Conformational selection of translation initiation factor 3 signals proper substrate selection

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During translation, initiation factor 3 (IF3) binds to the small (30S) ribosomal subunit and regulates the fidelity with which the initiator tRNA and mRNA start codon substrates are selected into the 30S initiation complex (30S IC). The molecular mechanism through which IF3 promotes the recognition and signaling of correct substrate selection, however, remains poorly defined. Using single-molecule fluorescence resonance energy transfer, we show that 30S IC–bound Escherichia coli IF3 exists in a dynamic equilibrium between at least three conformations. We found that recognition of a proper anticodon-codon interaction between initiator tRNA and the start codon within a completely assembled 30S IC selectively shifts this equilibrium toward a single conformation of IF3. Our results strongly support a conformational selection model in which the conformation of IF3 that is selectively stabilized within a completely and correctly assembled 30S IC facilitates further progress along the initiation pathway.

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RESULTS

Development of a functional, dual fluorescently labeled IF3

Labeling of an IF3 mutant carrying one cysteine at its NTD and one cysteine at its CTD with Cy3 FRET donor and Cy5 FRET acceptor fluorophores and subsequent purification generated a dual Cy3-Cys5-labeled IF3 (IF3(Cys65S38C97C)(Cy3-Cys5), hereafter referred to as IF3(Cy3-Cys5)) (Fig. 1b and Supplementary Fig. 1) that retained near-wild type biochemical function (Supplementary Fig. 2). Using 5′-biotinylated mRNAs, we assembled 30S ICs carrying IF3(Cy3-Cys5), tethered them to the polyethylene glycol–passivated and streptavidin-derivatized surface of a quartz microfluidic flow cell and imaged them at single-molecule resolution using a total internal reflection fluorescence (TIRF) microscope operating at an acquisition rate of 10 frames s⁻¹ (ref. 12). Control experiments demonstrated that under our experimental conditions, 80–95% of the individual, surface-localized IF3(Cy3-Cys5)s were bound to the flow cell surface by their interaction with a 30S IC carrying a biotinylated mRNA (Supplementary Fig. 3). For further details regarding sample preparation, TIRF imaging, control experiments and data analyses, see the Supplementary Note.

30S IC–bound IF3 is conformationally dynamic

We designed our initial experiments to probe the conformation of IF3 bound to a 30S subunit in the absence of the other initiation factors and tRNA (30S IC−tRNA, where the −tRNA superscript and −1/−2 subscript denote the lack of a tRNA within the P site and the lack of IF1 and IF2, respectively). The resulting smFRET efficiency ($E_{\text{FRET}}$) versus time trajectories sampled three distinct FRET states centered at $E_{\text{FRET}}$ values of 0.23 ± 0.01, 0.42 ± 0.01 and 0.87 ± 0.01, with 29.9% ± 8.6% of the trajectories showing fluctuations between at least two FRET states before photobleaching and 70.1% ± 17.3% of the trajectories sampling only one of these FRET states before photobleaching (Fig. 2a and Supplementary Fig. 4). Assuming rapid, isotropic tumbling of one or both fluorophore transition dipoles and a Förster radius of ~55 Å for the Cy3-Cy5 FRET pair, we interpret the $E_{\text{FRET}}$ values of 0.23, 0.42 and 0.87 (Supplementary Table 1) as corresponding to interdomain distances of ~67 Å, ~58 Å and ~40 Å, respectively. Notably, this range of distances is consistent with the interdomain distances accessible to IF3 when free in solution (28–65 Å)⁷. Hereafter, we refer to the conformations of 30S IC–bound IF3 that are associated with each of these $E_{\text{FRET}}$ values and distances as extended (IF3ext (0.23 and ~67 Å)), intermediate (IF3int (0.42 and ~58 Å)) and compact (IF3cpt (0.87 and ~40 Å)). Thermodynamic and kinetic analyses of the trajectories revealed equilibrium fractional occupancies of 54% ± 9%, 40% ± 10% and 6% ± 2% for IF3ext, IF3int and IF3cpt, respectively (Supplementary Table 2), and estimated the rates of interconversions between the three IF3 conformations as ranging between 0.002 and 0.11 s⁻¹ (Supplementary Table 3). Interpreted within the context of previously reported in vitro subunit joining experiments demonstrating that 30S ICs analogous to 30S IC−tRNA⁻1/−2 are substantially inhibited in their ability to undergo subunit joining⁴, these data suggest that IF3ext and IF3int are conformations of IF3 that are not conducive to rapid 50S subunit joining.

