to 30S PIC loaded with fMet-(Cy3)tRNA^{fMet}, simultaneously tracking 4 different labeled components in either GTP or GDPNP. We determined the overlap times of the IF2 signal with the 50S signal and the relative timing of IF2 departure to the arrival of the elongator tRNA, as well as the frequency of tRNA binding on the 70S ribosome post subunit joining.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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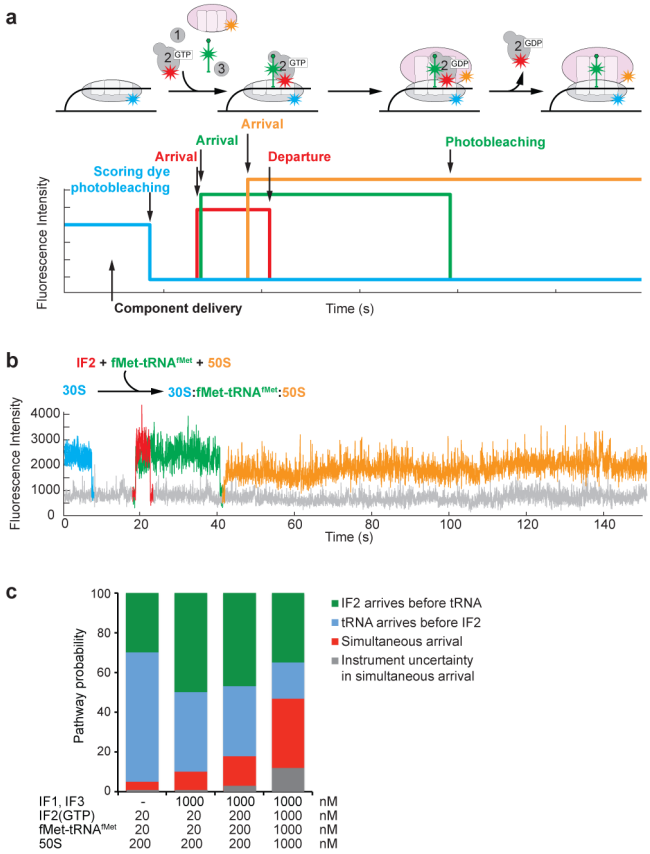

Figure 1. Pathways leading to 30S PIC formation

a. Single dye-labeled 30S complexes were immobilized on the bottom of zero-mode-waveguide (ZMW) wells and scored by fluorescence (Methods 3). Dye-labeled initiation factors, tRNAs, and 50S subunits were delivered at t = 7 s in all experiments. The appearance of fluorescence signals indicates arrival of the labeled molecules. Fluorescence signal disappears either due to dye-labeled molecule departure or photobleaching.

b. Productive initiation events were identified by stable 50S arrival (Methods 4). The order of arrival was determined by the sequence of the fluorescent pulses. Grey portions of the traces represent fluorescence background.

c. The ratios of possible 30S PIC formation pathways at different ligand concentrations were measured and plotted. See Methods 4 for an explanation of instrument uncertainty. From left to right, $n = 86$, $n = 51$, $n = 79$, and $n = 52$.

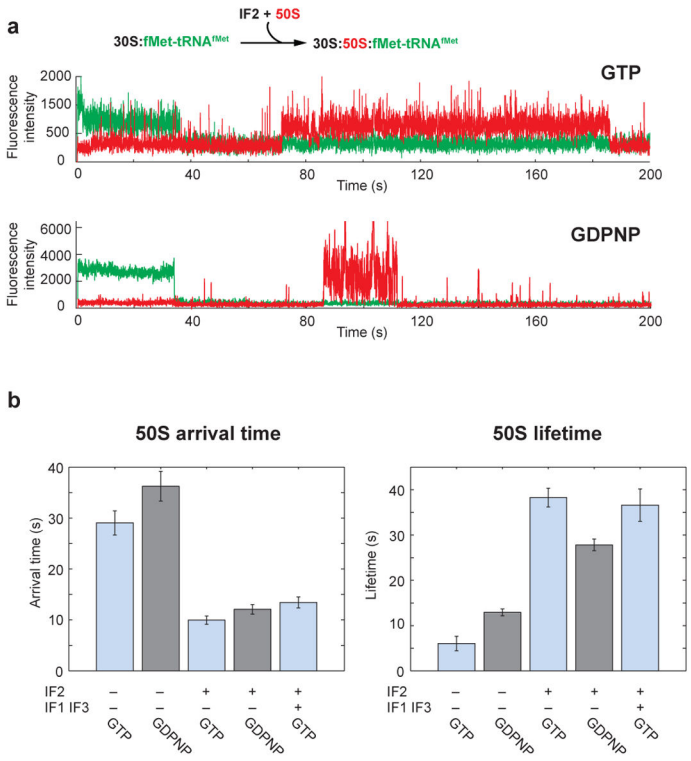

Figure 2. 50S subunit joining to 30S PIC

a. The appearance of a stable Cy5 signal is used to identify the arrival of the 50S subunit (Methods 5). The time until 50S arrival and the length of the 50S signal are then characterized.

b. The arrival times of a 50S subunit to and the observed 50S subunit lifetimes on a 30S PIC are fitted to single-exponential functions and plotted with s.d. error bars. The presence of IF2 is critical for efficiently and stably forming 70S complexes. From left to right for both panels, n = 246, n = 283, n = 451, n = 262, and n = 253.

