THE TRANSLATIONAL FIDELITY FUNCTION OF IF3 DURING THE TRANSITION FROM 30S TO 70S INITIATION COMPLEX

Christina Grigoriadou[1,2,¥], **Stefano Marzi**[1,2,†,¥], **Dongli Pan**[1], **Claudio O. Gualerzi**[2], and **Barry S. Cooperman**[1,*]

1 *Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104, USA*

2 *Laboratory of Genetics, Department of Biology MCA, University of Camerino, 62032 Camerino (MC), Italy*

SUMMARY

IF3 has a fidelity function in the initiation of translation, inducing the dissociation of fMet-tRNA^{fMet} from 30S initiation complexes (30SIC) containing a non-canonical initiation triplet (e.g., AUU) in place of a canonical initiation triplet (e.g., AUG). IF2 plays a complementary role, selectively promoting initiator tRNA binding to the ribosome. Here we use parallel rapid kinetics measurements of GTP hydrolysis, Pi release, light scattering, and changes in intensities of fluorophore-labeled IF2 and fMet-tRNA^{fMet} to determine the effects on both 30SIC formation and 30SIC conversion to 70S initiation complexes (70SIC) of a) substituting AUG with AUU, and/or b) omitting IF3, and/or c) replacing GTP with the non-hydrolyzable analogue GDPCP. We demonstrate that the presence or absence of IF3 has at most minor effects on the rate of 30SIC formation using either AUG or AUU as the initiation codon, and conclude that the high affinity of IF2 for both 30S subunit and initiator tRNA overrides any perturbation of the codon-anticodon interaction resulting from AUU for AUG substitution. In contrast, replacement of AUG by AUU leads to a dramatic reduction in the rate of 70SIC formation from 30SIC upon addition of 50S subunits. Interpreting our results in the framework of a quantitative kinetic scheme leads to the conclusion that, within the overall process of 70SIC formation, the step most affected by substituting AUU for AUG involves the conversion of an initially labile 70S ribosome into a more stable complex. In the absence of IF3, the difference between AUG and AUU largely disappears, with each initiation codon affording rapid 70SIC formation, leading to the speculation that it is the rate of IF3 dissociation from the 70S ribosome during IC70S formation that is critical to its fidelity function.

Keywords

translation initiation; IF3; fidelity; 30S initiation complex; 70S initiation complex

INTRODUCTION

Protein synthesis in prokaryotes begins with the interaction of the initiator fMet-tRNA^{fMet} and mRNA with the small (30S) ribosomal subunit. These two ligands bind stochastically to the 30S ribosomal subunit without initially interacting with each other, yielding a "30S pre-

initiation complex". A rate-limiting first-order conformational transition, which is kinetically controlled by all three initiation factors IF1, IF2 and IF3 (for reviews see references 1 and 2), transforms the 30S pre-initiation complex into a "30S initiation complex" (30SIC). In this transition, the canonical initiation codon (i.e., AUG or, more rarely, GUG or UUG)[3] moves from an initial "standby" site to the P-decoding site[4-8] where it base pairs with the 3'-UAC-5' anticodon of initiator tRNA.

The initiation factors play different roles in facilitating 30SIC[9]. IF3 influences both the on and off rates of fMet-tRNA^{fMet} binding, increasing the latter in atypical 30S complexes, such as those containing a non-canonical initiation triplet (e.g., AUU or AUC). The presence of IF3 introduces a translational bias which reduces or prevents translation starting from non-canonical initiation complexes, pseudo-initiation complexes, or complexes containing leaderless mRNA[1,2], preventing thereby formation of an "incorrect" 30SIC. This "fidelity function" of IF3 has also been described *in vivo*[3,10] and provides the molecular basis for the translational auto-regulation of *infC* expression by IF3[11-13]. fMet-tRNA^{fMet} binding to the 30SIC is reversible, but becomes essentially irreversible following 30SIC association with 50S subunits and formation of the 70S initiation complex (70SIC). Conversion of reversible to irreversible fMet-tRNA^{fMet} binding depends on release of IF3 from the ribosome[13], making IF3 release a key event in 70SIC formation.

IF2 plays a complementary role to that of IF3, selectively promoting the ribosomal binding of the initiator tRNA by increasing its on-rate, while having only a marginal effect on the binding of elongator aminoacyl-tRNAs[14-16]. Although IF2 forms a binary complex with fMet-tRNA^{fMet} via its C2 sub-domain (domain IV)[17,18], it remains unclear whether fMet-tRNA^{fMet} binds to 30S subunits as part of this binary complex[19], or rather selectively binds to a 30S complex already containing IF2[20], or whether both mechanisms are possible. The combined actions of IF2 and IF3 are thus responsible for the overall fidelity of the initiation process. Although IF1 might also have a fidelity function, recent *in vivo* experiments[21] have cast doubt on an earlier suggestion that IF1 binding within the A-site of the 30S subunit might prevent premature A-site binding of elongator aminoacyl-tRNA[22].

Previous studies have, in the main, focused on the effects of initiation factors on the fidelity of 30SIC formation, and only relatively recently has attention broadened to consider the "fidelity role" that such factors might play during the transition from 30SIC to 70SIC[23-27]. In the accompanying article[28], we present a quantitative kinetic scheme for 70SIC formation from 30SIC based on rapid kinetics measurements of five observables [light scattering, GTP hydrolysis, Pi release, and changes in the fluorescent intensities of fluorescent derivatives of IF2 (denoted IF2^C) and fMet-tRNA^{fMet} (denoted fMet-tRNA^{fMet}(prf20)]. According to this scheme (Figure 1), the initial 70S complex formed as a result of step 1, while sufficient to support IF2-dependent GTP hydrolysis (step 2), can rather readily dissociate into 30S and 50S subunits (step 2'). The subsequent conformational change that occurs in step 3 is crucial for the two most important aspects of 70SIC formation, the stabilization of the 70S particle and the subsequent accommodation of the 3'-end of fMet-tRNA^{fMet} within the 50S P-site (step 4). The latter is essentially the rate-determining step for 70SIC formation. The subsequent rapid and essentially irreversible loss of Pi (step 5) completes 70SIC formation.

