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IF2 Interaction with the Ribosomal GTPase-Associated Center During 70S Initiation Complex Formation

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Abstract

Addition of an E. coli 50S subunit ($50S^{Cy5}$) containing a Cy5-labeled L11 N-terminal domain (L11-NTD) within the GTPase-associated center (GAC) to an E. coli 30S initiation complex ($30SIC^{Cy3}$) containing Cy3-labeled initiation factor 2 complexed with GTP, leads to rapid development of a FRET signal during formation of the 70S initiation complex (70SIC). IF2 and EF-G induce similar changes in ribosome structure. Here we show that such similarities are maintained on a dynamic level as well. Thus, movement of IF2 toward L11-NTD after initial 70S ribosome formation follows GTP hydrolysis and precedes Pi release, paralleling movement of EF-G following its binding to the ribosome [Seo et al., (2006) Biochemistry 45, 2504 – 2514], and, in both cases, the rate of such movement is slowed if GTP hydrolysis is prevented. The $30SIC^{Cy3}$: $50S^{Cy5}$ FRET signal also provides a sensitive probe of the ability of initiation factor 3 to discriminate between a canonical and a non-canonical initiation codon during 70SIC formation. We employ B. stearothermophilus IF2 as a substitute for E. coli IF2 to take advantage of the higher stability of the complexes it forms with E. coli ribosomes. While Bst-IF2 is fully functional in formation of E. coli 70SIC, relative reactivities toward dipeptide formation of 70SICs formed with the two IF2s suggests that Bst-IF2·GDP is more difficult to displace from the GAC than E. coli IF2.GDP.

Initiation factor 2 (IF2) is a G-protein that plays a crucial role in the initiation of procaryotic protein synthesis, interacting directly with $fMet\text{-}tRNA^{fMet}$, favoring its decoding in the P-site, and physically linking the 30S and 50S subunits in the 70S initiation complex (70SIC) (1–5). It shares a common binding locus on the ribosome, the GTPase associated center (denoted GAC), with other G-proteins utilized in protein synthesis, such as elongation factor G (EF-G). The GAC includes 23 S rRNA helices 42–44, the associated proteins L11, L10, and at least one L7/L12 protein (6,7). Cryoelectron microscopy (cryo-EM) studies have indicated that both EF-G·GTP and IF2·GTP binding to the ribosome are accompanied by large conformational changes in the ribosome, and that, in both cases, further conformational changes in the ribosome·G-protein complex are seen following GTP hydrolysis (8–12).

The N-terminal domain of L11 (L11-NTD) is a particularly mobile portion of the ribosome that, following GTP hydrolysis, approaches the G' domain of EF-G (9,13,14). In earlier work utilizing single-turnover fluorescence resonance energy transfer (FRET) measurements, we determined that rapid movement of the G' domain toward L11-NTD within the E. coli ribosome requires prior GTP hydrolysis and, via branching pathways, either precedes Pi release (major pathway) or occurs simultaneously with it (minor pathway) (15). In this latter work fluorescent groups were placed on the Cys 38 within L11-NTD and on a suitable residue within the G' domain.

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Here we utilize a similar approach to determine whether there is a comparable movement toward L11-NTD of the G1-domain of IF2 (IF2 lacks a G' domain) during 70SIC formation from 30S initiation complex (30SIC) and 50S subunit. The cryo-EM structures of 70S complexes containing fMet-tRNA^{fMet}, mRNA and either IF2.GDPCP (a nonhydrolyzable analogue of GTP) or IF2·GDP (11) result in an estimated distance of 50 – 55 Å between the α-carbons of residue 378 in IF2 and residue 38 in L11 (Figure 1), quite suitable for probing by measurement of FRET efficiency. Accordingly, we employ 50S subunits containing protein L11 labeled with Cy5 at position 38 (denoted L11^{Cy5}) and B. stearothermophilus IF2 (Bst-IF2) labeled with Cy3 at position 378 (denoted Bst-IF2^{Cy3}) (16).

Substituting Bst-IF2 for Eco-IF2 has been shown to facilitate the characterization of translation intermediates, due to the higher stability of the complexes that Bst-IF2 forms with E. coli ribosomes and ribosomal subunits as compared with Eco-IF2 (16,17). Such substitution is reasonable in view of substantial evidence that Bst-IF2 is functionally interchangeable with Eco-IF2 in E. coli protein synthesis. Thus, Bst-IF2 complements an E. coli infB null mutation in vivo (E. Caserta and C. Gualerzi, private communication). In addition, in vitro studies demonstrate the near equivalence of Eco-IF2, Bst-IF2, and Bst-IF2^{Cy3} in binary complex formation with E. coli fMet-tRNA^{fMet} (18), in stimulating of AUG-dependent fMet-tRNA^{fMet} binding to E. coli 30S subunits and 70S ribosomes (19) and in kinetic measures of 70SIC formation (17, 20 and work reported herein).

We find that FRET efficiency increases as the 70S ribosomes formed initially from 30SIC and 50S subunits are transformed into the 70SIC, and that the rate of such increase depends on GTP hydrolysis, paralleling results obtained with EF-G. We further demonstrate that the increase in FRET efficiency can be used to monitor the fidelity function of the initiation factor IF3 during 70SIC formation.

MATERIALS AND METHODS

Proteins

E. coli IF1, IF2 and IF3 (17,21,22) and EF-Tu (23) were prepared as described. The B. stearothermophilus G378C-IF2 mutant (Bst-IF2) was prepared as described (20,22). Bst-IF2^{Cy3}, was prepared by reacting Bst-IF2 with Cy3-maleimide (GE Health Care) as described (20), except that, in addition to two hours of incubation at room temperature, the reaction is also kept overnight at 4 °C. Under these conditions, IF2 was labeled quantitatively with Cy3, as calculated using ε550nm (Cy3) equal to 150,000 $M^{-1}cm^{-1}$ (24) and estimating Bst-IF2 concentration by Bradford assay (25). MDCC-labeled phosphate-binding protein (PBP) (15) and N-terminally His-tagged L11 were prepared essentially as described (26). L11^{Cy5} was prepared by reacting L11 at its unique Cys (position 38), with Cy5-maleimide (GE Health Care) following Wang et al. (26). tRNA and mRNA. ^{35}S-labeled fMet-tRNA^{fMet}, fMet-tRNA^{fMet}(prf20), Phe-tRNA^{Phe}, and 022AUG and 022AUU mRNAs were prepared as described previously (23,27–29).

Ribosomes and subunits

Tight couple wild-type 70S ribosomes and 30S and 50S subunits were isolated from MRE600 as described (20). Mutant 70S ribosomes lacking L11 were isolated from E. coli strain AM77 as described (15) except that zonal gradient purification was carried out in a buffer containing 8 mM Mg^{2+} rather than 6 mM Mg^{2+}. AM77 50S subunits, denoted 50S^{-L11}, were prepared from AM77 70S ribosomes as described for wt-50S subunits (20).

Reconstituted 50S subunits

50S subunits containing Cy5-labeled L11 (denoted $50S^{Cy5}$) were prepared by incubating $L11^{Cy5}$ (1 nmol, measured as Cy5) with 1 nmole of $50S^{-L11}$ subunits in buffer A (25 mM Tris, pH 7.6, 30 mM NH_4Cl, 70 mM KCl, 7 mM $MgCl_2$, and 1 mM dithiothreitol) for 15 min at 37 °C. Unbound $L11^{Cy5}$ and any residual unincorporated dye were removed by ultracentrifugation for 18 hrs through a 1.1M sucrose cushion in buffer A in rotor Type 70.1Ti at 220,000g, yielding $50S^{Cy5}$ which contained 1.0 ± 0.2 Cy5/subunit. A control reconstitution experiment performed with wild-type MRE 600 50S subunits resulted in a Cy5:50S subunit ratio of <0.2. 50S subunits reconstituted from $50S^{-L11}$ subunits and unlabeled L11 (denoted $50S^{L11}$) were prepared similarly.

