Title: The complete, functional and dynamic cycle of the bacterial Initiation Factor 3

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Abstract

Initiation factor 3 (IF3) is an essential protein that enhances the fidelity and speed of bacterial initiation of mRNA translation. The dynamic interplay between the two independent IF3 domains, their alternative binding sites, and the mechanism that ensures translation initiation fidelity remains elusive. Here, we show that the functional positioning of IF3 domains occurs at velocities ranging over two orders of magnitude, driven by each 30S initiation ligand. IF1 and IF2 rapidly promote the accommodation of IF3 on the 30S platform with the C-terminal domain moving towards the P site. Reversion of this movement is triggered by decoding the mRNA start codon and rate limits translation initiation. Binding of the tRNA results in the concomitant accommodation of the N-terminal domain of IF3, largely dependent on the mRNA and initiator tRNA. 70S initiation complex formation promotes the closing and dissociation of IF3, recycling the factor for a new round of translation initiation. Altogether our results unveil the kinetic spectrum of IF3 conformations and highlight fundamental movements of the factor that ensure accurate translation initiation.

Introduction

IF3 is an essential bacterial protein, consisting of two domains (IF3C and IF3N) separated by a hydrophilic, lysine-rich, linker [1-3]. IF3 is involved in all steps of the bacterial translation initiation driving the 30S ribosomal subunit through the transition from the 30S initiation complex (30S IC) to a productive 70S initiation complex (70S IC) [4-6]. When bound to the 30S subunit, IF3 prevents the premature 50S subunit association and increases the rate of the P site codon-anticodon interaction between fMet-tRNA^{fMet} and the initiation triplet of the mRNA [5-8]. As a direct consequence, IF3 acts as an initiation fidelity factor by increasing the dissociation rate of non-canonical and pseudo-30S initiation complexes [8-10].

Although IF3 functions during the initiation phase of protein synthesis are well established, dynamic aspects of IF3 binding on ribosomal subunits and the release of the factor after the formation of productive 30S IC are still debated [11,12]. A model where IF3C and IF3N domains independently bind the 30S ribosomal subunit was initially proposed in the early '80s [13] and then confirmed by NMR analysis and time-resolved chemical probing experiments [14,15]. Several studies have dealt with the assignment of a topographical localization of IF3 on the 30S ribosomal subunit, producing conflicting conclusions [16-19]. Recently, CryoEM experiments demonstrated that IF3 undergoes large conformational changes to facilitate the accommodation of the initiator tRNA (fMet-tRNA^{fMet}) into the P site for start codon recognition [20]. These experiments, together with other recent structural reconstructions, contributed to the identification of a minimum of four different IF3 arrangements on the 30S ribosomal subunit and two alternative binding sites for each domain of the factor [20-22]. IF3C can interact with two distinct binding sites on the 30S subunit, either at the P site and in contact with IF1 (C2 position in Fig 1), or at helix 45 and helix 24, away from both IF1 and the P site (C1 position in Fig 1). Similarly, IF3N can

occupy two binding sites, either on the 30S platform near S11, or on the elbow of tRNA^{fMet}, herein called N_R and N_T, respectively. Despite these detailed structural data, a dynamic correlation between IF3 interaction sites, the ligands present on the 30S subunit and the functions of the factor remained elusive.

In this study, we use a combination of Förster Resonance Energy Transfer (FRET), pre-steady state kinetics techniques and molecular modeling to monitor IF3 movements on the ribosome and to construct a comprehensive model depicting the dynamics of IF3 domains along the full cycle of translation initiation, from the initial binding until the recycling of the factor (Fig 1).

Results and Discussion

To monitor the conformational rearrangements of IF3, we used a fluorescent derivative of the factor harboring donor and silent acceptor dyes specifically linked to the N and C terminal domains, respectively (Fig1, inset) [23]. Double-labeled IF3 (IF3_{DL}) allows to monitor intramolecular FRET changes resulting from the movement of IF3 domains with respect to each other. Donor fluorescence values were used to measure FRET changes of IF3_{DL} in a stopped-flow apparatus, allowing to monitor IF3 movements in real-time. An increase in time of donor fluorescence indicates that IF3 domains are moving apart and, *vice versa*, a decrease of the observed fluorescence indicates that the domains are getting close to each other (Supp. Fig 1) [23]. Binding of IF3_{DL} to the 30S ribosomal subunit resulted in an increase of fluorescence, consistent with the factor transiting from a closed conformation in solution to a more opened one while bound to the 30S subunit (Fig 2A). A biphasic dependence over time was observed with both apparent rates saturating with 30S concentration (Fig 2B, Supp. Fig 2A). Kinetic analysis of the 30S concentration dependence indicated that after the initial binding ($10^3 \mu\text{M}^{-1} \text{s}^{-1}$, [4]), IF3 stretches at an average velocity of 60s^{-1} . Our data is in agreement with time resolved chemical probing

100 and NMR spectroscopy analysis showing that IF3C and IF3N sequentially interact with the
101 30S ribosome [14,15].