In the present study we examine the effects on both 30SIC formation and 30SIC conversion to 70SIC of a) substituting the canonical AUG initiation codon with the non-canonical AUU codon, b) omitting IF3, and c) replacing GTP with the non-hydrolyzable analogue GDPCP. The effects of these changes, made singly or in combination, are determined by measurement of all or some of the same five observables used in formulating Scheme 1 (Figure 1), and the results obtained are interpreted using this scheme as a framework. We find that formation of stable 30SIC is little affected by the presence of IF3 regardless of which codon is present in

the P site, but that IF3 severely retards conversion of 30SIC to 70SIC when AUU replaces AUG.

RESULTS

Rapid high affinity IF2(GTP and fMet-tRNA^fMet binding to 30S complexes

In the accompanying article28 we used the fluorescence spectral change resulting from the binding of fluorescent IF2(GTP (IF2^C) to 30SIC* (defined as 30S subunits containing all ligands required to form 30SIC except for IF2) to demonstrate that such binding proceeds very rapidly and with high affinity via a two-phase reaction, with an overall K_d ($= k_{-1}k_{-2}/k_1k_2$) of 2.1 nM.

Substituting the non-canonical initiation triplet AUU for the canonical AUG present in the 022mRNA13 has very little effect on either the kinetics or level of IF2 binding (Figure 2A). The dependence of IF2 binding on 30SIC* concentration is similarly unaffected by the AUU for AUG substitution, with the results presented in Figure 2B permitting calculation of an overall K_d of 2.7 nM. These results are in good agreement with recent data obtained by more traditional binding studies29. The results of IF2^C binding studies similar to those displayed in Figure 2B are summarized in Table 1. They show that, in the presence of fMet-tRNA^fMet, the presence or absence of IF3 has only minor effects on IF2^C.GTP binding to 30S subunits programmed with either AUG or AUU as the start codon, with the values determined for K_d varying over a limited range (1.9 − 5.8 nM) for all four combinations investigated. However, the affinity of IF2^C.GTP for the subunit is decreased almost 20-fold in the absence of fMet-tRNA^fMet and >100-fold in the absence of both IF3 and fMet-tRNA^fMet (Table 1). These results, which are mainly accounted for by marked effects (up to a >30-fold increase) seen on k_{-1}, are consistent with earlier demonstrations that both fMet-tRNA29 and IF330 increase the affinity of IF2 for the 30S subunit.

fMet-tRNA^fMet(prf 20) also displays a biphasic spectral change on binding to 30S subunits to form a 30SIC complex. The rate and extent of such binding are, as in the case of IF2, virtually unaffected by substituting 022mRNA(AUU) for 022mRNA(AUG) (Figure 2C, Table 1). La Teana et al.13 also showed that fMet-tRNA^fMet binds with comparable stoichiometry both to 022mRNA(AUG) and 022mRNA(AUU) programmed 30S subunits under conditions similar to those we employ, but that, in the presence of IF3, fMet-tRNA dissociates more readily on dilution of the AUU complex. With respect to 30SIC formation, it is likely that the high affinity of IF2 for both 30S subunit and initiator tRNA overrides any perturbation of the codon-anticodon interaction resulting from AUU for AUG substitution. Indeed, even in the complete absence of mRNA, fMet-tRNA bound to the 30S subunit in the presence of IF2 becomes puromycin reactive upon addition of 50S subunits23.

Rapid kinetic measures of canonical 70SIC formation

Association of a 50S subunit with the canonical 30SIC containing 022mRNA(AUG) results in the formation of the canonical 70SIC. This process is accompanied by several uni- or multi-phase changes: GTP is hydrolyzed in a single phase, Pi release and light scattering (LS) changes occur in two phases, and the fluorescence changes of both IF2^C and fMet-tRNA^fMet(prf20) proceed in three phases. The apparent rate constants for the changes occurring in these phases are denoted here as: GTP1 (GTPase); Pi1, Pi2 (Pi release); LS1, LS2 (light scattering); IF2−1, IF2−2, IF2−3 (IF2^c fluorescence); and fMet1, fMet2, fMet3 [fMet-tRNA$_f$^Met(prf20) fluorescence].

These apparent rate constants, presented in Table2 (experiment #1), fall within three broad chronologicalphases that can be related to the specific steps described in Scheme 1 (Figure 1).

The rapid phase (step 1), corresponding to the initial binding of 50S subunit to 30SIC, has a net rate constant (127 s^{-1}) as measured by LS1, IF2−1, or fMet1. The subsequent intermediate phase includes steps 2, 2' and 3 [corresponding to GTP hydrolysis, reversible dissociation into subunits (K_d, 2.5 μM), and conformational change within 70S with IF2 fluorescence change, respectively] and is monitored by four different measures (LS2, IF2−2, fMet2, and P1) which provide similar estimates of a net rate constant ($5−6 \text{ s}^{-1}$). GTP hydrolysis occurs early in phase 2, and is considerably faster (GTP1 is 33 s^{-1}) than these other events. The final, slow phase includes steps 4, 5, 5' and 5" (corresponding to conformational change of fMet within 70S, Pi release, and reversible IF2^C dissociation from 70S and binding to free 50S, respectively). Its net rate constant can be measured by fMet3 and Pi2 (2 s^{-1}), or by IF2−3 (1.2 s^{-1}). The latter rate constant is somewhat lower since it includes changes due to steps 5' and 5"; which occur late in the slow phase and are not directly related to 70SIC formation, being important only in the presence of excess free 50S subunits.