It is unlikely that the interdomain dynamics of IF3 that we found here arise from a scenario in which one IF3 domain is tightly bound to the 30S IC and the other IF3 domain remains free in solution, undergoing restricted diffusion through the interdomain linker. Dynamic exchange between different interdomain conformations of IF3 involving such restricted diffusion of a free IF3 domain would be expected to occur with rates that are ~7–11 orders of magnitude faster than our estimated rates of interconversions between IF3ext, IF3int and IF3cpt.⁴ Instead, we propose a scenario in which both IF3 domains can bind to the 30S IC and exchange between different interdomain conformations as a result of (i) active repositioning of one or both IF3 domains among several binding sites on the 30S IC; (ii) passive changes in the distance between the two IF3 domains resulting from dynamic rearrangements of the 30S IC; or (iii) a combination of (i) and (ii). To test whether IF3 actively or passively participates in the dynamics, we constructed a model of translation initiation in bacteria and fluorescent labeling of IF3. (a) A minimal model of translation initiation. 30S IC refers to a completely and correctly assembled 30S ribosomal initiation complex, and 70S IC refers to a 70S ribosomal initiation complex harboring an initiator fMet–tRNAfMet bound to an AUG start codon at the P site. The initiation factors, mRNA and fMet–tRNAfMet reversibly bind to the 30S ribosomal initiation complex to form the 30S IC. Subsequent joining of the 50S subunit to the 30S IC triggers GTP hydrolysis by IF2 and, ultimately, dissociation of the three initiation factors, E, P and A. (b) X-ray crystal structures of the NTD (Protein Data Bank (PDB) ID 1TIF) and CTD (PDB ID 1TIG) of IF3 from Bacillus stearothermophilus. The linker connecting the NTD and CTD was cartooned by hand. The residues on the B. stearothermophilus structures corresponding to those in a C65S, S38C and K97C triple mutant of E. coli IF3 that was prepared and fluorescently labeled with Cy3 and Cy5 at Cys38 and Cys97 are indicated in the depicted structure.
IF1 and IF2 modulate the conformational equilibrium of IF3

Because IF1 amplifies and IF2 counteracts the tRNA-dissociation and subunit anti-association activities of IF3 during translation initiation\(^6\), we investigated the effects of IF1 and IF2 on the conformational dynamics of 30S IC-bound IF3 by assembling and imaging 30S ICs in the presence of IF1 (30S IC\(^{-\text{IF1}}\)), IF2 (30S IC\(^{-\text{IF2}}\)) or both IF1 and IF2 (30S IC\(^{-\text{IF1,IF2}}\)) (Fig. 2b). We imaged 30S ICs carrying IF1 and/or IF2 under saturating, 1 \(\mu\)M concentrations of each of these components. Relative to 30S IC\(^{-\text{IF1,IF2}}\), the presence of IF1 slightly shifted the conformational equilibrium of 30S IC\(^{-\text{IF2}}\) away from IF3\(_{\text{ext}}\) and IF3\(_{\text{cpt}}\) and toward IF3\(_{\text{int}}\), yielding fractional occupancies of 45\% ± 3\% (IF3\(_{\text{ext}}\)), 52\% ± 3\% (IF3\(_{\text{int}}\)) and 3\% ± 1\% (IF3\(_{\text{cpt}}\)) (Fig. 2b and Supplementary Table 2). In contrast, relative to 30S IC\(^{-\text{IF2}}\), the presence of IF2 markedly shifted the conformational equilibrium of 30S IC\(^{-\text{IF1}}\) toward IF3\(_{\text{ext}}\) and IF3\(_{\text{int}}\) and toward IF3\(_{\text{cpt}}\), yielding fractional occupancies of 23\% ± 17\% (IF3\(_{\text{ext}}\)), 11\% ± 4\% (IF3\(_{\text{int}}\)) and 66\% ± 17\% (IF3\(_{\text{cpt}}\)) (Fig. 2b and Supplementary Table 2). Notably, the effect of IF2 on the conformational equilibrium of 30S IC-bound IF3 was almost completely suppressed when IF1 was included together with IF2 in 30S IC\(^{-\text{IF1,IF2}}\) (Fig. 2b and Supplementary Table 2). Thus, in the most physiologically relevant scenario in which all three initiation factors are present on the 30S IC\(^{16}\), the conformational equilibrium of 30S IC\(^{-\text{IF1,IF2}}\)-bound IF3 favors IF3\(_{\text{ext}}\) and IF3\(_{\text{int}}\), almost exclusively, yielding fractional occupancies of 56\% ± 7\% (IF3\(_{\text{ext}}\)), 42\% ± 6\% (IF3\(_{\text{int}}\)) and 2\% ± 1\% (IF3\(_{\text{cpt}}\)) (Fig. 2b and Supplementary Table 2) and transition rates that were similar to those observed for 30S IC\(^{-\text{IF1,IF2}}\) (Supplementary Table 3).
fMet-tRNAfMet shifts the equilibrium toward IF3cpt