102 To position each domain of IF3_{DL} on the 30S subunit, we modeled the dyes on each IF3
103 domain at all possible binding combinations from recent 3D reconstructions [20] (Supp. Fig
104 3 and 4). The resulting accessible volumes for the dyes allowed to calculate the theoretical
105 distances between fluorophores and domains (Supp. Fig 3). The structural modeling of
106 IF3_{DL} combined with FRET changes as a function of 30S concentration suggests that in
107 the absence of any other ligand, IF3 acquires the most extended layout with IF3C
108 occupying the C1 site and IF3N moving into the N_R site with an interdomain distance of
109 about 60 Å (Fig 1, step 1, Supp. Table 1). This IF3 layout is affected by all ligands of the
110 30S subunit; however, none promotes an IF3 opening as extended as that in the 30S–
111 IF3_{DL} complex (see below).

112 Binding of IF1 to the 30S–IF3_{DL} complex resulted in a decrease of fluorescence over time,
113 indicating that IF3 domains transit from an extended to a more compact layout on the 30S
114 subunit (Fig 2C) in agreement with single molecule analysis [24]. Analysis of FRET time
115 traces obtained at increasing concentrations of IF1 indicated that IF3_{DL} closes at an
116 average speed of 0.8 s⁻¹ (Fig 2D, Supp. Fig 2C). Structural modeling of IF3_{DL} on the 30S
117 subunit suggests that IF1 enhances the movement of IF3C from the C1 to the C2 binding
118 site (Fig 1, step 3), partially accounting for the observed reduction of interdomain
119 distances. The extent of fluorescence reduction suggests that also IF3N is displaced
120 towards IF3C. Binding of IF2 also promoted a closure of IF3 at over 10-fold faster velocity
121 (10 s⁻¹) than those observed for IF1 (Fig 1, step 2, Fig 2F); however, with a different
122 amplitude dependencies on the concentration of the factor (Fig 2C, Supp. Fig 2B). IF1 and
123 IF2 cooperatively maximized the extent of IF3 closing (Fig 2E). Altogether, our data
124 indicates that the intermediate 30S–IFs complex promotes an IF3 layout where IF3N

125 dissociates from the N_R binding site and IF3C moves towards the C2, near the P site.

126 While occupation of the C2 binding site would interfere with the formation of inter-subunit

127 bridges (reviewed in [25]), positioning of IF3N would contribute to shape the tRNA^{fMet}

128 binding site.

129 Thus, the 30S–IFs complex assembles in ≈ 30 ms [4] and rearranges in ≈ 1 s (this work).

130 This intermediate of translation initiation is characterized by a high affinity of IF3, and

131 consequently, it prevents the binding of the 50S subunit [4] [26]. On the other hand, when

132 a canonical start codon of the mRNA is present in the P site, the initial interaction of fMet-

133 tRNA^{fMet} with the 30S complex is followed by codon-anticodon formation. The resulting

134 30S IC, in comparison to the pre-IC, displays an increased dissociation rate constant (k_{off})

135 of IF3 from the 30S subunit [4,5]. To probe how IF3 interdomain dynamics relate to the

136 differential off rates of the factor, we first measured fMet-tRNA^{fMet} binding to 30S

137 complexes containing IFs but lacking the mRNA. The resulting fluorescence traces

138 increased with time, indicating a re-accommodation of either (or both) IF3 domains,

139 moving away from each other (Fig 3A). Kinetic analysis obtained at increasing

140 concentrations of fMet-tRNA^{fMet} showed a linear dependence of velocities with tRNA

141 concentrations, consistent with IF3_{DL} monitoring the initial interaction of the fMet-tRNA^{fMet}

142 with 30S pre-IC lacking the mRNA (Fig 3B). Our results indicate that the fMet-tRNA^{fMet}

143 rapidly binds to the 30S complex, $k_1 = 17 \mu\text{M}^{-1} \text{s}^{-1}$, and can readily dissociate, $k_{-1} = 1 \text{s}^{-1}$.

144 Both kinetic parameters are in agreement with previous studies where different FRET pairs

145 were used to monitor fMet-tRNA^{fMet} binding to 30S pre-ICs [27]. Thus, the initial interaction

146 of fMet-tRNA^{fMet} entails the partial opening of IF3, probably as a consequence of

147 displacing IF3N rather than IF3C. Altogether, the formation of the 30S pre-IC lacking the

148 mRNA entails an IF3 layout where IF3C is positioned at the C2 site and IF3N is pushed by

149 the initiator tRNA towards the N_R site. The reaction is rapid and under our experimental

150 conditions ($1\ \mu\text{M}$ of fMet-tRNA^{fMet}) IF3 accommodation during 30S pre-IC formation shall
151 take less than 50 ms.