Below we examine the effects on these apparent rate constants of imposing several changes, either singly or in tandem, on canonical 70SIC formation.

Interplay between IF3 and the initiation codon

Replacing 022mRNA (AUG) by 022mRNA(AUU) during 70SIC formation has little effect on LS1, fMet1 or GTP1, but leads to a dramatic decrease (18-fold) in LS2 (Table 2, experiment #2), as well as to a reduction in the magnitude of the initial phase of light scattering change (Figure 3B). These effects are most straightforwardly interpreted as reflecting a large reduction in the rate with which the labile 70S complex is converted into stable 70SIC via steps 3 and 4, resulting in a shift toward dissociation of 70S ribosome via the reversible reaction 2' into 30S and 50S subunits. Since the value of LS2 is considerably lower than Pi2 (Table 2), Pi release must occur from the labile 70S complex and/or from the 30S.IF2.GDP.Pi.fMet complex produced via step 2', albeit somewhat more slowly than during canonical 70SIC formation. The similarity in the values of IF2−3 and Pi2 indicates that such release is accompanied by an IF2^C fluorescence change.

Other marked differences are seen in the fluorescence changes of IF2^C (Figure 3C) and fMet-tRNA^{fMet}(prf 20) (Figure 3D). For IF2^C, the rapid change upon initial 50S binding is not seen, while the 2nd and 3rd phase changes are reduced in magnitude. For fMet-tRNA^{fMet}(prf 20), a rapid though smaller increase is still seen upon mixing 30SIC with 50S subunits (phase 1), but the further, slower increase seen in the presence of 022mRNA (AUG) is replaced by a barely detectable fluorescence decrease in the presence of 022mRNA (AUU), even as 70SIC formation is slowly completed (Figure 3B). These effects presumably reflect alterations in the binding of fMet-tRNA^{fMet} and IF2^C to the 70S ribosome and/or to each other as a consequence of replacing cognate codon-anticodon interaction with AUG by near-cognate interaction with AUU.

When, however, IF3 is omitted, the effects caused by substituting AUU for AUG in 022mRNA are much less pronounced (Figure 4, Table 2, experiments #3 and #4). The apparent rate constants obtained with both mRNAs are similar to each other in magnitude and also similar to those observed in the formation of the canonical 70SIC, with (LS1,IF2−1,fMet1) > GTP1> (LS2, IF2−2, fMet2, P1)>(fMet3, Pi1>IF2−3). The main differences are those observed for the intermediate phase, which proceeds somewhat more rapidly in the presence of 022mRNA (AUU) ($\sim 8.5 − 10 \text{ s}^{-1}$) than in the presence of 022mRNA(AUG) ($5−6 \text{ s}^{-1}$). With either mRNA there is also a somewhat larger difference between fMet2, Pi2 ($2.5 − 3 \text{ s}^{-1}$) and IF2−3 ($1.3 − 1.6 \text{ s}^{-1}$) compared to what is found during canonical 70SIC formation.

There are also differences vis-à-vis canonical 70SIC formation in the fluorescence changes of IF2^C (Figure 4C) and fMet-tRNA^{fMet}(prf 20) (Figure 4D). Such differences are likely to reflect

changes in the immediate environments of each fluorophore when IF3 is absent. Indeed, a cryoelectronmicroscopy reconstruction of an IF2-GDPNP-stalled 70S complex has been interpreted as indicating that IF3 is bound in a position that is very close to, and possibly in direct contact with, both fMet-tRNA^{fMet} and IF2[31]. In addition, at the single 50S concentration employed, the total change of light scattering in the absence of IF3 is greater for 022mRNA (AUU) than for 022mRNA(AUG) (Figure 4B), suggesting that differences of the codon-anticodon interaction may have a direct effect on the strength of 50S binding to 30SIC.

Replacement of GTP by GDPCP

Replacement of GTP by GDPCP has relatively modest effects on 70SIC formation, leading to no change in either the rate or magnitude of light scattering increases[26,28] and reducing only somewhat the rate and magnitude of the fluorescence increase in fMet-tRNA^{fMet}(prf 20) and the magnitudes of the IF2^C fluorescence intensity changes during the 2nd and 3rd phases[26].

Similarly, the IF3-dependent effects seen on substitution of 022mRNA(AUG) by 022mRNA (AUU) are retained when GTP is replaced by GDPCP. Thus, substitution of AUU for AUG in the presence of GDPCP leads to large decreases both in LS2 (11-fold, Table 2, experiments #s 5, 6), and in the magnitude of the initial phase of light scattering change (Figure 5A). In addition, the effect on fMet-tRNA^{fMet}(prf20) fluorescence change is very similar to that seen with GTP (compare Figures 5C and 3D). The effect on IF2^C fluorescence change is even more dramatic than in the presence of GTP (compare Figures 5B and 3C), signaling a possible difference in the conformation of IF2.GDPCP as compared with IF2.GDP.Pi bound within the noncanonical 70S complex (see Scheme 1 in Figure 1). As is the case when GTP is present, these dramatic changes induced by substituting 022mRNA(AUU) for 022mRNA(AUG) are largely reversed when IF3 is omitted (Figure 5, Table 2, experiment #7).

The interplay between IF3 and fMet-tRNA^{fMet}

Omission of fMet-tRNA^{fMet} weakens the binding of IF2 to 30S subunits programmed with cognate mRNA (Table 1) and prevents 70S formation (Figure 6A), with the result that the reduced GTPase activity (Figure 6B), monotonic decrease in IF2^C fluorescence (Figure 6C) and very slow Pi release (Figure 6D) that result from 50S addition are due mostly to direct IF2.GTP binding to 50S subunits[28]. However, when both IF3 and fMet-tRNA^{fMet} are omitted, the changes in light scattering, IF2^C fluorescence, Pi release, and GTPase occurring upon 50S addition indicate that, despite much-weakened binding of IF2^C to 30S subunits (Table 1), 70S formation has been at least partially restored. These results show that IF3 destabilizes the 70S structure even when the ribosomes are programmed with a canonical AUG start codon, and suggest that the associative activity of fMet-tRNA^{fMet} counteracts such destabilization.