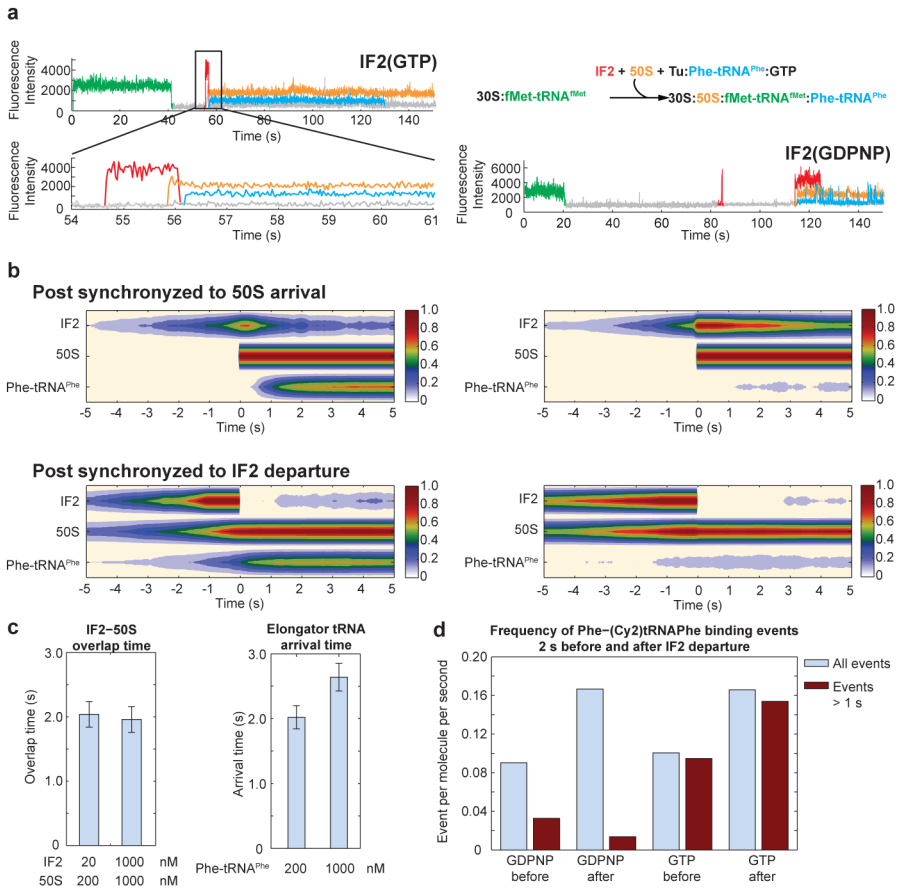

Figure 3. Timing of IF2 departure and elongator tRNA arrival after 70S complex formation

a. See Methods 6 for experimental setup. The timing of IF2 departure is determined by the disappearance of the Cy5 signal.

b. The panels represent postsynchronization plots on 50S subunit arrival and IF2 departure at 1 μM dye-labeled ligand concentrations. With GTP (n = 161), there is a ~2 s overlap between the IF2 and 50S subunit signals with a strong elongator tRNA density. With GDPNP (n = 87), the overlap between IF2 and 50S is longer (~10 s) but there is little elongator tRNA density.

c. The exponential lifetimes of the IF2-50S subunit overlap (left panel) in did not depend on IF2 or 50S subunit concentrations. Increasing the elongator tRNA concentration also did not reduce the wait time until tRNA arrival (right panel). From left to right for both panels, n = 169 and n = 161, error bars are s.d.

d. The event frequencies per molecule 2 s before and after IF2 departure are similar in both GTP (n = 161) and GDPNP (n = 87). Most events in GDPNP were removed by only counting events >1s. Most of the events in GTP were longer-lived tRNA binding events.

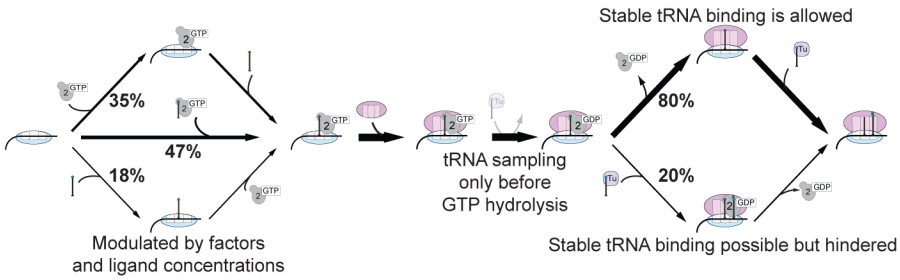

Figure 4. The heterogeneous pathways of translation initiation

Multiple pathways are possible to reach the important stages of initiation as the ribosome converges to an elongation-competent 70S IC. Concentrations of initiation factors, tRNAs, and ribosomal subunits all modulate the flux through the possible pathways that lead to successful initiation. Post 30S subunit binding to the mRNA, IF2 plays a central role in channeling the ribosome towards elongation. At physiological concentrations of initiation factors and tRNAs, the majority of 30S PICs may be formed by IF2 bringing in the initiator tRNA to the 30S. IF2 also guides rapid and stable 50S subunit joining, while GTP hydrolysis by IF2 and its departure from the ribosome gates the stable binding of the first elongator tRNA.