Complexes

Preformed complexes were incubated for 15 min at 37 °C prior to use. 30SIC was always formed by incubation of 1.0 equivalent of 30S subunits with 1.5 equivalents of IF1, IF3, and fMet-tRNAfMet, 3.0 equivalents of mRNA and various amounts of IF2. 30SICs made up with Eco-IF2, Bst-IF2, or Bst-$IF2^{Cy3}$ are denoted $30SIC^{Eco}$, $30SIC^{Bst}$ and $30SIC^{Cy3}$, respectively. Ternary complex (TC) was formed by incubation of 1.0 equivalent of [^{3}H]-Phe-$tRNA^{Phe}$ with 1.5 equivalents of EF-Tu and 200 M GTP. TC concentration is calculated as the concentration of [^{3}H]-Phe-$tRNA^{Phe}$. 70SIC was preformed by incubation of 30SIC (0.3 M) with 50S subunits (0.5 M) in buffer A.

Equilibrium and kinetic measurements—All concentrations specified in the text and figure legends refer to final concentrations after mixing unless otherwise specified. All equilibrium and kinetic experiments involving fluorescent measurements, GTP hydrolysis, and peptide bond formation were performed in buffer A at 20 °C. Equilibrium fluorescence. Solutions were excited at 540 nm and emission was monitored from 560 nm to 720 nm (SPEX Fluorolog-3, Jobin Yvon Inc.)

Light scattering, $IF2^{Cy3}$ fluorescence, $50S^{Cy5}$ fluorescence, Pi release. Measurements were performed in either an SX.18MV (Applied Photophysics) or a KinTec SF-2004 stopped-flow spectrophotometer by rapid mixing of 30 complexes with 50S subunits. Light scattering and Pi release were measured as described (20). For $IF2^{Cy3}$ and $50S^{Cy5}$ fluorescence, excitation was at 540 nm and output was monitored at 567 nm and either at 670 nm or using a 680 ± 10 nm bandpass filter (Figure 7), respectively.

GTPase activity, fMet-puromycin formation, and fMetPhe-$tRNA^{Phe}$ formation. Measurements were performed in a KinTec RQF-3 apparatus. Rates of GTPase were determined by rapid mixing of 30 complexes with 50S subunits followed by rapid quenching, as described (20). Rates of [^{35}S]-fMet-puromycin formation were determined by rapid mixing of preformed 70SIC with the puromycin, followed by quenching with 0.3M sodium acetate (pH 5.2), extraction with ethyl acetate, and determination of the radioactivity in the ethyl acetate layer. Rates of fMetPhe-$tRNA^{Phe}$ formation were determined as described (20), by rapid mixing of either 30S complexes with 50S subunits and Phe-$tRNA^{Phe}$EF-Tu.GTP ternary complex, or preformed 70SIC with ternary complex, followed by rapid quenching.

Kinetic analyses

Apparent rate constants and microscopic constants for specific kinetic schemes were determined using the programs Igor-Pro (Wavemetrics – for single, double and triple exponential equations, Table 1 and Table 2, Figure 6) and Scientist (MicroMath Research, LC - for global fitting of multiple parameters to Scheme 1, Figure 5C).

Poly(U)-dependent poly (Phe) synthesis

The assay was carried out as described (26), except that 70S ribosomes were formed by pre-incubation of 50S subunits (0.5 M) with 30S subunits (1.0 M) for 15' at 37 °C.

R_o and FRET efficiency estimation

The Förster distance R_o was calculated from eq 1, where ϕ_D is the quantum yield of $30SIC^{Cy3}$, η is the refractive index of water (= 1.33; 30), κ^2 is a dipole orientation factor, and was set equal to 2/3 assuming random orientations of the fluorophores (31,32) and J(λ) is the spectral overlap integral. ϕ_D was determined by comparing the integrated fluorescence of $30SIC^{Cy3}$ to that of a standard, Rhodamine B, as described (31), yielding a value of 0.57 ± 0.03. J(λ), equal to 4.66×10^{-13} $M^{-1}cm^3$, was determined from the fluorescence and absorption spectra of $30SIC^{Cy3}$ and $50S^{Cy5}$, respectively. These values permit calculation of R_o equal to 60 ± 5 Å, in good agreement with other reported values of 50 – 60 Å for the Cy3:Cy5 pair (33,34).

$$R_o\,(\text{Å}) = [8.8 \times 10^{-5} \cdot \kappa^2 \cdot \eta^{-4} \cdot \phi_D \cdot \mathbf{J}(\lambda)]^{1/6} \quad (1)$$

FRET efficiency, E, was calculated from eq 2, where F_{DA} and F_A are the background-corrected fluorescences of the donor/acceptor pair and the acceptor alone, respectively, on irradiation at the excitation wavelength ($_{ex}$) of the donor (540 nm) and detection at the emission maximum of the acceptor, $_A$ (670 nm), f_D is a fractional labeling of donor (equal to 1.0), and $_A$ and $_D$ are the extinction coefficients of donor and acceptor at $_{ex}$.

$$E = \left(\frac{\varepsilon_A(\lambda_{ex})}{\varepsilon_D(\lambda_{ex})}\right)\left(\frac{F_{DA}(\lambda_A)}{F_A(\lambda_A)} - 1\right)\left(\frac{1}{f_D}\right) \quad (2)$$

RESULTS

In the work described below we use the FRET signal between a 30SIC containing Bst-$IF2^{Cy3}$ and a 50S subunit containing $L11^{Cy5}$ to monitor the relative movement of IF2 with respect to L11 during 70SIC formation. Below we first demonstrate that, with minor qualifications, Bst-$IF2^{Cy3}$ and $L11^{Cy5}$ are acceptable functional analogues of Eco-IF2 and unlabeled L11, respectively, before presenting our FRET results.

Bst-IF2 and Bst-$IF2^{Cy3}$ as functional analogues of Eco-IF2 with respect to 70SIC formation and reactivity

In the presence of IF3, 30S association with 50S subunits to form 70SIC is completely dependent on the presence of IF2 (20). Here we carry out four rapid kinetics measures (increase in light scattering, IF2-dependent single turnover GTP hydrolysis and subsequent Pi release, increase in fMet-$tRNA^{fMet}$ (prf20) fluorescence) that we previously employed in formulating a detailed quantitative kinetic scheme for 70SIC formation from 30SIC and 50S subunit (20) to compare the functionalities of Eco-IF2 ($30SIC^{Eco}$), BSt-IF2 ($30SIC^{Bst}$), and Bst-$IF2^{Cy3}$ ($30SIC^{Cy3}$) in this process. We also compare the reactivities of the three 70SICs formed in dipeptide formation. Our results show that all three 30SICs have similar rates of 70SIC formation, but that the reactivity of the 70SIC in dipeptide formation is somewhat faster in the presence of Eco-IF2 vs. either of the Bst-IF2s.

The increase in light-scattering on addition of 30SIC to 50S subunit is well described as a 2-phase process (Figure 2A), with the first corresponding to initial binding of the 50S subunit to 30SIC to form a labile 70S complex and the second reflecting conversion to the more

stable 70SIC. These two phases have apparent rate constants LS1 and LS2. Initial 70S formation is followed by GTP hydrolysis (Figure 2B), with an apparent rate constant (GTP1) that is similar to or somewhat smaller than LS1. Pi release proceeds via a lag phase, with an apparent rate constant Pi1, similar in magnitude to LS2, followed by the Pi release step, with an apparent rate constant Pi2 (Figure 2C). The increase in fluorescence of fMet-tRNA^{fMet}(prf20) on 70SIC formation, which could not be measured for Bst-IF2^{Cy3} because of fluorophore interference, is also preceded by a lag phase (Figure 2D), with apparent rate constants for both phases, fMet1 and fMet2, that are similar in magnitude to the values of Pi1 and Pi2.

Values for each of the apparent rate constants mentioned above are collected in Table 1. From these values, as well as by direct inspection of Figures 2A–D, we conclude that 70SIC formation proceeds in a very similar manner with either 30SIC^{Eco}, 30SIC^{Bst}, or 30SIC^{Cy3}. Some small quantitative differences include larger overall light scattering changes falling in the order Bst-IF2^{Cy3} > Bst-IF2 > Eco-IF2, and GTP1 values decreasing in the order Eco-IF2>BSt-IF2>Bst-IF2^{Cy3}.