152 The extent of IF3_{DL} signal as a function of fMet-tRNA^{fMet} recruitment depended on the
153 presence of the mRNA. As indicated in Fig 3A, mRNA-programmed 30S complexes
154 produced larger amplitudes of IF3_{DL} opening if compared with complexes without mRNA.
155 In addition, the fluorescence trace appeared biphasic in time, indicating a further
156 accommodation of IF3_{DL} following the initial binding of fMet-tRNA^{fMet} (Fig 1, step 4, Fig
157 3A). Kinetic analysis of time courses obtained at increasing concentrations of fMet-
158 tRNA^{fMet} showed that the velocities of the fMet-tRNA^{fMet} accommodation step in 30S pre-
159 ICs containing the mRNA tended to saturate at $4\ \text{s}^{-1}$ (Fig 3C, Supp. Fig 2D). These
160 experimental data, coupled with the structural modeling, support a model where IF3
161 accommodates the fMet-tRNA^{fMet} on the P site, most likely through IF3N evaluating
162 intermediates of initiator tRNA and IF3C moving back towards the C1 position (Fig 1, steps
163 4,5). Both reactions push IF3 domains away from each other.

164 However, the complexity of the above reactions precluded us to obtain accurate
165 information on the kinetics of mRNA start site decoding. To overcome this limitation, we
166 set up an experiment where 30S pre-ICs were pre-bound to fMet-tRNA^{fMet} and mixed with
167 increasing concentrations of mRNA. Mixing of the mRNA with 30S pre-ICs resulted in an
168 increase of IF3_{DL} fluorescence, indicating an increase of distance between IF3 domains
169 with velocities saturating at $0.3\ \text{s}^{-1}$ (Fig 3D,E, Supp. Fig 2E). The increase of fluorescence
170 observed in this experimental setup is likely the result of the displacement of IF3C from C2
171 towards the C1 binding site (Fig 1, step 5) rather than repositioning IF3N, which is affected
172 by the presence of the pre-bound initiator tRNA (see above). Accordingly, structural
173 modeling of IF3_{DL} on 30S IC structures indicate that the IF3C displacement accounts for
174 $10\ \text{\AA}$ change of distance between the fluorophores on IF3 domains (Supp. Fig 3 and 4). A

175 comparison of the velocities for all IF3 movements suggests that the displacement of IF3C
176 towards the C1 binding site is the slowest, likely occurring during decoding of the mRNA
177 start site. Thus, our results show that the IF3C displacement is the slowest step, rate-
178 limiting the progression of 30S IC formation and taking about 3 s.

179 The progression towards 30S IC formation entails an isomerization or locking step
180 between the 30S pre-IC and 30S IC (reviewed in [28]). Both complexes are identical in
181 terms of bound ligands; yet, they differ in their biochemical properties. While the off rates
182 of IF1, IF2, mRNA, and initiator tRNA are greatly reduced in the locked 30S IC, that of IF3
183 is increased [4]. Thus, the alternative binding sites of IF3C can be associated to the
184 differential IF3 off rates observed in the unlocked and locked 30S complexes. While the C1
185 binding site would represent fast off rates, the C2 can be responsible of the slow
186 dissociation rates for IF3. The C1 binding site can be proposed as both, the entry and exit
187 position for IF3C, while the C2 site can be proposed as the active site for IF3C.

188 Joining of the 50S to 30S ICs results in an unstable 70S pre-IC which upon release of IFs
189 allows the formation of a 70S IC capable of translating the in-frame mRNA [5,11]. The
190 reaction appears to be sequential [11] and to be tightly regulated by IF3 [5,26,29]. 50S
191 joining to 30S ICs with IF3_{DL} resulted in a fluorescence decrease over time, indicating a
192 closure of the factor upon dissociation (Fig 1, step 6, Fig 4A). Kinetic analysis of time
193 courses at increasing concentrations of 50S suggests that the overall rate of dissociation
194 and subsequent closure of IF3_{DL} occurs at 4 s⁻¹, a rate very similar to previous reports
195 where IF3 dissociation was measured with FRET between the factor and fMet-tRNA^{fMet}
196 (Fig 4B, Supp. Fig 2F) [5,11].

197 Previous reports showed that the efficiency and velocity of 70S IC formation (i.e. subunit
198 joining, GTP hydrolysis, Pi release, and IFs dissociation) strictly depended on the tRNA
199 and programmed mRNA [5,6,11,26,30]. When we measured the concomitant arrival of

200 fMet-tRNA^{fMet} and 50S to 30S pre-ICs with and without mRNA, we could observe a time
 201 dependence of IF3_{DL} fluorescence change indicating the opening of the factor due to
 202 initiator tRNA binding (increase from 0.5 to 1.0) and the following closing due to IF3
 203 ejection from the resulting 70S IC (decrease from 1.0 to 0.0) (Fig 4C). The rate of the latter
 204 was very similar to that monitored upon mRNA binding to 30S pre-ICs, indicating that
 205 decoding of the start codon and the subsequent movement of IF3C to the C1 site, rate
 206 limits 70S IC formation. In the absence of the mRNA, IF3_{DL} in the 30S pre-IC still sensed
 207 the arrival of fMet-tRNA^{fMet}; however, IF3 dissociation was missing and the factor
 208 appeared to adopt its initial layout (Fig 4C). Whether initiator tRNA stays bound or
 209 dissociates from these complexes, remains an open question.