A completely and correctly assembled 30S IC that is primed for rapid 50S subunit docking contains an fMet-tRNAfMet that is correctly base paired to a start codon within the 30S IC P site. Driven by this, we assembled and imaged a 30S IC containing IF1, IF2, IF3(Cy3-Cy5) and fMet-tRNAfMet on an mRNA containing an AUG start codon (30S ICfMet) using saturating, 1 µM concentrations of IF1, IF2 and fMet-tRNAfMet. Notably, the average number of surface-tethered, 30S ICfMet–bound IF3(Cy3-Cy5) molecules per field of view did not vary appreciably relative to the number of surface-tethered, 30S IC–bound IF3(Cy3-Cy5) molecules in any of the other identically prepared and imaged 30S ICs we studied. Thus, the presence of an fMet-tRNAfMet that is correctly base paired to a start codon within a 30S IC shifts the conformational equilibrium of IF3 markedly away from IF3ext and IF3int toward IF3cpt (ref. 4), suggesting that IF3cpt is a conformation of IF3 that is conducive to rapid dissociation of IF3 from the 30S IC within the timescale of our experiments (~10 min). Instead we found that relative to 30S IC−tRNA, the presence of an fMet-tRNAfMet that is correctly base paired to an AUG start codon within 30S ICfMet shifts the conformational equilibrium of IF3 markedly away from IF3ext and IF3int and toward IF3cpt (Fig. 3), yielding fractional occupancies of 15% ± 13% (IF3ext), 17% ± 7% (IF3int) and 68% ± 17% (IF3cpt) (Supplementary Table 2). This equilibrium shift seems to be driven primarily by the destabilization of IF3ext and IF3int, as evidenced by the large increases in the estimated rates of IF3ext→IF3int and IF3int→IF3cpt transitions in 30S ICfMet relative to 30S IC−tRNA (Supplementary Table 3). On the basis of these results, we hypothesized that the observed shift in the conformational equilibrium of 30S IC−bound IF3 toward IF3cpt in 30S ICfMet relative to 30S IC−tRNA forms the molecular and structural basis for signaling fMet-tRNAfMet and start-codon selection within the 30S IC and the associated relaxation of the subunit anti-association activity of IF3. Consistent with this hypothesis, previously reported in vitro 50S subunit–joining experiments have shown that 30S ICs analogous to 30S ICfMet undergo rapid 50S subunit joining relative to 30S ICs analogous to 30S IC−tRNA (ref. 4), suggesting that IF3cpt is a conformation of IF3 that is conducive to rapid 50S subunit joining. In the context of this hypothesis, IF3ext and IF3int are not only conformations of IF3 that are not conducive to 50S subunit joining, as discussed above, but also conformations of IF3 that prevent IF3 from populating IF3cpt and consequently from undergoing rapid 50S subunit joining, until the 30S IC has properly selected an fMet-tRNAfMet and the start codon.