The functionality of the three 70SIC complexes in fMetPhe-tRNA^{Phe} formation on addition of the Phe-tRNA^{Phe}.EF-Tu.GTP ternary complex was determined either following rapid mixing of each of the pre-formed 30SICs with 50S subunits and the cognate TC, or following rapid mixing of each of the pre-formed 70SICs with cognate TC (Figure 2E, Table 2). Comparable reactivities (k'_{dp}) were found following the first protocol, with apparent rate constants falling in the order 30SIC^{Eco} ($0.28 \pm 0.06\ s^{-1}$) > 30SIC^{Cy3} ($0.18 \pm 0.06\ s^{-1}$) ~ 30SIC^{Bst} ($0.14 \pm 0.03\ s^{-1}$). On the other hand, the second protocol, while leaving the apparent rate constant (k^*_{dp}) for 30SIC^{Bst} essentially unchanged ($0.12 \pm 0.02\ s^{-1}$), leads to a marked increase k^*_{dp} found with 30SIC^{Eco} ($1.6 \pm 0.2\ s^{-1}$).

The latter difference may be related to the more stable binding within the 70SIC of Bst-IF2·GDP than Eco-IF2·GDP (17,35). Thus, productive binding of TC leading to dipeptide formation requires, at a minimum, movement of IF2 away from its canonical position within the 70SIC (11), allowing TC binding to the GAC, if not full IF2 dissociation from the ribosome. Such movement (or dissociation) might be partially rate-determining for dipeptide formation when 50S subunit and TC are added simultaneously to 30SIC, consistent with earlier results of Tomsic et al. (36), but might already be completed, at least for the more weakly bound Eco-IF2, when TC is added to pre-formed 70SIC, resulting in more rapid dipeptide formation.

50S^{Cy5} as a functional analogue of wt-50S with respect to 70SIC formation and reactivity

Having established the functionality of 30SIC^{Cy3}, we next use 30S^{Cy3} to compare 50S^{Cy5} with wt-50S with respect to three single-turnover kinetic (GTP1 and the apparent rate constants for dipeptide and fMet-puromycin formation) and three single-turnover stoichiometric (GTPase, dipeptide, fMet-puromycin) measures of 70SIC formation and reactivity. The results, as displayed in Figure 3A,B and Table 2 (along with more limited results for 50S^{L11} and 50S^{-L11}) show 50S^{Cy5} to be a good functional analogue of wt-50S, with the only significant difference in these six measures being found for the value of k'_{dp} (fMet-Phe formation), $0.18 \pm 0.06\ s^{-1}$ and $0.07 \pm 0.01\ s^{-1}$ for wt-50S and 50S^{Cy5}, respectively. Furthermore, pre-formed 70S ribosomes have virtually identical activities in poly(U)-dependent poly(Phe) synthesis, whether made with wt-50S or 50S^{Cy5} subunits (Figure 3C).

There is a significant decrease in the GTPase stoichiometry obtained with 50S^{-L11} subunits (Figure 3A, Table 2), consistent with earlier results showing that 50S^{-L11} subunits are somewhat defective in forming 70SIC from 30SIC (37, 38). However, even the 50S^{-L11}

subunits show appreciable activity in polyPhe synthesis (Figure 3C), in agreement with earlier results (39, 40).

FRET changes accompanying 70SIC formation from 30SIC^{Cy3} and 50S^{Cy5}

The experiments described above establish the basic functionality of 30SIC^{Cy3} and 50S^{Cy5} in 70SIC formation, making it likely that the FRET experiments described below that measure FRET during the combination of these modified subunits will be relevant for understanding the process of 70SIC formation from native, unmodified subunits. Long term incubation (15 min, 37 °C) of 30SIC^{Cy3}, containing a fluorescent donor (D), with 50S^{Cy5} subunits containing a fluorescence acceptor (A) results in formation of the double-labeled 70SIC (DA sample) and the generation of a strong FRET signal (Figure 4A), with considerable decreases and increases in donor and acceptor fluorescence signals, respectively, as compared with the fluorescence of the D (30SIC^{Cy3} plus 50S^{L11}) and A (30SIC^{Bst} plus 50S^{Cy5}) samples. In fact, the donor decrease shown in Figure 4A underestimates FRET efficiency, given the intrinsic rise in donor fluorescence that accompanies 70SIC formation when 30SIC^{Cy3} is rapidly mixed with unlabeled 50S^{L11} subunits (Figure 4B). By contrast, there is no corresponding change in acceptor fluorescence when 30SIC^{Bst} replaces 30SIC^{Cy3} (Figure 4B). The similarity in acceptor fluorescence intensity at the two 30SIC^{Cy3} concentrations employed in Figure 4A indicates that essentially all of 50S^{Cy5} is taken up within the 70SIC at the higher 30SIC concentration. These results permit calculation of a FRET efficiency in the 70SIC complex of ~50% by application of eq 2 (Materials and Methods).

Changes in FRET vs. changes in light scattering during 70SIC formation

Strong evidence that the FRET signals seen in Figure 4 are a direct consequence of 70SIC formation is provided by results in Figure 5A showing no such appearance of a FRET signal, measured as the increase in Cy5 fluorescence, when fMet-tRNA^{fMet} is omitted from the reaction mixture. This is in accord with results showing that, in the presence of IF3, fMet-tRNA^{fMet} is required for 70SIC formation (20,41,42).

The increase in light scattering on formation of 70SIC from 30SIC and 50S subunit provides another measure of 70SIC formation (20). Comparison of the time dependence of the increase in FRET efficiency for the complete system (i.e., including fMet-tRNA^{fMet}) with the corresponding increase in light scattering, measured on identical samples (Figure 5A), clearly shows that both increases proceed in more than one phase, with both showing an initial rapid phase that is somewhat more pronounced for the light scattering change (20) than for the FRET change. As with FRET change, the rapid increase in light scattering is abolished when fMet-tRNA^{fMet} is omitted.

The experiments shown in Figure 5A were conducted with both IF2^{Cy3} and 50S^{Cy5} subunits present in excess over 30S subunits. Under these conditions, the time courses of FRET and light scattering increases are directly comparable, with virtually all 30S subunits in the sample containing IF2^{Cy3} as part of the 30SIC and all 30SIC^{Cy3} interacting with 50S^{Cy5}. Other conditions, such as those employed in Figure 5B (30S > IF2^{Cy3} > 50S^{L11}; 50S > 30S > IF2^{Cy3}), lead to multiphasic increases in light scattering that reflect sample heterogeneity and are unsuitable for directly comparing increases in FRET and light scattering. Thus, slow phases of light scattering are observed when either 50S subunit concentration is limiting, due to the presence of a minor fraction (< 20 %) of 50S subunits that lack L11 (50S^{-L11}) and form 70SIC complexes only very slowly (data not shown –see also 37, 38), or when IF2^{Cy3} is limiting, since 30S initiation complexes formed in the presence of IF3 but lacking IF2 also form 70SIC very slowly (20). Such sample heterogeneity is not a problem for FRET measurements, which only measure rapid 70SIC formation events between 30SICs

containing Bst-IF2^{Cy3} and 50S subunits containing L11^{Cy5}. The rate of Pi release is also shown in Figure 5B for direct comparison with the rate of FRET increase.

The results in Figures 5A and 5B provide clear evidence that 70SIC formation, culminating with Pi release, proceeds in a minimum of three phases. Thus, the change in the ratio of normalized FRET change to normalized light scattering change, which is completed within 0.1 s (Figure 5A inset), demonstrates an initial rapid phase, while the slower overall increase in the FRET signal, indicative of phase 2, clearly proceeds more rapidly than Pi release (Figure 5B), which proceeds in phase 3. These results can be globally fit to the minimal Scheme 1 (Figure 5C), in which an initial binding reaction to form $70S_I$ ribosomes (step 1), giving rise to a FRET signal with concomittant GTP hydrolysis (Figure 2B, Figure 3A), is followed by a conformational change (step 2), resulting in formation of $70S_{II}$ ribosomes, from which Pi is released (step 3). In carrying out this fitting the light scattering increase due to $70S_I$, $70S_{II}$, or 70SIC formation is assumed to be identical (41). Constraining the FRET efficiencies to also be the same for all three 70S species led to poor fits to the FRET data. However, setting the relative FRET efficiency (RFE) for 70SIC equal to 1.0 and allowing the RFEs of the other 70S species to be different led to best-fit values of 0.57 and 1.00 for $70S_I$ and $70S_{II}$, respectively. This point is made graphically in the inset to Figure 5A which shows that the ratio, normalized FRET change: normalized light scattering change, increases as $70S_I$ is converted to $70S_{II}$ and does not change thereafter. We conclude that the L11-NTD gets closer to the G1 domain of IF2 as $70S_I$ is converted to $70S_{II}$, and that this movement follows GTP hydrolysis and precedes Pi release.