210 Our intramolecular FRET approach to measure IF3 interdomain layouts during 30S IC
 211 formation and comparison with theoretical distance changes between fluorophores
 212 revealed a timely model for the functional IF3 cycle: from binding to dissociation (Fig 5).
 213 The combination of both approaches indicates subsequent movements of IF3 domains, in
 214 response to the recruitment of each 30S ligand and within a detailed temporal framework.
 215 While IF1 and IF2 cooperatively allow IF3C sensing start codon decoding, movements of
 216 IF3N appear to be coupled to fMet-RNA^{fMet} binding and subsequent accommodation. Our
 217 study reveals the kinetics associated to each movement of the factor with remarkable
 218 insights. First, IF3C moves to the C2 site in 0.1 - 1 s, induced by IF1 and IF2 recruitment,
 219 (Fig 1, Steps 2,3) likely responsible for preventing the premature joining of the 50S
 220 subunit. Second, IF3N dissociates from the 30S platform to interact with fMet-RNA^{fMet}, with
 221 a subsequent tRNA accommodation towards the P site (Fig 1, Step 4,5). Third, decoding
 222 of the mRNA start site by the P-site initiator tRNA displaces IF3C towards the C1 site. This
 223 event, which is a rate-limiting step for the formation of the 30S IC (≈ 3 s) allows joining of
 224 the 50S subunit (Fig 1, Step 5). Finally, IF3 dissociates during 70S IC formation in less

225 than 250 ms and the factor is now ready to bind a newly available 30S subunit (Fig 1, Step
226 6, Fig 5).

227 A model where each IF3 domain moves independently can fully accommodate our current
228 understanding of the dynamics of the factor, on and off the ribosome (movie in
229 supplementary materials). In this perspective we report the dynamics and directionality of
230 IF3 movements resulting from the interaction of each translation initiation ligand with the
231 30S subunit. We observe the sequential reduction of inter-domain distance towards the
232 assembling of the 30S pre-IC lacking the initiator tRNA. Although, from our FRET studies
233 we cannot derive the position of IF3 domains on the ribosome, we observe an interdomain
234 distance trend similar to the hypothetical progression of available 30S IC structures and
235 modeling of IF3_{DL} (Fig 5, steps 1 to 3) [20]. The reduction of IF3 interdomain distances
236 appears more evident in the FRET data (step 3, sky blue) if compared to that derived from
237 modelling of the corresponding structures (Fig 5, complex 3 (orange)). This difference is
238 likely to arise from the C2 to C1 movement of IF3C, or from the concomitant movement of
239 IF3N. The latter is an appealing model as it would entail a progressive induced fit of the
240 fMet-tRNA^{fMet} binding architecture. In this context, IF3C moving to the C2 site would be
241 coupled to the dissociation of IF3N from the N_R site to adopt a layout that is ready to
242 interact with the initiator tRNA. This alternative model is supported by single molecule
243 studies where interdomain distances were shown to be influenced by IF1 and IF2 [24]. In
244 this context, binding of fMet-tRNA^{fMet} and decoding of the mRNA start site would uncouple
245 IF3 movements, promoting IF3C to move towards the C1 position while the IF3N remains
246 tRNA-bound.

247 In a cellular context, the progression of translation initiation would result in at least two
248 kinetically stable intermediate complexes, 30S–IFs and the 30S pre-IC. The former would
249 ensemble in around 1 s, being able to reject the 50S by maximal occupancy of the C2 site

250 by IF3C. On the other hand, the 30S–IFs complex can rapidly recruit the initiator tRNA at
 251 the P site by providing a functional binding pocket with IF3C at the C2 site and IF3N ready
 252 to interact with the tRNA elbow. Coordinately, IF2 through its Carboxyl-terminal domain
 253 interacts with the formyl group of the initiator tRNA. The transition towards a 30S pre-IC,
 254 harboring all ligands, would be limited by the availability of mRNA translation initiation
 255 regions (TIRs). Finally, upon decoding of the mRNA start site, the displacement of IF3C
 256 from the C2 site induces 30S IC locking, limiting the overall progression rate of translation
 257 initiation. This step would allow to build 70S pre-ICs in less than 250 ms followed by a
 258 rapid and directional cascade of reactions that ultimately leads to a productive and ready-
 259 to-elongate 70S IC [11]

260 In summary, we describe the functional conformations of IF3 defining with high accuracy
 261 the distances and temporal framework (movie in supplementary materials) covered by
 262 both domains of the factor in correlation with all events taking place during the translation
 263 initiation process. Altogether, this work allows to accurately assign the speed and nature of
 264 elemental movements of IF3 behind its essential fidelity function.