The shift toward IF3cpt requires fMet-tRNAfMet and an AUG codon

If the hypothesis outlined in the previous section is correct, then we would expect the shift in the conformational equilibrium of IF3 to depend on the identity of the tRNA and/or codon within the 30S IC P site, as signaling of proper substrate selection and the associated rapid 50S subunit joining occur only within a 30S IC that is specifically carrying an fMet-tRNAfMet that is properly base paired to a start codon. Thus, to further test our hypothesis, we assembled and imaged complete 30S ICs in which the identities of the tRNA and/or codon at the P site were varied. In line with our hypothesis, 30S ICs assembled and imaged using saturating, 1 µM concentrations of either Phe-tRNA Phe or Lys-tRNA Lys and an AUG start codon at the P site (30S IC Phe and 30S IC Lys, respectively) did not undergo the shift in conformational equilibrium of IF3 toward IF3cpt relative to 30S IC−tRNA that we observed for 30S ICfMet relative to 30S IC−tRNA (Fig. 3). Instead, the fractional occupancies of IF3ext, IF3int and IF3cpt within 30S IC Phe and 30S IC Lys were comparable to those observed within 30S IC−tRNA despite the presence of saturating concentrations of Phe-tRNA Phe or Lys-tRNA Lys (Fig. 3 and Supplementary Table 2). Comparison of 30S IC−tRNA, 30S ICfMet, 30S IC Phe and 30S IC Lys showed that the shift in the conformational equilibrium of IF3 toward IF3cpt depends not only on the presence of an aa-tRNA at the 30S IC P site but also on the identity of that aa-tRNA. Nevertheless, altering the identity of the aa-tRNA, but not the AUG start codon, caused the P sites of 30S IC Phe and 30S IC Lys to contain incorrectly selected elongator aa-tRNAs as well as mismatched GAA-AUG (in the case of 30S IC Phe) and UUU-AUG (in the case of 30S IC Lys) anticodon-codon interactions. Thus, the failure of 30S IC Phe and 30S IC Lys to undergo a shift in the conformational equilibrium of IF3 toward IF3cpt relative to 30S IC−tRNA could potentially be caused by the presence of the elongator aa-tRNA and/or the mismatched anticodon-codon interaction within the 30S IC P site.

To separate the effects that the identity of the aa-tRNA and the nature of the anticodon–codon base-pairing interactions have on the conformational equilibrium of IF3, we imaged completely assembled 30S ICs whose P sites contained either Phe-tRNA Phe at a UUC codon that is cognate for Phe-tRNA Phe (30S IC Phe UUC) or fMet-tRNAfMet at an AUU codon that is near cognate for fMet-tRNAfMet (30S ICfMet AUU) using saturating, 1 µM concentrations of Phe-tRNA Phe or fMet-tRNAfMet, respectively. Regardless of the Watson-Crick complementarity of the anticodon–codon interaction in 30S IC Phe UUC, IF3 showed thermodynamic and kinetic behavior that was comparable to that observed for IF3 within 30S IC−tRNA despite the presence of saturating concentrations of Phe-tRNA Phe (Fig. 3 and Supplementary Tables 2 and 3). Similarly, in the presence of a partially mismatched anticodon–codon interaction in 30S ICfMet AUU IF3 showed thermodynamic and kinetic behavior that was comparable to...
that of 30S IC–tRNA despite the presence of saturating concentrations of fMet-tRNA^{Met} (Fig. 3 and Supplementary Tables 2 and 3).

**DISCUSSION**

Taken together, the results we obtained with 30S IC{\textsuperscript{Phe}}, 30S IC{\textsuperscript{Lys}}, 30S IC{\textsuperscript{Phe,UUC}} and 30S IC{\textsuperscript{Cys,AUU}} suggest that relative to 30S IC–tRNA, the shift in the conformational equilibrium of IF3 toward IF3{\textsubscript{cpt}} is dependent on the specific presence of an fMet-tRNA{\textsuperscript{Met}} at the 30S IC P site, as well as proper base pairing between fMet-tRNA{\textsuperscript{Met}} and a start codon at the P site; these are precisely the conditions under which extensive in vitro biochemical experiments have shown that the subunit antiassociation activity of IF3 is relaxed and 50S subunit association to the 30S IC is accelerated{\textsuperscript{4}}. Specifically, rapid kinetic experiments have shown that within the context of a 30S IC containing all three initiation factors, the presence of an fMet-tRNA^{Met} and an AUG start codon increases the rate of 50S subunit joining by a factor of 1,200 relative to a 30S IC lacking an aa-tRNA at the P site{\textsuperscript{5}}, a factor of 400 relative to a 30S IC carrying a Phe-tRNA{\textsuperscript{Phe}} that is mismatched to an AUG start codon{\textsuperscript{5}} and a factor of 90 relative to a 30S IC carrying an fMet-tRNA^{Met} that is mismatched to a noncanonical start codon{\textsuperscript{2}}.