GTP hydrolysis accelerates the increase in FRET efficiency following 70S formation

The previous conclusion led us to examine whether GTP hydrolysis was required for the increase in FRET efficiency within the 70S complex by replacing GTP with its nonhydrolyzable analogue GDPNP. We initially attempted to compare light scattering and FRET changes in the presence of GDPNP under conditions (50S > IF2 >30S) exactly paralleling those we had used in the presence of GTP (Figure 5A). As seen in Figure 6A, replacement of GTP by GDPNP during 70SIC formation leads to only a small decrease in the magnitude of light-scattering increase, with little change in the rate of such increase. However, these conditions are not suitable for measuring FRET changes during 70SIC formation, since a rapid and significant change in FRET signal is seen even in the absence of 70S formation (i. e., with fMet-tRNA^{fMet} omitted, Figure 6B), reflecting direct binding of Bst-IF2^{Cy3}·GDPNP to 50S^{Cy5}. No such signal is seen in the presence of GTP (Figure 5A), because of the rapid hydrolysis of GTP within the 50S.IF2·GTP complex, and the relatively weak binding of IF2·GDP to 50S subunits (20).

Accordingly FRET experiments were carried out under conditions (30S > IF2 > 50S) minimizing 50S·IF2·GDPNP formation, as seen by the very small FRET change when fMet-tRNA^{fMet} is omitted (Figure 6C). Under these conditions, the total FRET change seen with GDPNP is similar to that seen with GTP, but the time development is different, with the initial rise occurring slightly faster with GDPNP and the full FRET change occurring much more slowly.

The light scattering results in the presence of GDPNP were well fit assuming biphasic kinetics. In contrast, fitting the FRET results required three phases, and show an approximate doubling in the apparent FRET efficiency during the slow third phase (k_{app} ~0.2 s^{-1}) when, on the basis of the light scattering results, little additional 70S formation would be expected. Taken together, the results in Figure 6A and 6C are consistent with a kinetic scheme for 70SIC formation in the presence of GDPNP that is similar to Scheme 1 for GTP (Figure 5C), except that step 3 would be reversible and the increase in FRET efficiency following 70S formation occurs later in the process. It would thus appear that

GTP hydrolysis accelerates, but is not essential for, the movement of L11-NTD toward the G1 domain of IF2 as the initial 70S complex formed is converted to 70SIC.

FRET monitoring of the fidelity function of IF3

Earlier we showed that the rate and magnitude light scattering increase provided a sensitive measure of the ability of IF3 to discriminate between a canonical (AUG) and a non-canonical (AUU) initiation codon in 70SIC formation (27). The increase in FRET efficiency accompanying 70SIC formation also provides a sensitive probe of such discrimination (Figure 7). Thus, in the presence of IF3, substituting AUU (trace 3) for AUG (trace 1) leads to large decreases in both the rate and extent of FRET efficiency increase, whereas in the absence of IF3 the apparent rate is little affected, and the extent of increase is somewhat increased (traces 2 and 4). Identical trends were observed for light scattering changes (27).

DISCUSSION

IF2 is a G-protein that is part of the 30SIC, and is retained within the 70SIC that is formed following reaction of the 30SIC with the 50S subunit (11,12). Here we show that FRET measurement of IF2 interaction with L11-NTD can be used to monitor the relative motions of IF2 and the GTPase activation center during the process of 70SIC formation. We measure a FRET efficiency of 50% for the 70SIC complex, corresponding to an approximate fluorophore-fluorophore distance of 60 Å, equal to R_o (see Materials and Methods). This value is in reasonable accord with the distances of 50 – 55 Å between the α-carbons of residue 378 in Bst-IF2 and residue 38 in L11 that can be estimated from cryo-EM structures of 70S complexes containing fMet-tRNA^{fMet}, mRNA and either IF2.GDPCP (another nonhydrolyzable analogue of GTP) or IF2·GDP (11), since the distances between the dyes would be expected to be somewhat greater than the distances between the α-carbons to which they are attached.

Although IF2·GDP binds to 70S ribosomes less tightly than IF2·GTP (20), consistent with the cryo-EM results of Myasnikov et al. (11) that indicate substantial differences in the overall interaction of IF2 with the 70S ribosome following GTP hydrolysis and Pi release, the results presented in Figure 5 indicate that the G1 domain of IF2 moves closer toward L11-NTD as part of the process by which the complex initially formed from 50S association with the 30SIC, $70S_I$·GDP·Pi in Scheme 1 (Figure 5C), is converted into the $70S_{II}$·GDP·Pi complex, preceding Pi release and 70SIC formation. Based on the relative FRET efficiencies of $70S_I$·GDP·Pi and $70S_{II}$·GDP·Pi, we estimate this distance reduction as ~ 12 Å (eq 3); i.e., from 72 Å to 60 Å.

$$R=R_o\left(\frac{1}{E}-1\right)^{1/6} \quad (3)$$

Scheme 1 is a minimal kinetic scheme that accounts quantitatively for the results presented in Figure 5. It is fully consistent with the more complete scheme for 70SIC formation that we proposed earlier (20,27), with the one minor change that the binding of 30SIC^{Cy3} to 50S^{Cy5} is 2 – 3 fold weaker than to wt-50S. This earlier work, which employed a coumarin derivative of Bst-IF2, labeled at position 451, and fMet-tRNA^{fMet}(prf) (Figure 2D), demonstrated that conversion of the initially formed 70S complex to 70SIC required two conformational changes, corresponding approximately to steps 2 and 3 in Scheme 1, with step 2 involving a change in IF2 fluorescence and step 3 involving a change in fMet-tRNA^{fMet} fluorescence, the latter occurring at a rate very close to that of Pi release.

Results presented in Figure 7 show that FRET changes can be used to demonstrate the ability of IF3 to discriminate between a canonical (AUG) and a non-canonical (AUU) initiation codon in 70SIC formation and are consistent with the notion, proposed by us earlier (27), that such discrimination occurs during 70SIC formation. This notion has recently received direct support from some rate measurements of Milon et al. (43) showing that 70S formation from 30SIC precedes IF3 dissociation, and contrasts with results of Antoun et al. (42) indicating that IF3 dissociation from 30SIC precedes 70S formation. It is likely that the reason for this apparent disagreement has to do with the strong dependence of the rates of these two processes on mRNA sequence. Thus, both our earlier (27) and current studies and those of Milon et al. (43) employed ribosomes programmed with 022mRNA, which has a relatively short Shine–Dalgarno sequence (4 nt) separated from the AUG initiation codon by a long spacer (9 nt) and affords relatively rapid 70SIC formation. However, Milon et al. also found that use of 002mRNA, which has a long Shine–Dalgarno sequence (9 nt) and a shorter spacer (5 nt), leads to much slower 70S formation, which proceeds at the same rate as IF3 dissociation, consistent with the results of Antoun et al. (42), who used an mRNA similar to 002mRNA.

The 70S ribosome is a labile structure that undergoes conformational changes on the binding of the G-proteins EF-G and IF2. Cryoelectron microscopy studies (11,12) have shown not only that G-proteins bind to the same site on the 70S ribosome via their G(GTPase)-domains, but also that the conformational changes that result from G-protein binding as a GTP complex and from hydrolysis of the ribosome-bound GTP to GDP are similar for such proteins. This has led to the suggestion that ribosomal GTPases take advantage of the intrinsic flexibility of the ribosome to induce conformational changes that promote movement of mRNA and tRNA across the ribosome surface during the various steps of the protein synthesis cycle (11,44,45).