Rates of dipeptide formation

Rates of fMetPhe formation were measured by rapidly mixing various forms of 30SIC with 50S subunits and ternary complex Phe-tRNA^{Phe}-EF-Tu-GTP (Figure 7, Table 3). In this experiment, 70SIC formation is followed by ternary complex binding and dipeptide formation. The overall rate constant for dipeptide formation (0.18 s^{-1}), which was determined using the canonical 30SIC complex, is virtually identical to that measured earlier[24], and provides an almost direct measure of the specific rate constant for dipeptide formation, since both 70SIC formation [overall rate constant of ~ 1.4 s^{-1} (Table 2)] and ternary complex binding to 70SIC[32] proceed much more rapidly. When 022mRNA(AUG) is substituted by 022mRNA (AUU) the rate of peptide bond formation is decreased almost 2-fold. This relatively small decrease arises from the very large decrease in LS2 on switching to the non-canonical near cognate initiation codon (from 6.5 s^{-1} to 0.36 s^{-1}, Table 2). As a result, the rates both of 70SIC formation and of the transpeptidation reaction make significant contributions to the overall rate constant for dipeptide formation. Omission of IF3 in the presence of non-canonical AUU triplet

largely restores the rate constant for dipeptide formation, to 0.16 s^{-1}, in line with the large increase in LS2. However, this rate constant is still smaller than what is seen with canonical triplet AUG in the absence of IF3 (0.24 s^{-1}). The difference is perhaps due to a small change in the positioning of bound fMet-tRNA^{fMet}, as reflected in the spectral changes observed in Figure 4D.

In the accompanying paper we showed that substituting GDPCP for GTP in the canonical 30SIC complex leads to no change in overall rate constant for dipeptide formation (~0.2 s^{-1}) but with a reduced stoichiometry[28]. The same pattern is seen for the noncanonical complex, in which once again the lower rate constant (0.1 s^{-1}) vs. canonical complex reflects a large decrease in LS2 (Table 2, experiments#2 and #6).

Effect of IF3 concentration on rates of light scattering change

The results presented above provide strong evidence for the involvement of IF3 in distinguishing between canonical vs. non-canonical mRNA within the 70S initiation complex, requiring that 70S formation precede IF3 dissociation. Consistent with this view, we find the two apparent rate constants describing light scattering increase on 50S addition to 30SIC to be essentially independent of IF3 concentration over the range 0.0−3.0 μM (Figure 8), having average values similar to those shown in Table 2 (Experiment # 1). Since the second phase of light scattering change includes Scheme 1 through step 3, these results suggest that IF3 dissociation occurs during or subsequent to step 3.

DISCUSSION

As shown by our present results and in the accompanying paper[28], conversion of a canonical 30SIC into a canonical 70SIC can be conveniently described as a three-phase process. In the first phase, rapid subunit association leads to formation of a labile 70S complex and induces a first conformational change within IF2, thereby activating its GTPase activity. In the second phase, the labile 70S complex either dissociates reversibly into 30S and 50S subunits or is converted to a more stable form, with the latter conversion being coupled to a second IF2 conformational change. The third phase, resulting in 70SIC formation involves movement of fMet-tRNA^{fMet}, most plausibly to occupy the tRNA P-site within the 50S subunit, and a third IF2 conformational change largely associated with release of Pi. The second and/or third changes likely coincide with a progressive weakening of the interaction between subdomain C2 of IF2 and the acceptor end of fMet-tRNA^{fMet}[18]. This flow of events is in good agreement with recent cryo-EM studies showing that IF2 bound within a 70S complex remains in strong contact with fMet-tRNA^{fMet} prior to GTP hydrolysis[31] and undergoes major conformational changes following conversion of IF2.GTP to IF2.GDP[33].

During the course of 70SIC formation, IF2 is bound to 70S ribosomes in three different forms: IF2.GTP, IF2.GDP.Pi, and IF2.GDP (Figure 1). Our experiments, together with those of others, suggest that, in the presence of IF3, rapid conversion of the labile 70S complex to a more stable form has two requirements: first, that the canonical AUG initiation codon be present, rather than the noncanonical AUU initiation codon; second, that IF2 be bound as the IF2.GDP.Pi complex, or its putative structural homologue, IF2.GDPCP[33]. Thus, rapid conversion is achieved with ribosomes containing an AUG initiation codon and either bound IF2.GDP.Pi, formed from added IF2.GTP (Scheme 1), or bound IF2.GDPCP (Table 2, experiments #1 and #5). In contrast, such conversion is slow when AUU replaces AUG, even with bound IF2.GDPCP (Table 2, experiments #2 and #6). Further, replacement of IF2.GTP with IF2.GDP slows 70SIC formation from 30SIC by ~ 10-fold, even with AUG as the initiation codon[25].

The observed differences between AUG- and AUU-programmed ribosomes with respect to 70SIC formation depend for the most part on the presence of IF3 (Figures 3 and 4, Table 2),

consistent with the well-established role of this factor in ensuring the overall fidelity of translation initiation by rejecting non-cognate or other non-standard initiation complexes1-3. Here we show that IF3 discrimination between the cognate, canonical AUG and near-cognate, non-canonical AUU initiation codons, which had been shown to occur within the 30S subunit, is also exercised during 70SIC formation. A functional role for IF3 within 70S ribosomes is supported by the results of three other studies. First, time-resolved chemical probing studies of the 30S-50S association process show that inter-subunit bridges are formed sequentially34 and that IF3 remains at least partially bound to the 30S subunit during the formation of at least some of the bridges35. Second, as already noted, there is cryo-electronmicroscopy evidence that IF3 remains ribosome-bound within an IF2-GDPNP-stalled 70S complex31. Third, monitoring of FRET signals between fluorescently-labeled IF3 and other 30S ligands, including fMet-tRNA, demonstrate that IF3 is dissociated from the 30S subunit during, rather than prior to, formation of the 70SIC (Milon, P., Konevega, A.L., Gualerzi, C.O. and Rodnina, M.V., manuscript in preparation).