Integrating our current findings regarding the conformational dynamics of 30S IC–bound IF3 with the results of the biochemical studies described in the previous paragraph allows us to propose a structure-based mechanistic model for how IF3 recognizes and signals fMet-tRNA^{Met} and start-codon selection within the 30S IC (Fig. 5).

**Figure 4** Observation of an intermolecular IF3–50S subunit smFRET signal. The cartoon shows the 70S IC that is formed through stopped-flow delivery of a solution containing 50 nM of the Cy5-labeled 50S subunit (labeled at L9Q18C as previously described{\textsuperscript{19}} into a flow cell containing a surface-tethered 30S IC carrying IF3{\textsuperscript{C65S S38C} (Cy3)). Results in an intermolecular IF3–50S subunit smFRET signal that reports on 50S subunit joining and the formation of a 70S IC. (a) A representative example of single-molecule Cy3 (green line) and Cy5 (red line) intensity versus time trajectories (top) and an E{\textsubscript{FRET}} versus time trajectory (bottom) for the 70S IC. AU, arbitrary units. (b) A normalized, one-dimensional E{\textsubscript{FRET}} histogram constructed using the full 120 s of 25 E{\textsubscript{FRET}} versus time trajectories. The FRET state centered at an E{\textsubscript{FRET}} value of ~0.45 arises from time points in the E{\textsubscript{FRET}} versus time trajectories during which the Cy5-labeled 50S subunit has joined to a 30S IC carrying the Cy3-labeled IF3. The FRET state centered at an E{\textsubscript{FRET}} value of 0 arises from time points in the E{\textsubscript{FRET}} versus time trajectories before joining of the Cy5-labeled 50S subunit to the 30S IC carrying the Cy3-labeled IF3 and from time points after photobleaching of Cy5 on the Cy5-labeled 50S subunits.

**Figure 5** A structure-based mechanistic model for how IF3 recognizes and signals fMet-tRNA^{Met} and start-codon selection within the 30S IC. 30S IC–bound IF3 exists in a conformational equilibrium in which it can dynamically exchange between at least three distinct conformational states, IF3{\textsubscript{ext}}, IF3{\textsubscript{int}} and IF3{\textsubscript{cpt}}. Specific recognition of an fMet-tRNA^{Met} that is properly base paired to a start codon within the P site of a 30S IC carrying all three initiation factors shifts the conformational equilibrium of IF3 strongly toward IF3{\textsubscript{ext}}, a conformation of 30S IC–bound IF3 that exposes and/or optimally positions ribosomal RNA and/or ribosomal protein residues on the 30S IC that are crucial for intersubunit bridge formation and is thus conducive to rapid and productive 50S subunit joining (top). In contrast, the absence of a aa-tRNA, the presence of an elongator aa-tRNA or the presence of a noncanonical start codon within the P site of a 30S IC carrying all three initiation factors shifts the conformational equilibrium of IF3 strongly toward IF3{\textsubscript{ext}} and IF3{\textsubscript{int}}, conformations of 30S IC–bound IF3 that occlude and/or misorient residues involved in intersubunit bridge formation and are thus not conducive to efficient 50S subunit joining (bottom).
fMet-tRNA\textsuperscript{Met} that is mismatched to a noncanonical start codon within the 30S IC P site, the conformational equilibrium of IF3 is strongly shifted toward IF3\textsubscript{ext} and IF3\textsubscript{int}, which are conformations of IF3 that are not conducive to rapid 50S subunit joining. Specific recognition of fMet-tRNA\textsuperscript{Met} that is properly base paired to a start codon within the P site of a 30S IC carrying all three initiation factors, in contrast, strongly shifts the conformational equilibrium of IF3 toward IF3\textsubscript{cpt}, which is a conformation of IF3 that signals proper substrate selection and is conducive to rapid 50S subunit joining. It is interesting that IF3 predominantly occupies IF3\textsubscript{cpt} in both the completely and correctly assembled 30S IC (30S IC\textsuperscript{Met}) as well as the incompletely assembled 30S IC lacking IF1 and fMet-tRNA\textsuperscript{Met} (30S IC\textsuperscript{tRNA}) (Figs. 2b and 3). Thus, in the absence of IF1 and fMet-tRNA\textsuperscript{Met}, the presence of IF2 on the 30S IC can shift the conformational equilibrium of IF3 toward IF3\textsubscript{cpt}. This is notable in light of rapid kinetic data indicating that 50S subunit joining to a 30S IC that is analogous to 30S IC\textsuperscript{tRNA} is 145-fold slower than joining to a 30S IC that is analogous to 30S IC\textsuperscript{Met} (ref. 3). Thus, although our model stipulates that IF3\textsubscript{cpt} is conducive to and permits rapid 50S subunit joining, it is probable that additional factors, such as the presence of IF2 and fMet-tRNA\textsuperscript{Met} on the 30S IC\textsuperscript{2,3}, are required to actualize rapid 50S subunit joining. Furthermore, the finding that IF3\textsubscript{cpt} is rarely sampled within 30S IC\textsuperscript{tRNA} suggests that IF1 has a key role in negatively regulating the conformational dynamics of IF3 such that 30S IC–bound IF3 does not substantially populate IF3\textsubscript{cpt} in the absence of fMet-tRNA\textsuperscript{Met} that is correctly base paired to a start codon.