Our current and earlier related studies provide strong evidence that the analogy between the structures of the complexes that at least two G-proteins, IF2 and EF-G, make with the ribosome is maintained on a dynamic level as well. In particular, the kinetics of FRET efficiency increase between fluorescent derivatives of L11-NTD and either IF2 (labeled in the G1 domain, this work) or EF-G [labeled in the G’-domain (15)] have two important points in common: 1) FRET efficiency increases, indicating a movement of the G-proteins toward L11-NTD, following GTP hydrolysis and prior to Pi release (Figure 5); and 2) the rate of attainment of the higher FRET efficiency state is considerably decreased on substitution of a nonhydrolyzable analogue for GTP, although the total increase in FRET efficiency is maintained (Figure 6C). These two points provide strong evidence that at least some of the conformational changes attributed to GTPase activity are triggered by the hydrolysis step itself, rather than by Pi release, in accord with earlier suggestions (36,46,47), and that the high FRET efficiency state is the preferred mode of G-protein binding to the 70S ribosome.

Acknowledgments

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ABBREVIATIONS

Bst-IF2	G378C-IF2 from B. stearothermophilus
Bst-IF2^{Cy3}	Bst-IF2 labeled with Cy3 at position 378
cryo-EM	cryoelectron microscopy

Eco-IF2	IF2 from E. coli
FRET	fluorescence resonance energy transfer
GAC	GTPase associated center
GDPCP, GDPNP	GTP analogues in which the oxygen connecting the β- and γ–phosphorous atoms is replaced by $–CH_2-$ or –NH-, respectively
L11-NTD	L11 N-terminal domain
$L11^{Cy5}$	L11 labeled at position 38 with Cy5
MDCC	7-diethylamino-3-((((2-maleimidyl)ethyl)amino)carbonyl) coumarin
PBP	phosphate binding protein
RFE	relative FRET efficiency
TC	ternary complex, $Phe\text{-}tRNA^{Phe}$·EF-Tu·GTP
30SIC	30S initiation complex
$30SIC^{Bst}$, $30SIC^{Cy3}$, $30SIC^{Eco}$	30SICs made with Bst-IF2, Bst-$IF2^{Cy3}$, or Eco-IF2, respectively
50S-L11, 50SL11, 50SCy5	50S subunits isolated from AM77 cells and used directly, or reconstituted with L11 or $L11^{Cy5}$, respectively
70SIC	70S initiation complex

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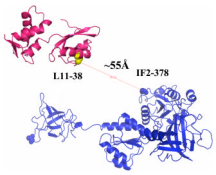

Figure 1. Distance between L11 residue 38 and IF2 residue 378 in a 70S.IF2
GMPPCP fMet-tRNA^{fMet}.mRNA complex. According to a cryoelectron microscopy structure (EMD-1172, 11).

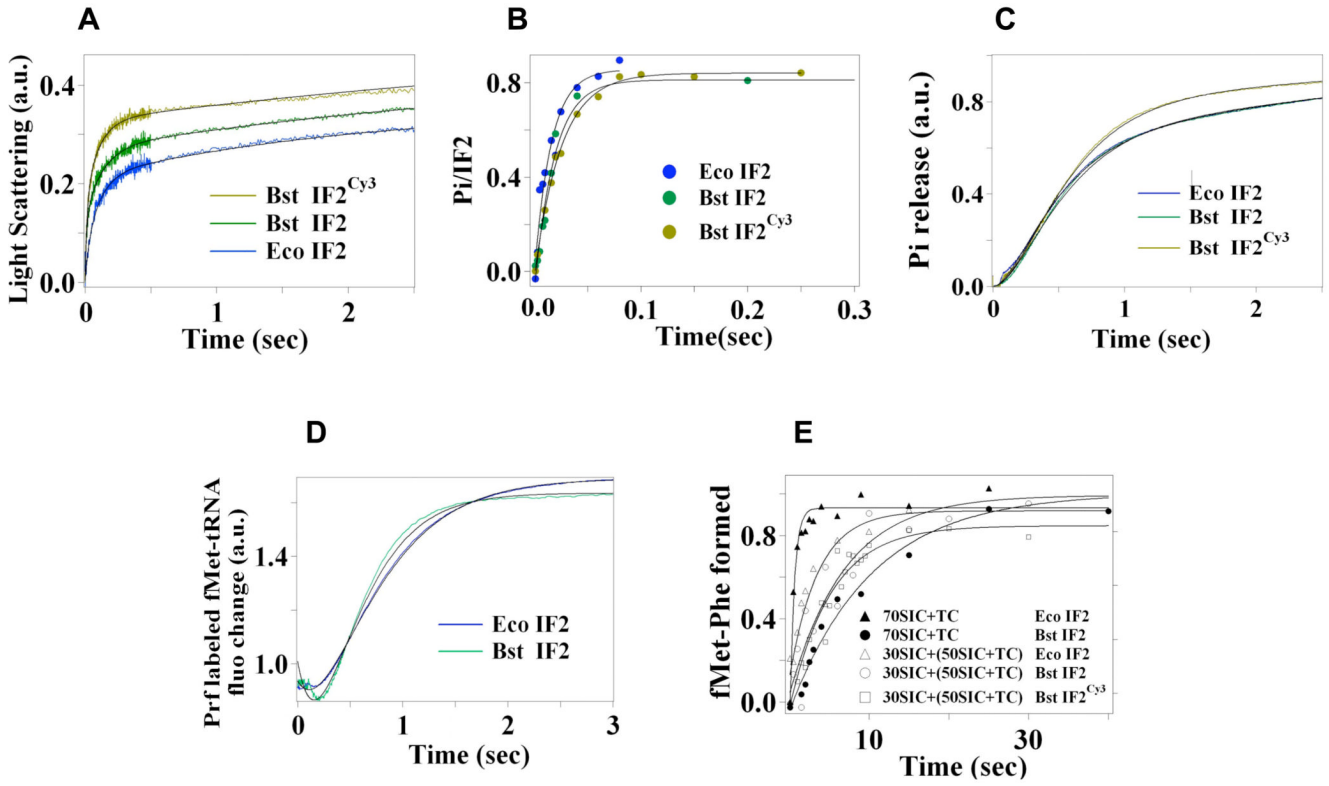

Figure 2. Measures of 70SIC formation and reactivity using different 30SICs

(A) Light scattering. (B) GTP hydrolysis; (C) Pi release; (D) fMet-tRNA^{fMet}(prf) fluorescence; (E) fMetPhe-tRNA^{Phe} formation. Experiments in (A), (C), and (D) were carried out by rapid mixing of the various 30SICs with wt-50S subunits in a stopped-flow spectrofluorometer. In all experiments except those in (E) with pre-formed 70SIC, 30S and 50S subunits were present in final concentrations of 0.3 M and 0.5 M, respectively. Preformed 70SIC in (E) was present at 0.3 M. Other final concentrations were: IF2: 0.15 M (A), (E); 0.45 M (B) – (D). GTP: 100 M (A), (C), (D); 36 M (B); 200 M (E). TC: 1.0 M (E).