A role for IF3 within 70S subunits is also consistent with the lack of effect that raising IF3 concentration has on formation of the 70S complex on addition of 50S subunits to 30SIC (Figure 8). In contrast to these results and the conclusions reached in this work, Antoun et al. 27 reported that excess IF3 strongly inhibits 70S formation and concluded that IF3 dissociation must precede 70S formation. At present we can only speculate as to the reasons for this apparent discrepancy. Differences in the upstream sequences from the AUG initiation codon in the mRNA employed in both studies may be significant. The 022 mRNA used here has a relatively short Shine-Dalgarno sequence (4 nts) separated by a long spacer (9 nts) from AUG13. By contrast, the MFTI-mRNA used by Antoun et al.27 has a relatively long Shine-Dalgarno sequence (8 nts) separated by a short spacer (5 nts) from AUG. Recent results (Milon, P., Konevega, A.L., Gualerzi, C.O. and Rodnina, M.V., in preparation) demonstrate that such differences strongly impact rates of 30SIC association with 50S subunits and of initiation factor dissociation during 70SIC formation.

How does IF3 exercise its fidelity role within the 70S ribosome? In the absence of IF3, there is little codon selection between AUG and AUU (Table 2). Both initiation codons lead readily to 70SIC formation, with, somewhat surprisingly, overall reaction proceeding slightly faster with AUU than with AUG (experiments #3 and #4). This situation changes dramatically in the presence of IF3, which, while having little effect on the rate of 70SIC formation with the AUG codon (experiments #1 and #3), strongly inhibits the rate of 70SIC formation with the AUU codon (experiments #2 and #4). The similarity of the apparent 2nd phase rate constants (LS2, IF2−2, Pi1, Fmet2) for the 70S AUG complex in the presence and absence of IF3 (experiments #1 and #3) strongly implies that the conformational change taking place in this phase, corresponding to reversible step 3 in Scheme 1, precedes IF3 release. The product of step 3 is a stable 70S ribosome. As a stable 70S ribosome is sterically incompatible with bound IF336 we hypothesize that, for the 70S AUG complex, the conformational change in step 3 is followed by rapid IF3 release and the formation of additional bridges between the 30S and 50S subunits, thereby driving the reaction forward. According to this hypothesis, the inhibitory effect of IF3 on step 3 for the 70S AUU complex is due to its failure to rapidly dissociate from the ribosome as a part of step 3. This is consistent with recent results (Milon, P., Konevega, A.L., Gualerzi, C.O. and Rodnina, M.V., in preparation) showing that substituting AUU for AUG as the initiation codon retards IF3 release during 70SIC formation.

Irrespective of the precise kinetic mechanism, the question remains as to the structural basis for the IF3 fidelity function within the initially formed labile 70S ribosome. At the level of 30SIC formation IF3 could discriminate against non-canonical complexes via direct inspection of the peculiar features of the anticodon stem-loop of initiator tRNA16,37, so direct interaction is one possibility. On the other hand, long-range, ribosome-mediated conformational change

has been suggested as an explanation for IF3 discrimination against "non-standard" 30SIC 2, 35,37-40. The same might be true for discrimination within a 70S ribosome. Resolution of this issue will likely require careful structural studies comparing both the 30SIC and 70SIC formed in the presence of canonical and noncanonical initiation codons.

MATERIALS AND METHODS

Materials

All materials were prepared as described previously28. 022AUU mRNA has been described12 and was prepared as previously described for 022AUG mRNA28.

Rapid kinetics measurements

All measurements were performed in 25 mM Tris-HCl pH 7.5; 70 mM NH_4Cl; 30 mM KCl; 7 mM $MgCl_2$; 1 mM DTT at 20 °C. IF2 refers to *B. stearothermophilus* V451C-IF2. Stopped-flow fluorescence (IF2[c], fMet-tRNA[fMet], Pi release) and light-scattering measurements were carried out in an SX.18MV stopped-flow spectrophotometer. Quenched flow measurements (GTPase, dipeptide formation) were carried out in a KinTec RQF-3 apparatus. All measurements were carried out as described previously28.

IF2[c] and fMet-tRNA[fMet](prf20) binding to 30S subunits

All 30S-containing solutions were preincubated for 15 min at 37 °C prior to mixing with either IF2[C] or fMet-tRNA[fMet](prf20). For IF2[C] binding experiments, IF2[C] was preincubated with GTP for 15 min at 37 °C, and then rapidly mixed with increasing concentrations of 30SIC*. Final concentrations of IF2[C] and GTP were 0.15 μM and 100 μM, respectively. Final concentrations of 30S were: 0.05 μM; 0.1 μM; 0.30 μM. 30SIC* contained, relative to 30S subunit: IF1, IF3 (when present), fMet-tRNA[fMet](when present), 1.5 equivalents; 022mRNA (AUG or AUU), 3.0 equivalents. For fMet-tRNA[fMet](prf20) binding experiments, final concentrations were: fMet-tRNA[fMet](prf20), 0.18 μM; 30S subunits, 0.3 μM; IF1, IF2, IF3, 0.45 μM; 022mRNA (AUG or AUU), 0.9 μM; GTP 100 μM.