265

266 **Materials and Methods**

267

268 *Escherichia coli* strains, expression vectors, cell growth and protein expression

269 Expression vectors pET24c containing *InfA*, *InfB*, *InfC*, and *InfC-E166C* were acquired
 270 commercially (GenScript, USA). Competent *E. coli* BL21 (DE3) cells were CaCl₂
 271 transformed (Mix & Go, ZymoResearch, USA) with expression vectors coding for IF1 *wt*,
 272 IF2 with an NTD His-tag, IF3 *wt* or IF3_{E166C}. Typically, 2 L of Luria Bertani (LB) medium
 273 were used to grow the BL21 (DE3) strains to an OD_{600 nm} of 0.5. Protein expression was
 274 induced by adding 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, ThermoScientific,

USA). Cells were allowed to express the factors for 3 hours prior to harvesting by centrifugation at 5000 x g at 4°C. Cells were lysed by sonication (20 cycles of 10 s followed by 30 s without sonication) in Buffer A (50 mM Hepes (pH 7.1), 100 mM NH₄Cl, 10 mM MgCl₂, 10 % Glycerol, 6 mM 2-mercaptoethanol) for IF1 and IF3; or Buffer B (50 mM Sodium Phosphate buffer (pH 7.7), 300 mM NaCl, 30 mM Imidazole) for IF2. Debris and supernatant were separated by centrifugation at 15,000 x g for 30 min at 4 °C.

281

282 *Purification of Initiation Factors*

Initiation factors IF1 and IF3 were purified by Cation exchange chromatography on a HiTrap™ SP HP column (GE Healthcare Life Sciences, Uppsala, Sweden). Cleared lysates were loaded onto the column (1 mL column volume, CV) and subsequently subjected to a linear NH₄Cl gradient (0.05 – 1 M) in Buffer A using a HPLC system (Jasco, Japan). IF3 and IF1 eluted at 700 mM and 400 mM NH₄Cl, respectively. Best separation conditions were found at 1 mL/min flow rate and 20 CVs long gradient, collecting fractions of 1 mL each. Protein elution was followed by absorbance at 290 nm and SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE, 15 %). While IF3 eluted with an elevated degree of purity, IF1 fractions contained high molecular weight contaminants. Protein contaminants were eliminated by subjecting the combined IF1 fractions to Amicon® Ultra 30K Da centrifugal filters (Merck, Germany) followed by concentration on a HiTrap™ SP HP (GE Healthcare Life Sciences, Uppsala, Sweden), single step eluted with 1 M NH₄Cl in Buffer A. Eluted proteins were dialyzed against Storage buffer (50 mM Tris-HCl (pH 7.1), 200 mM NH₄Cl, 10 % Glycerol, 6 mM 2-mercaptoethanol) and small aliquots were stored at – 80 °C. Purity was assayed by 15% SDS-PAGE and total protein staining using blue coomassie. Initiation factor IF2 was purified by affinity chromatography on a Histrap FF crude column (1 mL). Supernatants were manually loaded to the column, followed by 5 mL of wash buffer (50 mM Imidazole in Buffer B) and to 2.5 mL of elution buffer (250 mM Imidazole in

301 Buffer B). The first 1.5 mL were collected and subjected to dialysis in Buffer A. Finally, IF2
302 was further purified by Cation exchange chromatography as explained above for IF1 and
303 IF3.

304 *Double labeling of IF3*

305 IF3_{E166C} was subjected to extensive dialysis in Labeling buffer (50 mM Hepes (pH 7.1),
306 100 mM NH₄Cl, 10 % Glycerol and 0.5 mM TCEP) in a D-Tube™ Dialyzer Maxi (EMD
307 Millipore Corp, Billerica, USA) to remove traces of 2-mercaptoethanol as the reducing
308 agent strongly inhibits the coupling to maleimide linked dyes to cysteines. First, the C-
309 terminal was labeled at the recombinant cysteine (C166) as it is exposed and efficiently
310 reacts with maleimide derivatives. A 10-fold excess of Atto-540Q maleimide (Atto-Tec
311 GmbH, Siegen, Germany) over IF3_{E166C} was incubated in Labelling buffer for 20 minutes.
312 The reaction was stopped by the addition of 6 mM 2-mercaptoethanol. The modified
313 IF3_{C540Q} was purified from unreacted dyes on a HiTrap SP HP column as described above.
314 After 10 CV washes with Buffer A containing 100 mM NH₄Cl, a single step elution was
315 applied using 3 mL of 1 M NH₄Cl in Buffer A. Typically, full protein recovery is achieved in
316 0.5 mL and elution of the labeled protein is readily visible. IF3_{C540Q} was subsequently
317 dialyzed as mentioned above in labeling buffer containing 2 M UREA. The partial
318 denaturation of IF3 results in the exposure of the otherwise buried cysteine at position 65
319 of the NTD. The unfolded protein was incubated with a 10-fold molar excess of Alexa488
320 maleimide (Invitrogen, USA) for 1h at RT, mild shaking was applied. IF3_{C540Q-N488} (IF3DL)
321 was purified from the unreacted dye as described above using HiTrap SP HP column.
322 Eluted proteins were dialyzed against Storage buffer and small aliquots were stored at -
323 80°C. Purity and efficiency of labeling was assayed by 15% SDS-PAGE where
324 fluorescence was observed under a UV trans-illuminator and total protein was determined
325 by Blue Coomassie staining.