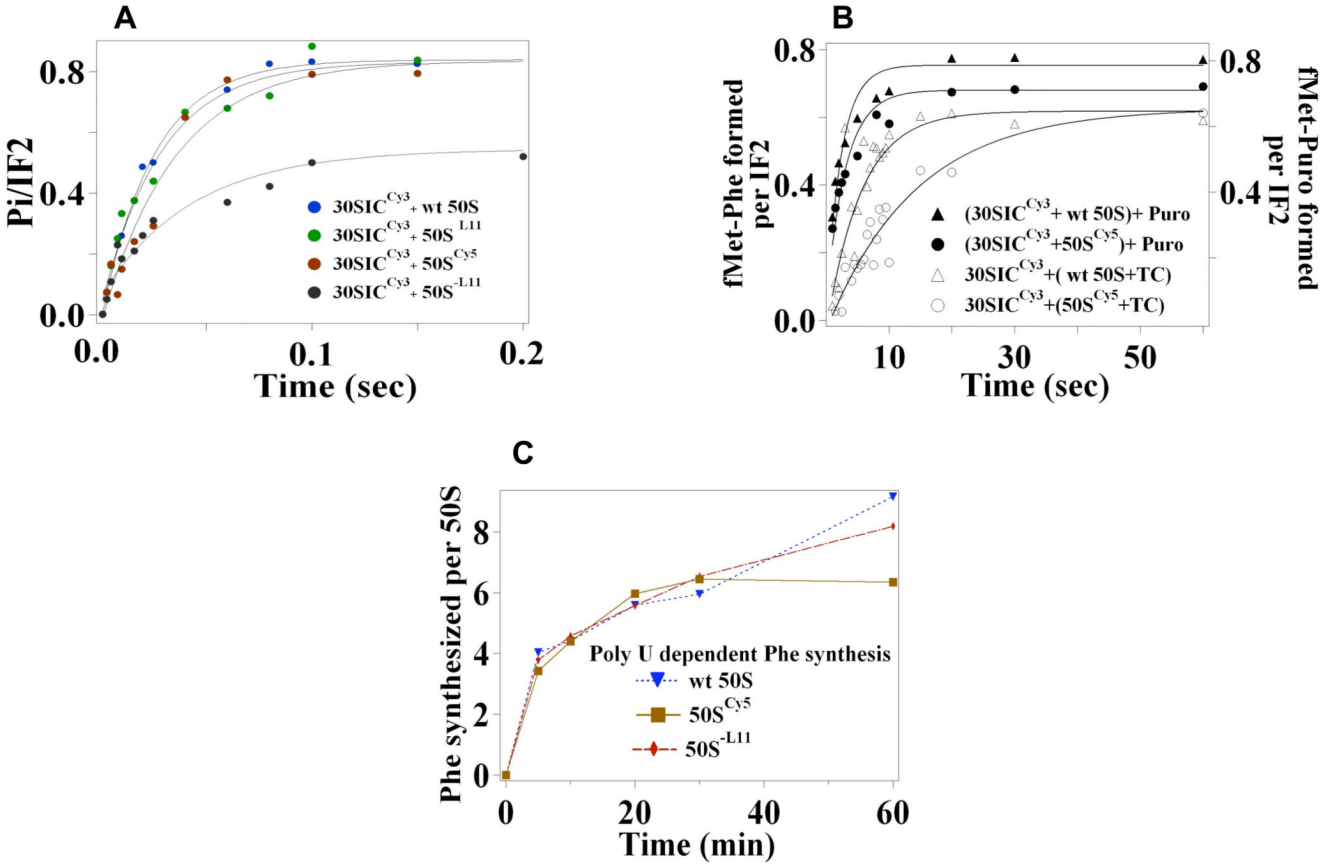

Figure 3. Measures of 70SIC formation and reactivity using different 50S subunits

(A) GTP hydrolysis; (B) fMetPhe-tRNA^{Phe} and fMet-puromycin formation; (C) Poly (U)-dependent polyPhe synthesis. fMetPhe-tRNA^{Phe} formation was carried out by rapid mixing of 30SIC^{Cy3} with 50S subunits and Phe-tRNA^{Phe}EF-Tu.GTP ternary complex. Final concentrations were: 50S, 0.5 M; 30S, 0.3 M; IF2, 0.15 M. Other final concentrations were: GTP: 36 M (A); 200 M (B); puromycin, 2.5 mM (B). (C) see Materials and Methods. Results with wt-50S and 50S^{-L11} parallel those reported earlier (40).

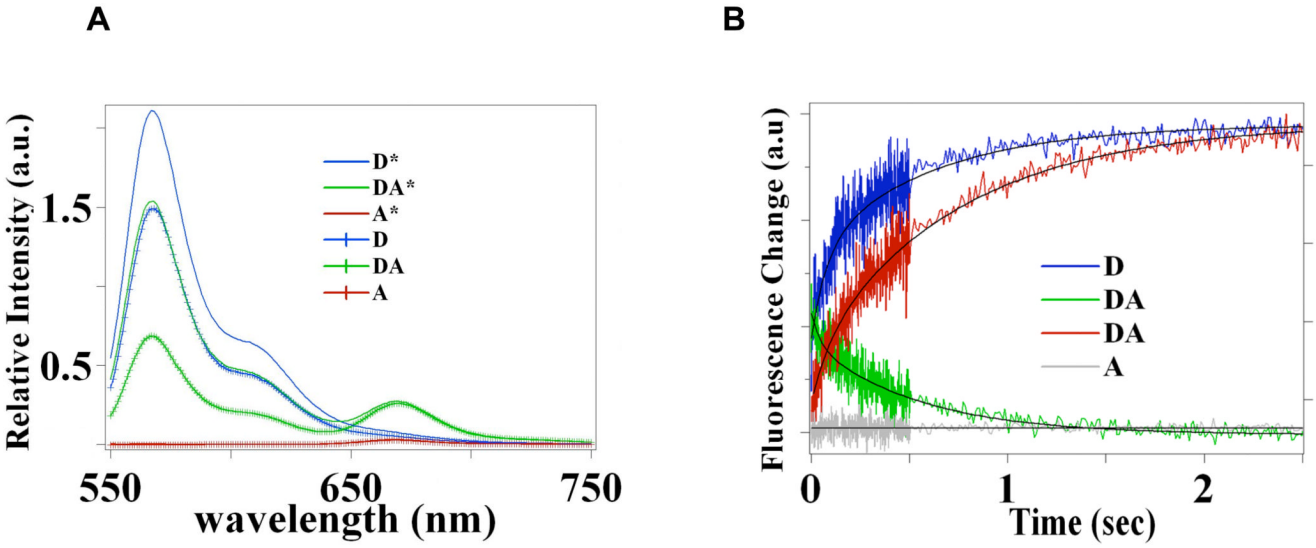

Figure 4. FRET between L11^{Cy5} and Bst-IF2^{Cy3} in the 70SIC complex

(A) After 70SIC formation. (B) During 70SIC formation. Excitation was at 540 nm. DA samples contain 30SIC^{Cy3} and 50S^{Cy5}; D samples contain 30SIC^{Cy3} and 50S^{L11}; A samples contain 30SIC^{Bst} and 50S^{Cy5}. In (A) 30SIC and 50S subunits were incubated at 37 °C for 15’ prior to the taking of fluorescence spectra. Final concentrations were: 30S, 0.3 M (hatched lines) or 0.6 M (smooth lines); Bst-IF2 or Bst-IF2^{Cy3}, 0.15 (hatched lines) or 0.3 M (smooth lines); 50S^{L11} or 50S^{Cy5}, 0.14 M; GTP, 100 M. In (B) 30SICs were rapidly mixed with 50S subunits. The D and A samples were monitored at 567 nm and 670 nm, respectively. The DA samples were monitored at both wavelengths, as indicated. Final concentrations were: 30S, 0.30 M; IF2, 0.25 M; 50S, 0.18 M; GTP, 100 M.