Measurements during 70S formation

30S complexes were preincubated for 37 °C for either 15 min (IF2[C] and fMet-tRNA[fMet](prf20) fluorescence, light scattering, Pi release) or 10 min (GTPase) and rapidly mixed with 50S subunits. Final 50S concentration was 3.0 μM for measurements of GTPase, light scattering, and IF2[C] fluorescence, and 1.2 or 3 μM for measurements of Pi release and fMet-tRNA[fMet](prf20) fluorescence. The standard final concentrations of 30SIC components were: 30S, 0.3 μM; IF1, IF2, IF3 (when present), fMet-tRNA$_f$[Met] (when present), 0.45 μM; 022mRNA (AUG or AUU), 0.9 mM; GTP or GDPCP 100 mM. Deviations from these standard concentrations are noted below.

- GTPase activity. The final concentrations of 30SIC components were 30S, 0.45 μM; IF2, 0.3 μM; [g-32P] GTP (~1000 d.p.m./ pmol), 36 mM. GTP hydrolysis stoichiometries reported in Figures 3, 4, and 6 were normalized to the stoichiometry obtained with 70S ribosomes in the canonical system (AUG022mRNA in the presence of IF3 and fMet-tRNA[fMet]). This value varied from 0.7−0.9 /IF2.

- Light scattering. The final concentration of IF2 was 0.15 μM.

- IF2[C] fluorescence. IF2 was replaced by IF2[C] at a concentration of 0.15 μM.

- fMet-tRNA[fMet](prf20) fluorescence. fMet-tRNA[fMet] was replaced by fMet-tRNA[fMet](prf20) at a final concentration of 0.18 μM.

Dipeptide formation

30S complexes were preincubated for 37 °C for 15 min and rapidly mixed with a solution containing 50S subunit and EF-Tu.Phe-tRNA^{Phe}.GTP ternary complex. Final concentrations were: 50S subunit, 0.6 mM; ternary complex, 1.0 μM; 30S, 0.3 μM; IF1, fMet-tRNA$_f^{Met}$, IF3 (when present) 0.45 μM; IF2 0.15 μM; 022mRNA (AUG or AUU), 0.9 μM; GTP or GDPCP, 200 μM.

Kinetic analyses

Igor-Pro (Wavemetrics) was used to determine best fit values for GTP1 (single exponential), LS1 and LS2 (double exponential); IF-1, IF-2, and IF-3 (triple exponential), dipeptide formation (single exponential), and fMet-tRNA^{fMet}(prf20) binding to 30S subunits (double exponential). Global fitting using the program Scientist (MicroMath Research, LC) was employed to fit values for Pi1, Pi2, fMet1, fMet2, and fMet3 and for IF2^C·GTP interaction with 30SIC* via a two-step binding reaction.

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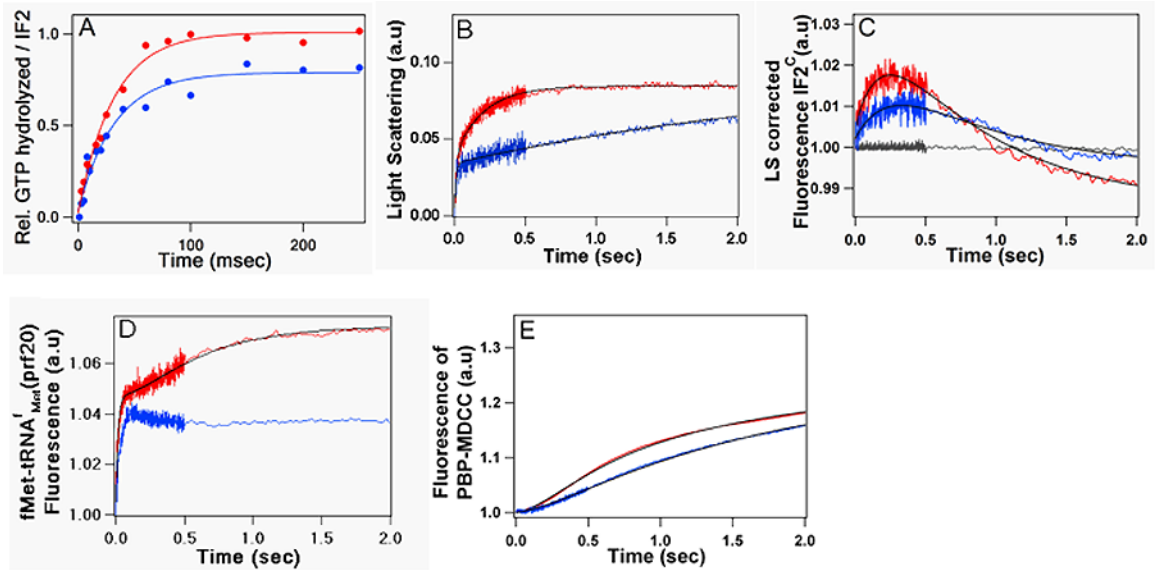
SCHEME 1 - 70SIC Formation

$$50S.F_F.GDP$$

$$0.6\pm0.1 \ s^{-1} \ \big\| \ 5\pm1\mu M^{-1}$$
$$+ 50S \ \big\| \ s^{-1} \ (5')$$

$$30S.F_A.GXP.M_N \qquad 30S.F_{A'}.GDP.Pi.M_{N'}$$
$$+ 50S \qquad\qquad\qquad + 50S$$

$$70S.M_P + F_E.GDP$$

$$35\pm4s^{-1} \ \big\| \ 34\pm14 \ \mu M^{-1}s^{-1} \quad 12\pm3 \ s^{-1} \ \big\| \ 4.8\pm0.4 \ \mu M^{-1}s^{-1} \qquad\qquad 4\pm2s^{-1} \ \big\| \ 200\pm100\mu M^{-1}$$
$$(1) \qquad\qquad\qquad\qquad (2') \qquad\qquad\qquad\qquad\qquad\qquad s^{-1} \ (5')$$