326 *Ribosomal subunits, fMet-tRNA^{fMet} and mRNAs*

30S and 50S ribosomal subunits were purified from tight coupled 70S ribosomes by sucrose gradient centrifugation under dissociative conditions (3.5 mM MgCl₂), essentially as described [31]. 30S subunits were reactivated by 20 mM MgCl₂ treatment for 30 min at 37 °C prior to use. fMet-tRNA^{fMet} was *in vitro* aminoacylated, formylated, and purified by reverse phase HPLC as described [31]. The model mRNA was chemically synthesized and commercially acquired from Trilink (USA) or Microsynth (Switzerland) with the following sequence: AAA CAA UUG GAG GAA UAA GGU AUG UUU GGC GGA AAA CGA.

Stopped-Flow measurements and analysis

Fluorescence stopped-flow measurements were performed using a SF-300X stopped-flow apparatus (KintekCorp, USA) or a SX20 (Applied Photophysics, UK) by rapidly mixing equal volumes of each reacting solution. The excitation wavelength for Alexa 488 was 470 nm. The emission signal was measured after a long-pass optical filter with a 515 nm cut-off. 1000 points were acquired in each measurement. 7 to 10 replicates were recorded for each reaction and subsequently averaged. All stopped flow reactions were performed in TAKM₁₀ buffer (50 mM Tris (pH 7.5), 70mM NH₄Cl, 30 mM KCl, 10 mM MgCl₂, 6 mM 2-Mercaptoethanol) at 25°C. Unless otherwise stated, 30S complexes were prepared by mixing 0.1 μM 30S, 0.08 μM IF3_{DL}, 0.3 μM IF1,2, 100 μM GTP, 0.5 μM mRNA or fMet-tRNA^{fMet}. Mixtures were incubated at 37 °C for 30 min and centrifugated at 14000 x g for 5 min prior to the measurement. Fluorescence time courses were baseline removed, and to highlight the overall conservation of the signal along the full cycle of IF3, an amplitude factor corresponding to the previous reaction was added. All graphical representations have the same y axis range and represent change of Volts as measured by the Instrument Photomultiplier. Time courses were analyzed by non-linear regression with one or two exponential terms (equations 1 and 2) using the Prism Software v. 7.0 (Graphpad, USA).

351

$$F = F_0 + F_1 e^{-k_{app1}t} \quad \text{Equation [1]}$$

352

353 With t being the time; k_{app1} , the apparent rate constant; F , the fluorescence; F_0 , the initial

354 fluorescence value; and F_1 , the fluorescence change amplitude related to k_{app1} .

355

$$F = F_0 + F_1 e^{-k_{app1}t} + F_2 e^{-k_{app2}t} \quad \text{Equation [2]}$$

356

357 With t being the time; k_{app1} , an apparent rate constant for a first phase; k_{app2} , an apparent

358 rate constant for a second phase; F , the fluorescence; F_0 , the initial fluorescence value; F_1 ,

359 the fluorescence change amplitude related to k_{app1} ; and F_2 , the fluorescence change

360 amplitude related to k_{app1}

361

362 30S, IF2, IF1, and mRNA showed conformational changes of IF3_{DL} slower than their

363 respective bimolecular reactions [4], allowing to fit the obtained rates as a function of titrant

364 concentration with a hyperbolic equation (equation 3) where V_{max} and K_S could be

365 calculated.

366

$$Y = \frac{V_{max}[C]}{K_S + [C]} \quad \text{Equation [3]}$$

367

368 With Y being the reaction rate; $[C]$, the concentration; V_{max} , the maximum rate; and K_S , the

369 rapid equilibration constant.

370

$$k_{AV} = \frac{F_1 k_{app1} + F_2 k_{app2}}{F_1 + F_2} \quad \text{Equation [4]}$$

371

With k_{AV} being the averaged apparent rate constant calculated from equation 2. Standard errors of k_{AV} were calculated according to error propagation functions for addition and product using the mean and standard deviation of each term of equation 4.

Structural modelling

Structures of the 30S pre-IC and 30S IC were obtained from the Protein Data Bank (PDB s: 5lmn, 5lms, 5lmt, 5lmu, 5lmv) [20]. IF3 was extracted into new PDB files from each complex using Chimera software [32]. IF3_{E166C} was then modelled with the Swiss Modeler server using each IF3 pdb file as template [33]. Finally, the modeled IF3_{E166C} replaced the original IF3 in the reported 30S IC structures. fMet-tRNA^{fMet} was removed from all PDB files to generate a subset of structures lacking the tRNA. The accessible volume (AV) of each fluorophore on the 30S ICs structures was determined with FRET-restrained Positioning and Screening software [34]. The fluorophore dimension parameters were: linker length: 15 Å; Width: 4.5 Å; Dye radius: 4.5 Å as defined from a model generated with Maestro software (Schrödinger, NY, USA). The distances between points of the AV of donor and acceptor dyes were calculated in Matlab (Mathworks, USA) using an analytical geometry approximation. The distance distribution between AVs was determined with Prism 7.0 (Graphpad Software, USA).

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Author Contributions

AF and PM conceived the project; JAN and AF performed experiments; JAN, AK, RS, AF, and PM analyzed the data; AF, RS and PM wrote the manuscript with the inputs of JAN and AK.