Although we will have to wait until X-ray crystallographic structures of the 30S IC are solved to confirm our suspicions, we suspect that IF3\textsubscript{cpt} is a conformation of 30S IC–bound IF3 that exposes and/or optimally positions ribosomal RNA and/or ribosomal protein residues on the 30S IC that are crucial for intersubunit bridge formation, thus enabling rapid and productive 50S subunit joining, whereas IF3\textsubscript{ext} and IF3\textsubscript{int} are conformations of 30S IC–bound IF3 that occlude and/or misorient these residues, thereby blocking 50S subunit joining. In contrast with models in which a selective increase in the rate of spontaneous dissociation of IF3 from the 30S IC is required for productive 50S subunit joining\textsuperscript{3}, the model presented here predicts that efficient 50S subunit joining can occur on a completely and correctly assembled 30S IC that contains IF3. Indeed, preliminary smFRET data collected using Cy3-labeled IF3 and Cy5-labeled 50S subunits (labeled at ribosomal protein L9) revealed that the 50S subunit can rapidly and productively join to a completely and correctly assembled 30S IC containing IF3 that is presumably in the IF3\textsubscript{cpt} conformation (30S IC\textsuperscript{Met}) (Fig. 4). Nevertheless, our model (Fig. 5) and intermolecular 50S–IF3 smFRET data do not exclude the possibility that IF3 in the IF3\textsubscript{cpt} conformation is more weakly bound to the 30S IC than is IF3 in the IF3\textsubscript{ext} and IF3\textsubscript{int} conformations, such that IF3 in the IF3\textsubscript{cpt} conformation is easily displaced from the 30S IC during or shortly after productive 50S subunit joining. Indeed, kinetic measurements\textsuperscript{5,18} have suggested that 50S subunit joining to the 30S IC is slightly faster than the rate of IF3 dissociation from the 30S IC. Given recent studies demonstrating that differences in the translation initiation region of individual mRNAs, such as the sequence of the Shine-Dalgarno element and the length of the spacer between the Shine-Dalgarno element and the start codon, can influence the rate of 50S subunit joining to 30S ICs assembled on different mRNAs\textsuperscript{5,17}, it is possible that translation initiation region–mediated regulation of the conformational equilibrium of 30S IC–bound IF3 will be an effective mechanism for regulating the efficiency with which individual mRNAs are initiated and translated in the cell.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
M.M.E. and R.L.G. contributed to the experimental design, data interpretation and manuscript writing. M.M.E. performed the experiments and carried out data analyses.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Sample preparation. E. coli ribosomes and translation factors were purified as previously described20. 5′-biotinylated mRNA with a sequence derived from the mRNA encoding gene product 32 from T4 bacteriophage was purchased from Thermofisher, Inc. See the Supplementary Note for details on the sequence of this mRNA. tRNA_Amt was purchased from MP Biomedicals, and tRNA_Phe and tRNA_Asn were purchased from Sigma. All tRNAs were aminoaacetylated and, in the case of tRNA_Asn, formylated as previously described20.