$$70S.F_B.GXP.\ M_O \xrightarrow{44\pm10 \ s^{-1}} 70S.F_B.GDP.Pi.\ M_O \xrightleftharpoons[2.1\pm0.8 \ s^{-1}]{24\pm 10 \ s^{-1}} 70S.F_C.GDP.Pi.\ M_O \xrightarrow{2.3\pm0.3 \ s^{-1}} 70S.F_C.GDP.Pi.\ M_P \xrightarrow[-Pi]{12\pm1 \ s^{-1}} 70S.F_D.GDP.M_P$$
$$(2) \qquad\qquad\qquad\qquad (3) \qquad\qquad\qquad\qquad (4) \qquad\qquad\qquad (5)$$

$$\big\downarrow \ + TC$$

dipeptide
formation
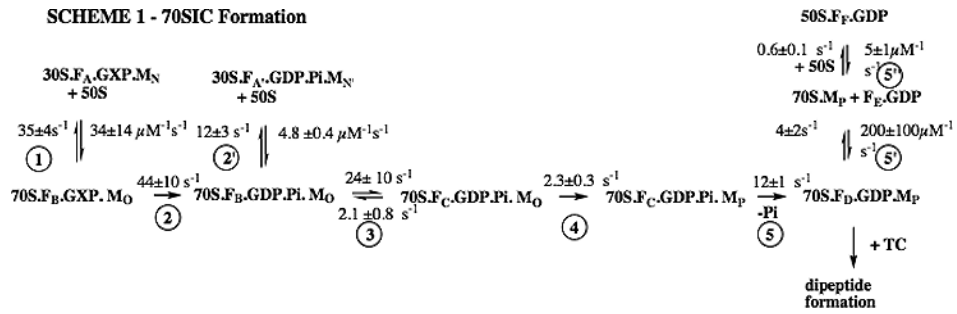
Figure 1. A quantitative scheme for 70SIC formation, emphasizing the role of IF228

Here GXP is either GTP or GDPCP; F is IF2; M is fMet-tRNA^{fMet}; 30S is 30S containing IF1, IF3, and mRNA; and TC is ternary complex (Phe-tRNA^{Phe}.EF-Tu.GTP). The subscripts A-F and N-P refer to different conformations of IF2^C and fMet-tRNA^{fMet}(prf20), respectively, that have different fluorescent intensities.
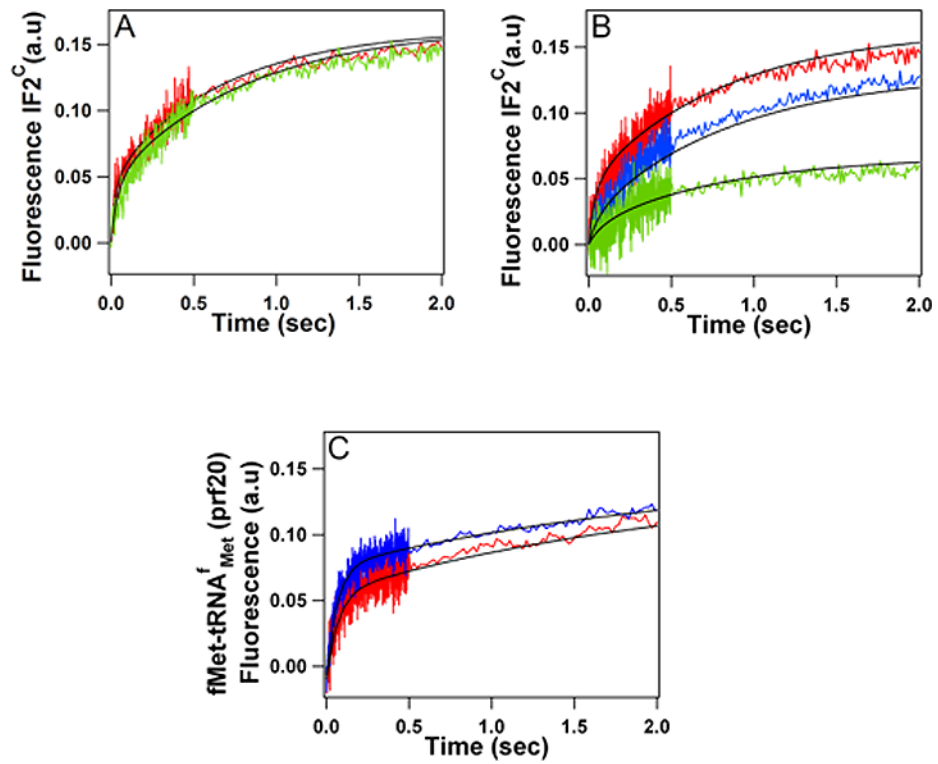
Figure 2. IF2^C and fMet-tRNA^{fMet}(prf20) binding to 30S subunits
(A) IF2^C·GTP binding to 30SIC* subunits programmed with AUG022mRNA (red trace) or AUU022mRNA (green trace). (B) IF2^C·GTP binding to varying concentrations of 30SIC* programmed with AUU022mRNA: 0.3 μM, red trace; 0.1 μM, blue trace; 0.05 μM, green trace. (C) fMet-tRNA^{fMet} binding to 30S subunits programmed with AUG022mRNA (red trace) or AUU022mRNA (blue trace).