Conflict of interest

The authors declare no conflict of interest

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511 Figure Legends

513 Figure 1 - Scheme of IF3 motions during bacterial translation initiation.

514 Conformational accommodations of IF3 along the full cycle of translation initiation was
515 monitored by intramolecular FRET between dyes specifically linked to each domain of the
516 factor (IF3_{DL}, inset) and structural modeling of IF3_{DL} on available 30S IC structures [20].
517 Round arrow (sky blue) indicates the direction of complex progression by measuring IF3_{DL}
518 accommodation as a function of the 30S subunit (white), IF2 (green), IF1 (pink), fMet-
519 tRNA^{fMet} (yellow), the mRNA (red), and the 50S subunit. Sky blue numbers indicate the
520 main reactions studied here. Red arrows and values indicate the directionality and rates of
521 IF3 movements measured here. Alternative binding sites for IF3N are: N_R (30S-bound) and
522 N_T (fMet-tRNA^{fMet}- bound). Alternative binding sites for IF3C are indicated as C1 or C2.

523 30S pre-IC indicates the pre-initiation complex while mRNA is indicated by a red line in the
524 30S and 70S ICs.

525

526 **Figure 2 - IF1 and IF2 rapidly promote a compact layout of IF3 on the 30S platform.**

527 Stopped-flow analysis of IF3_{DL} conformational transitions during binding to the 30S subunit
528 and binding of IF1 and IF2 to the 30S–IF3_{DL} complex.

529 A Time course of donor fluorescence change of 0.04 μM IF3_{DL} during binding to 0.12
530 μM 30S (sky blue).

531 B IF3_{DL} binding velocities as a function of 30S concentration. Averaged rates were
532 used to estimate the overall velocity of IF3 opening (Equation 4).

533 C Time courses of 0.5 μM IF1 (pink) or IF2 (green) binding to 0.05 μM 30S–IF3_{DL}
534 complexes.

535 D IF1 concentration dependence of the averaged apparent rates of IF3_{DL} closure upon
536 binding of IF1 to the 30S–IF3_{DL}–IF2 complexes.

537 E Time traces of IF3_{DL} donor fluorescence change as function of 0.5 μM IF1 (pink) or
538 IF2 (green) binding to 0.05 μM 30S–IF3_{DL}–IF2 or 30S–IF3_{DL}–IF1 complexes, respectively.

539 F IF2 concentration dependence of the apparent rates of IF3_{DL} closure upon binding
540 of IF2 to the 30S subunit.

541 Grey lines in (A), (C), and (E) represent reactions with IF3 labelled only with the donor
542 fluorophore at IF3N, without the quencher molecule at IF3C (no acceptor) to verify that the
543 fluorescence signal of IF3_{DL} depended on distance changes between fluorophores.

544 Continuous lines represent non-linear fitting with exponential (A, C, E, Equations 1-2) or
545 hyperbolic (B, D, F, Equation 3) functions. Each time trace results from the average of 7-

546 10 independent measurements. Error bars in (B), (D), and (F) represent standard
547 deviations.

548

549 **Figure 3 - fMet-tRNA^{fMet} and decoding of the mRNA start site promote an open**
550 **layout of IF3 on the 30S platform.**

551 A Time course of donor fluorescence change of IF3_{DL} during binding of 0.5 μM fMet-
552 tRNA^{fMet} to 0.05 μM 30S pre-IC in the presence (orange) or absence (yellow) of the
553 mRNA.

554 B, C fMet-tRNA^{fMet} concentration dependence of the first (B) and second (C) apparent
555 rates of IF3_{DL} opening upon binding of the initiator tRNA to 30S pre-ICs.

556 D Time courses of donor fluorescence change of IF3_{DL} during binding of 0.5 μM
557 mRNA to 0.05 μM 30S pre-IC in the presence (red) or absence (blue) of fMet-tRNA^{fMet}.

558 E mRNA concentration dependence of the apparent rates of IF3_{DL} closure upon
559 binding to the 30S pre-ICs containing fMet-tRNA^{fMet}.

560 The control curves, indicated by grey lines in (A) and (D), represent reactions carried out in
561 the presence of IF3 lacking the acceptor at the C-terminus subunit (no acceptor) to assess
562 whether the fluorescence signal was dependent on the distance change between
563 fluorophores. Continuous lines represent the fitting with exponential (A) and (D) (Equations
564 1-2), linear (B) or hyperbolic (C) (E) (Equation 3) functions. Each time trace results from
565 the average of 7-10 independent measurement. Error bars in (B), (C), and (E) represent
566 standard deviations.

567

568

Figure 4 - 70S IC formation is accompanied by IF3 closing during dissociation.

A Time course of donor fluorescence change of IF3_{DL} during binding of 0.5 μ M 50S to 0.05 μ M 30S IC (sky blue). Grey line indicates a control using an IF3 without the acceptor dye (as in Fig 2 and 3).

B 50S concentration dependence of the average rates of IF3_{DL} closure upon binding of the large subunit. Error bars represent standard deviations.

C Time courses of donor fluorescence change of IF3_{DL} during the concomitant binding of 0.5 μ M 50S and fMet-tRNA^{fMet} to 0.05 μ M 30S pre-IC in the presence (red) or absence (sky blue) of the mRNA.