The gene encoding E. coli IF3 was cloned into the pProEx-HTb plasmid vector (Invitrogen), which encodes a hexahistidine (His6) affinity purification tag and a TEV protease cleavage site at the N-terminal end of the gene encoding IF3. Mutagenesis of IF3 in the pProEx-HTb plasmid vector was performed using the QuikChange Site-Directed Mutagenesis System (Stratagene). DNA primers for mutagenesis were designed following the recommendations provided by the QuikChange Site-Directed Mutagenesis System and were purchased from Integrated DNA Technologies. No further purification of the DNA primers was performed. Using this approach, the single Cys65 in wild-type IF3 was mutagenized to serine, Ser38 in the NTD was mutagenized to cysteine, and Lys97 in the CTD was mutagenized to cysteine, yielding a triple-mutant IF3 variant (IF3_C65S_S38C_K97C). Note that the residue numbering for IF3 used in this study is based on wild-type E. coli IF3 numbering. Mutations were verified by DNA sequencing of the plasmid purified from an ampicillin-resistant clone (Genewiz).

The pProEx-HTb plasmids encoding all of the IF3 variants used in this study were transformed into BL21-DE3 cells for protein overexpression, and the overexpressed His6-tagged IF3 variants were purified using Ni2+-nitrilotriacetic acid affinity purification, treated with TEV protease to remove the His6 tags and further purified using cation exchange chromatography. Further details regarding the cloning, overexpression and purification of the IF3 variants used in this study can be found in ref. 20. The N terminus of the IF3 variants used in this study consists of a GlyAlaMetAlaLys2 sequence, where GlyAlaMetAla denotes four non–wild type amino acids resulting from the cloning strategy, and Lys2 denotes the beginning of the wild-type E. coli IF3 sequence.

IF3_C65S_S38C_K97C was labeled with Cy3- and Cy5-maleimide (GE Healthcare) following the manufacturer’s recommendations. See the Supplementary Note for further details.

The unlabeled, monolabeled and dual-labeled IF3_C65S_S38C_K97C products were separated using a TSKgel Phenyl-SPW hydrophobic interaction chromatography (HIC) column ( Tosoh Biosciences) that had been pre-equilibrated with HIC Buffer A (Supplementary Table 4). A 0–100% linear gradient of HIC Buffer B applied over 20 column volumes enabled separation of the various unlabeled and labeled IF3 species (Supplementary Fig. 1). The biochemical activities of mutagenized and fluorescently labeled IF3_C65S_S38C_K97C (IF3(Cy3-Cy5)) and IF3_C65S_S38C_K97C_V75S (IF3(Cy3-Cy5)) were tested using a primer extension inhibition, or toeprinting, assay and a TIRF microscope–based RNA dissociation assay (Supplementary Fig. 2).

30S ICs for smFRET studies were prepared by incubating 1.8 μM 5′-biotinylated mRNA, 0.9 μM IF1, 0.9 μM IF2, 0.9 μM tRNA, 0.6 μM 30S subunits and 0.6 μM IF3(Cy3-Cy5) at 37 °C for 10 min in Tris-Polymyx buffer (Supplementary Table 4). 30S ICs were then aliquoted, flash frozen in liquid nitrogen and stored at −80 °C until further use.

TIRF microscopy. 30S ICs for imaging by TIRF microscopy were thawed, diluted to ∼200 pM in Tris-Polymyx buffer (Supplementary Table 4), introduced into a microfluidic flow cell that had been passivated with a mixture of polyethylene glycol and biotinylated PEG and derivatized with streptavidin as previously described21, and incubated at room temperature for 5 min. 30S ICs that did not tether to the surface of the flow cell at the end of the 5-min incubation were removed by flushing the flow cell with Tris-Polymyx buffer containing an enzymatic oxygen scavenger system, a triplet-state quencher cocktail and, as specified in the individual experiments, mixtures of initiation factors and aa-tRNAs (Supplementary Table 4).