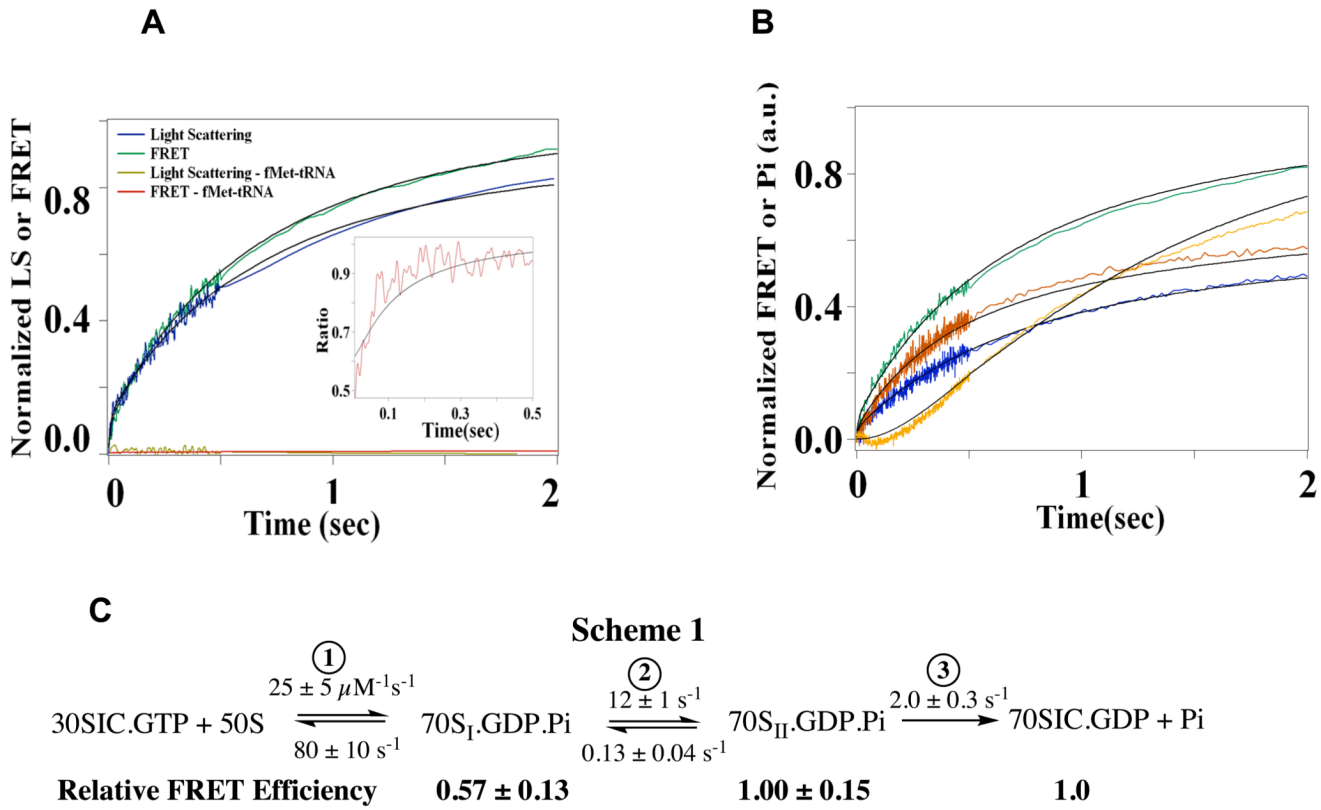

Figure 5. Measures of 70SIC formation on combining 30SIC^{Cy3} and 50S^{Cy5}: FRET, light scattering, and Pi formation

(A) Direct comparison of FRET (green trace, acceptor fluorescence – excitation 540 nm; monitoring 670 nm) and light scattering (blue trace – irradiation at 436 nm; monitoring via a 455 nm cutoff filter) changes during 70SIC formation. Both traces were determined for an identical solution having the following final concentrations: 30S, 0.3 μM; Bst-IF2^{Cy3}, 0.45 μM; 50S^{Cy5}, 0.60 μM. For ease of comparison, the changes in each value were normalized to the total change seen at the plateau for each measurement (~ 10 s). The ratio of normalized FRET change to normalized light scattering change is plotted in the inset. When fMet-tRNA^{fMet} was omitted, virtually no changes were seen in either FRET (red trace) or light scattering (yellow trace). (B) FRET changes (green, red, and blue traces) and Pi formation (orange trace). Final concentrations employed: green and orange traces: 30S, 0.3 μM; Bst-IF2^{Cy3}, 0.45 μM; 50S^{Cy5}, 0.60 μM; blue trace: 30S, 0.6 μM; Bst-IF2^{Cy3} 0.50 M; 50S^{Cy5}, 0.18 μM; red trace: 30S, 0.3 μM; Bst-IF2^{Cy3} 0.25 M; 50S^{Cy5}, 0.50 μM. FRET changes are normalized for the total change seen at the plateau for the green trace, as in (A). The Pi release is normalized for the total change seen at the plateau, achieved at ~ 5s. Final GTP concentration in (A) and (B), 100 M. All solid black lines are fits of the data to Scheme 1, with $R^2 = 0.997$ (R^2 is the square of the sample correlation coefficient between the outcomes and their predicted values). Attempts to fit the results in (A) and (B) to a two-step model resulted in significantly lower R^2 values. (C) Scheme 1, the minimal scheme accounting quantitatively for 70SIC formation in the presence of GTP.