Continuous lines represent the fitting with exponential (A) and (C), or hyperbolic (B) functions. Each time trace results from the average of 7-10 independent measurements. Error bars in (B) represent standard deviations.

Figure 5: Intramolecular distances between IF3 domains during progression of translation initiation. Comparison between the amplitude changes of IF3_{DL} for all the reactions measured here (sky blue, left axis) and distances between the fluorophores on the corresponding complexes as analyzed by molecular modeling (orange, right axis). Sky blue numbers indicate the reactions measured here (refer to Fig 1). Orange numbering refer to the 30S PICs 1A, 3, and 4 complexes from [20]. x-axis represent the progression time of each reaction as calculated from the saturating rates measured here. Error bars represent standard deviations from the total amplitudes for each reaction (sky blue) or from the distance distribution of IF3_{DL} fluorophores calculated by structural modelling of the complexes mentioned above (orange, Supp. Figure 3 and 4, Supp. Table 1).

F

Supplementary Information for:

The complete, functional and dynamic cycle of the bacterial Initiation Factor 3

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Supplemental Figure 1. Experimental approach. IF3_{DL} for dynamic measurements of the factor on the 30S platform. **(A)** Absorption and emission spectra of Alexa488 fluorescent dye (green) and Atto540Q quencher (magenta). **(B)** Schematic example of IF3_{DL} arrangements. IF3 surface is shown in blue while meshed volumes indicate the spatial volume that dyes occupy (colors are as (A)). **(D)** Scheme of Stopped-Flow experimental set-up and the typical signal read out upon mixing 30S-IF3_{DL} with a 30S binder (sky blue trace). In order to assign the signal as FRET, the same experiment is performed in the absence of the acceptor, in this case IF3_N Alx488 (grey trace).

Supplementary figure 2. Time traces of IF3_{DL} donor fluorescence change upon formation of all initiation intermediates. **(A)** Time courses of donor fluorescence change of 0.04 μM IF3_{DL} during binding to varying concentration of 30S (0.15-0.5 μM). **(B)** Time courses of IF2 (0.15- 0.5 μM) binding to 0.05 μM 30S–IF3_{DL} complexes. **(C)** Time courses of IF1 (0.15-0.5 μM) binding to 0.05 μM 30S–IF2–IF3_{DL} complexes. **(D)** Time courses of fMet-tRNA^{fMet} (0.15-0.5 μM) binding to 0.05 μM 30S–IF3_{DL}–IF1–IF2 complexes. **(E)** Time courses of mRNA (0.15-1 μM) binding to 0.05 μM 30S PICs (IF3_{DL}). **(F)** Time courses of 50S (0.15-1 μM) binding to 0.05 μM 30SIC (IF3_{DL}) complexes. A control against buffer is shown in each titration (sky blue). Each time trace results from the average of 7-10 independent measurement.

Supplementary figure 3. Structural analysis of IF3_{DL} on 30S complexes. Frequency distribution of the distances between donor and acceptor dyes for IF3 occupying all combination of binding sites. The median plus its deviation is indicated.

Supplementary figure 4. Structural representation of IF3_{DL} on 30S complexes. IF3 domains in the N_r, N_{TIII}, C₁, and C₂ binding configurations in the 30S platform. Donor (Alexa 488) accessible volume (AV) is shown in green and acceptor (Atto540Q) AV in magenta. All structures are redrawn from [20] after modeling IF3 and the dyes attached to the factor (see methods).

Position	Distance between fluorophores (Å)*	Distance measurements (N x 10 ⁵)	Distance between domains (Å)**
C ₁ – N _R	57 ± 7	15.6	63
C ₂ – N _R	44 ± 7	14.9	61
C ₁ – N _{T_I}	54 ± 6	8.8	57
C ₂ – N _{T_I}	54 ± 5	7.3	51
C ₁ – N _{T_{II}}	54 ± 6	9.9	60
C ₂ – N _{T_{II}}	51 ± 5	6.2	50
C ₁ – N _{T_{III}}	39 ± 6	11.1	54
C ₂ – N _{T_{III}}	31 ± 8	9.7	46

Supplementary Table 1. Distances between fluorophores and domains on IF3_{DL} layouts. *Distances determined from the AV. **Distance between labelled cysteines in each domain.

	AV pts	Volume (Å ³)*	AV pts (no tRNA)	Volumen (Å ³)* (no tRNA)
C ₂	4338	3805.82	4338	3805.82
C ₁	4975	4364.68	4975	4364.68
N _R	3384	2968.86	3384	2968.86
N _{TI}	192	168.45	2341	2053.81
N _{TII}	216	189.50	2436	2137.16
N _{TIII}	223	195.64	2009	1762.54
Max	16114**	14137.17	16114**	14137.17

Supplementary Table 2. Calculated accessible volume (AV) for each fluorophore at all binding configurations of IF3_{DL}. AV spatial coordinates were generated with the FPS-software [34]. *Volume approximation by the number of coordinates to the AV to a sphere with a radius of 15 Å. **Maximum number of coordinates present in an AV with the following parameters: Linker length: 15 Å; Width: 4.5 Å; Dye radius: 4.5 Å.