A previously described, laboratory-built, wide-field, prism-based TIRF microscope22 was used to image the flow cells containing the surface-tethered 30S ICs. Briefly, a diode-pumped, solid-state, 532-nm laser (CrystaLaser) operating at a power of 18 mW (measured just before striking the prism) was used to directly excite Cy3, and a diode-pumped, solid-state, 643-nm laser (CrystaLaser) operating at a power of 18 mW (measured just before striking the prism) was used to directly excite Cy5. Fluorescence emissions from Cy3 and Cy5 were collected through a high numerical aperture objective (Nikon), wavelength separated into individual Cy3 and Cy5 fields of view using a Dual-View simultaneous imaging system (Photometrics, Inc.) and simultaneously imaged using the two halves of a back-thinned, 512 pixel × 512 pixel electron-multiplying charged-coupled device (EMCCD) camera (Cascade II 512-B, Photometrics, Inc.) operating with 2 pixel × 2 pixel binning and a frame rate of 10 frames s−1.

Within a 60 μm × 120 μm field of view, 200–400 spatially well separated 30S ICs were imaged. Direct excitation of Cy5 using the 643-nm laser during the first frame of each movie was used to record the spatial location of each Cy5 fluorophore in the field of view. The 643-nm laser was subsequently switched off and the 532-nm laser was switched on simultaneously to directly excite Cy3 and perform smFRET imaging starting with the second frame of each movie. Imaging continued until >95% of the Cy3 fluorophores had photobleached. Three independent data sets consisting of 12–15 movies each were collected on separate days using independently prepared samples and microfluidic devices for each 305 IC.

smFRET data analysis. Generation and selection of single-molecule E_FRET versus time trajectories from each movie were performed as previously described19,21,22, Briefly, the first frame of each movie, which was collected using direct excitation of Cy5 with a 643-nm laser, was used to identify single, diffraction-limited Cy5 spots. The locations of these spots were transferred to the Cy3 field of view to align the Cy5 field of view with the subsequent 532 nm–directly excited Cy3 field of view. The aligned Cy3 and Cy5 fields of view were used to identify pairs of Cy3 and Cy5 spots corresponding to single, surface-tethered 30S ICs carrying dual Cy3-Cy5–labeled IF3s, and Metorphor (Molecular Devices), Excel (Microsoft), Origin (OriginLab Corporation) and Matlab (The MathWorks) were used to plot Cy3 and Cy5 intensity versus time trajectories for each IF3. Trajectories with (i) time-averaged Cy3 and Cy5 intensity values characteristic of single Cy3 and Cy5 fluorophores, respectively, as determined by visual inspection; (ii) single-step photobleaching of Cy3 and/or Cy5 fluorophores, as determined by visual inspection; (iii) anticorrelated changes in Cy3 and Cy5 intensities, as determined by visual inspection; and (iv) Cy5 fluorescence lasting longer than 1 s before photobleaching, as determined by visual inspection, were kept for further analysis (see Fig. 2a for representative Cy3 and Cy5 versus time trajectories). In addition to these selection criteria, trajectories in which FRET could not be confirmed because of the simultaneous, single-step drop of both Cy3 and Cy5 intensities to baseline before undergoing an anticorrelated change in Cy3 and Cy5 intensities (<10% of the total number of trajectories per independently collected data set) were omitted from further analysis. Each of the Cy3 and Cy5 versus time trajectories selected for further analysis was baseline corrected by subtracting the average EMCCD readout over the last ten Cy3 time points (that is, after photobleaching of the Cy3 fluorophore) from each Cy3 time point and subtracting the average EMCCD readout over the last ten Cy5 time points (that is, after photobleaching of the Cy5 fluorophore) from each Cy5 time point. In addition, each Cy5 time point was corrected for bleed through of Cy3 intensity into the Cy5 field of view, which arises from the imperfect performance of emission filters, by subtracting 7% of the total Cy3 intensity (the experimentally determined average amount of Cy3 intensity that bleeds through into the Cy5 field of view in our TIRF microscope system) at each time point from the Cy5 intensity at the same time point. Each pair of baseline-corrected and bleed through–corrected Cy3 and Cy5 versus time trajectories was converted to a single, raw E_FRET versus time trajectory using the equation E_FRET = I_Cy5 / (I_Cy3 + I_Cy5), where E_FRET is the FRET efficiency at each time point and I_Cy3 and I_Cy5 are the baseline-corrected and bleed through–corrected Cy3 and Cy5 intensities at each time point, respectively. The raw E_FRET versus time trajectories were idealized by hidden Markov modeling using the vbFRET software package24 and further analyzed as described in Figure 2 and Supplementary Tables 1–3. All data are presented as the mean ± s.d.
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