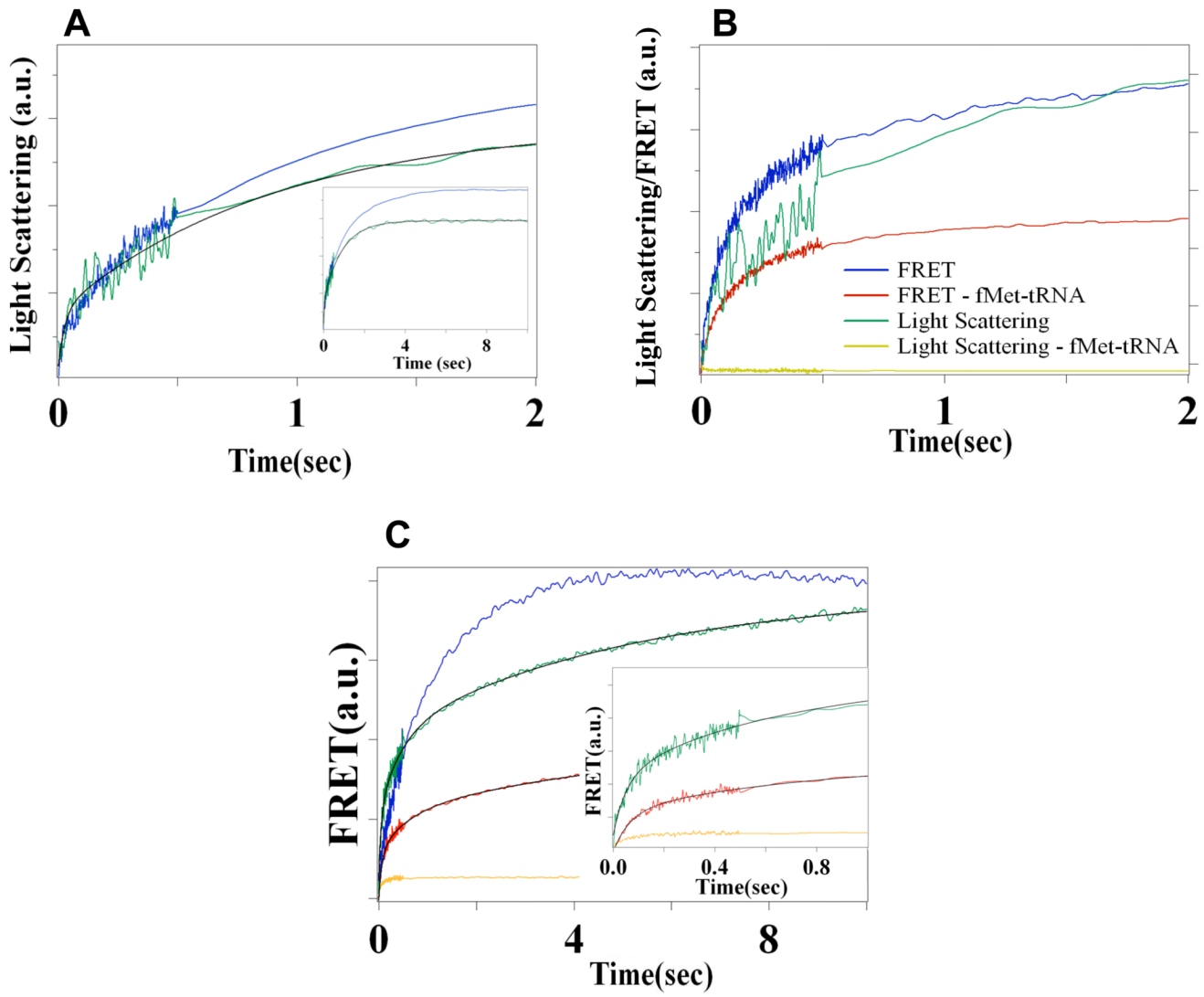

Figure 6. Measures of 70SIC formation when GDPNP replaces GTP

(A) Light scattering increase during 70SIC formation (blue trace, GTP; green trace, GDPNP). Inset: extension of results to 10 s. (B) FRET efficiency and light scattering increases during 70SIC formation measured in the presence of GDPNP. FRET efficiency, blue and red traces in the presence or absence of fMet-tRNA^{fMet}, respectively. Light scattering, green and yellow traces in the presence or absence of fMet-tRNA^{fMet}, respectively. (C) FRET efficiency increases during 70SIC formation. Blue trace: higher 30SIC concentration in the presence of GTP; green and orange traces, higher 30SIC concentration in the presence of GDPNP in the presence or absence of fMet-tRNA^{fMet}, respectively; red trace, lower 30SIC concentration in the presence of GDPNP. Inset: Expanded time scale. Final concentrations in (A) and (B) were 30S, 0.3 μM; Bst-IF2^{Cy3}, 0.45 μM; 50S^{Cy5}, 0.60 μM. Final concentrations in (C) were 30S, 0.6 μM; Bst-IF2^{Cy3}, 0.5 μM; 50S^{Cy5}, 0.18 μM (blue and green traces –higher 30SIC) or 30S, 0.3 μM; Bst-IF2^{Cy3}, 0.25 μM; 50S^{Cy5}, 0.18 μM (red trace – lower 30SIC). Final GTP or GDPNP concentration in (A) – (C), 100 M. Solid black lines are fits of the GDPNP results to either a two-phase (light scattering) or three-phase (FRET) reaction. Fitted parameter values are: light scattering - k_{app1}, 35 ± 5 s^{-1}, k_{app2}, 1.10 ± 0.03 s^{-1}; FRET, higher 30SIC - k_{app1}, 20 ± 1 s^{-1},

k_{app2}, 2.2 ± 0.1 s^{-1}, k_{app3}, 0.19 ± 0.01 s^{-1}: relative FRET efficiency amplitudes; phase 1, 0.46 ± 0.05: phase 2, 0.51 ± 0.05: phase 3, 1.00. FRET, lower 30SIC - k_{app1}, 16 ± 1 s^{-1}, k_{app2}, 1.9 ± 0.1 s^{-1}, k_{app3}, 0.19 ± 0.01 s^{-1}: relative FRET efficiency amplitudes; phase 1, 0.64 ± 0.08: phase 2, 0.47 ± 0.06: phase 3, 1.00.

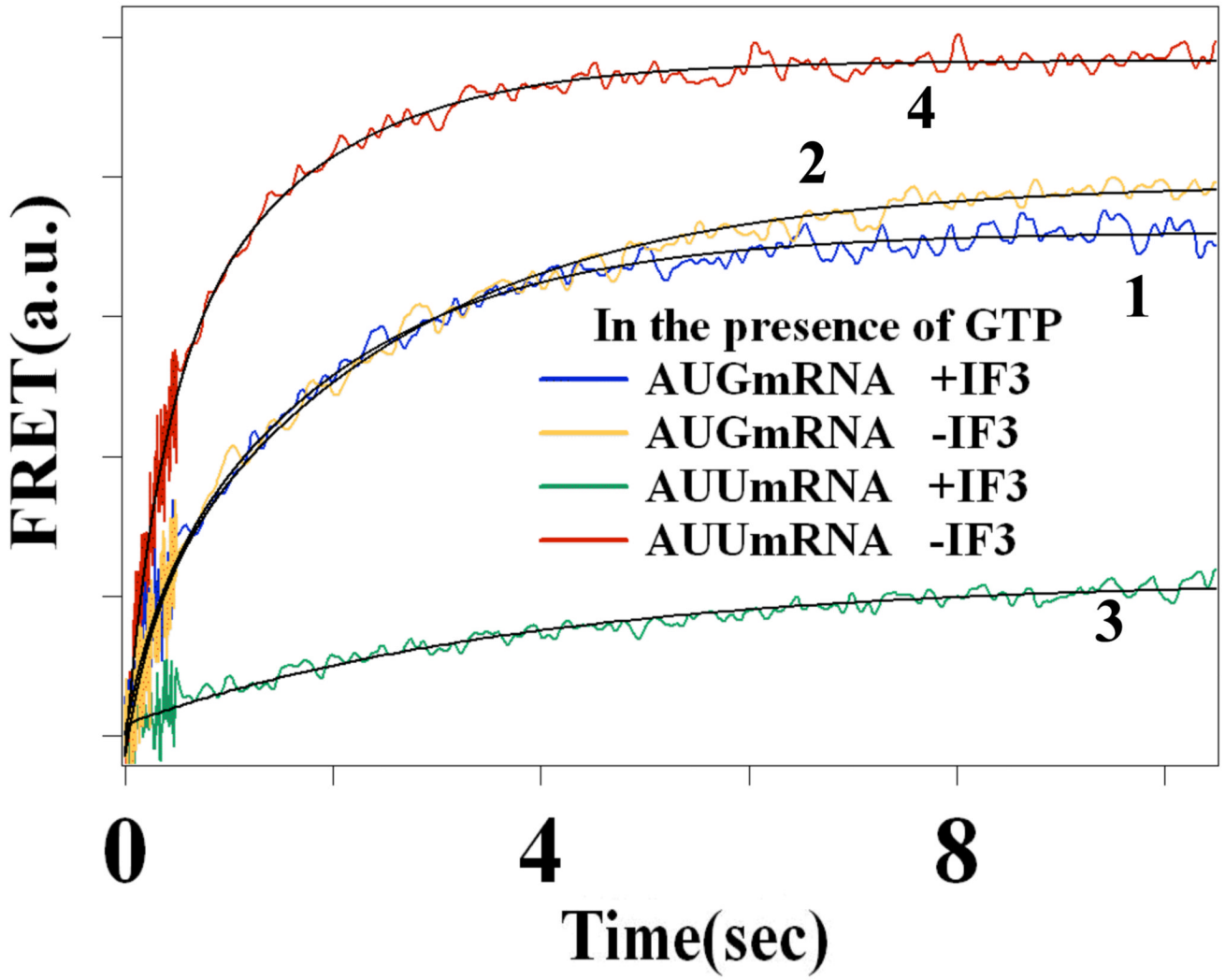

Figure 7. FRET monitoring of the fidelity function of IF3

The effect of IF3 on the rate and extent of FRET efficiency increase when the AUG initiation codon is replaced by AUU. 022AUG-mRNA, +IF3, blue trace; 022AUG-mRNA, -IF3, orange trace; 022AUU-mRNA, +IF3, green trace; 022AUU-mRNA, -IF3, red trace. Final concentrations were: $IF2^{Cy3}$, 0.15 M; 30S, 0.3 M; $50S^{Cy5}$, 0.14 M; GTP, 100 M.

Table 1

Functionality of different 30SICs with respect to 70SIC formation and reactivity[a]

Apparent rate constant (s^{-1})	$30SIC^{Eco}$	$30SIC^{Bst}$	$30SIC^{Cy3}$
LS1	61 ± 5	82 ± 5	65 ± 3
LS2	10 ± 1	8 ± 1	10 ± 1
GTP1	64 ± 13	53 ± 8	40 ± 2
fMet1	4.1± 0.4	5.2 ± 0.5	-
fMet2	1.6 ± 0.3	2.1 ± 0.2	-
Pi1	7.0 ± 0.5	7.0 ± 0.5	7.0 ± 0.5
Pi2	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
k'_{dp}[b]	0.28 ± 0.06	0.14±0.03	0.18±0.06
k^*_{dp}[c]	1.6 ± 0.2	0.12±0.02	-

[a] reaction conditions as described in the legend to Figure 2

[b] dipeptide formation, rapid mixing of 30SIC with 50S subunits and cognate TC

[c] dipeptide formation, rapid mixing of 70SIC with cognate TC

Table 2

50S subunit function in 70SIC formation: apparent rate constants and reaction stoichiometries

Parameter	wt-50S	$50S^{-L11}$	$50S^{L11}$	$50S^{Cy5}$
GTP1 (s^{-1})	41 ± 3	23 ± 6	37 ± 9	29 ± 6
Pi/IF2	0.85 ± 0.05	0.48± 0.04	0.83 ± 0.07	0.83± 0.04
$k_{,'dp}(s^{-1})$	0.18 ± 0.06	-	-	0.07 ± 0.01
fMetPhe/IF2	0.65 ± 0.07	-	-	0.66± 0.04
$k'_{puro}(s^{-1})$	0.42 ± 0.05	-	-	0.33 ± 0.04
fMet-puro/IF2	0.75 ± 0.03	-	-	0.65 ± 0.03