Figure 3. The effects of replacing AUG with AUU on measures of 70SIC formation in the presence of IF3

(A) GTPase. (B) Light Scattering. (C) IF2^C fluorescence change. (D) fMet-tRNA$^f_{Met}$(prf-20) fluorescence change. (E) Pi Release. AUG022mRNA (red traces), AUU022mRNA (blue traces). The gray trace in (C) is a control of 30SIC in the absence of added 50S.
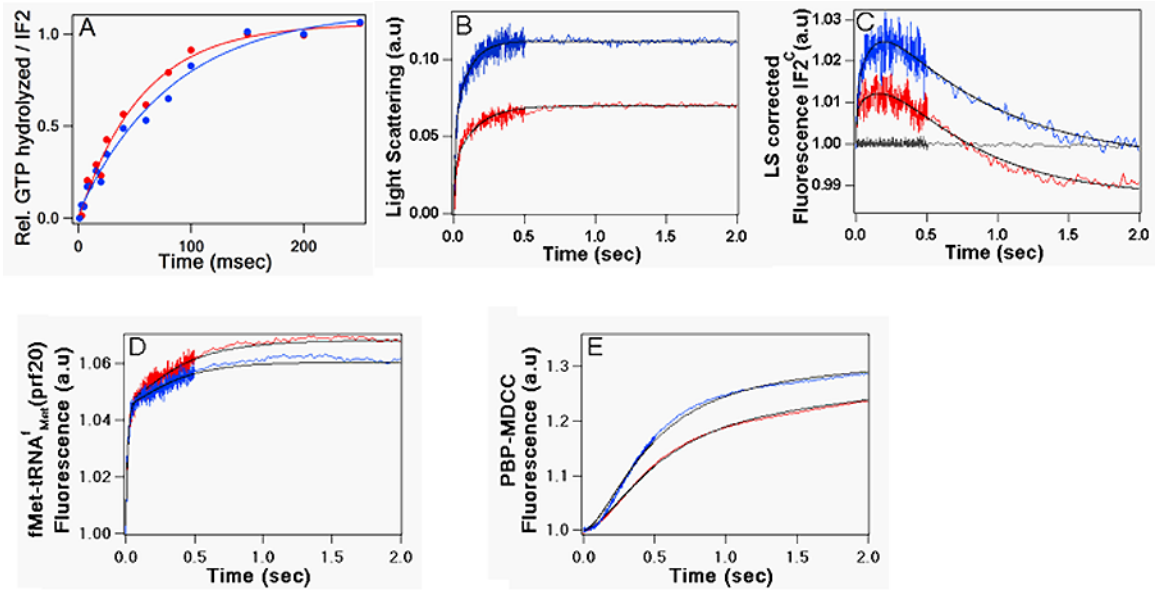
Figure 4. The effects of replacing AUG with AUU on measures of 70SIC formation in the absence of IF3

(A) GTPase. (B) Light Scattering. (C) IF2^C fluorescence change (D) fMet-tRNA$^f_{Met}$(prf-20) fluorescence change and (E) Pi Release. AUG022mRNA (red traces), AUU022mRNA (blue traces). The gray trace in (C) is a control of 30SIC in the absence of added 50S.
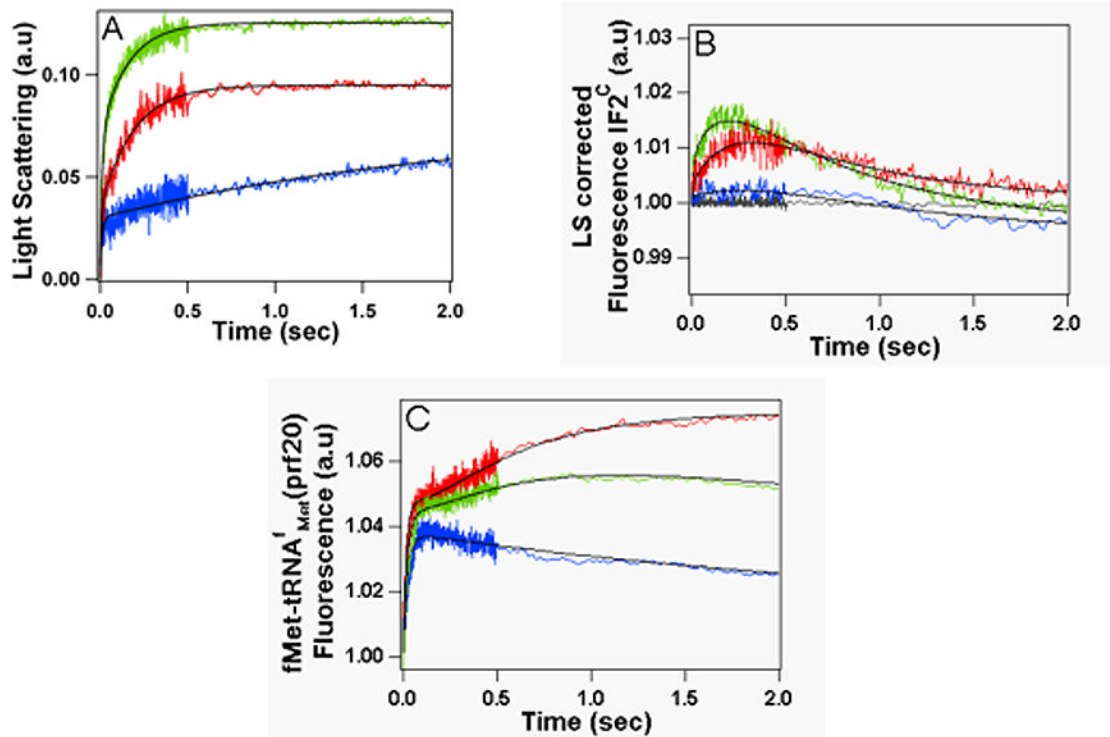
Figure 5. The effects of replacing GTP with GDPCP on measures of 70SIC formation
(A) Light Scattering. (B) IF2^C fluorescence change (C) fMet-tRNA^{fMet}(prf20) fluorescence
change. Red traces AUG022mRNA + IF3; Blue traces, AUU022mRNA + IF3; Green traces,
AUU022mRNA - IF3. The gray trace in (C) is a control of 30SIC in the absence of added 50S.
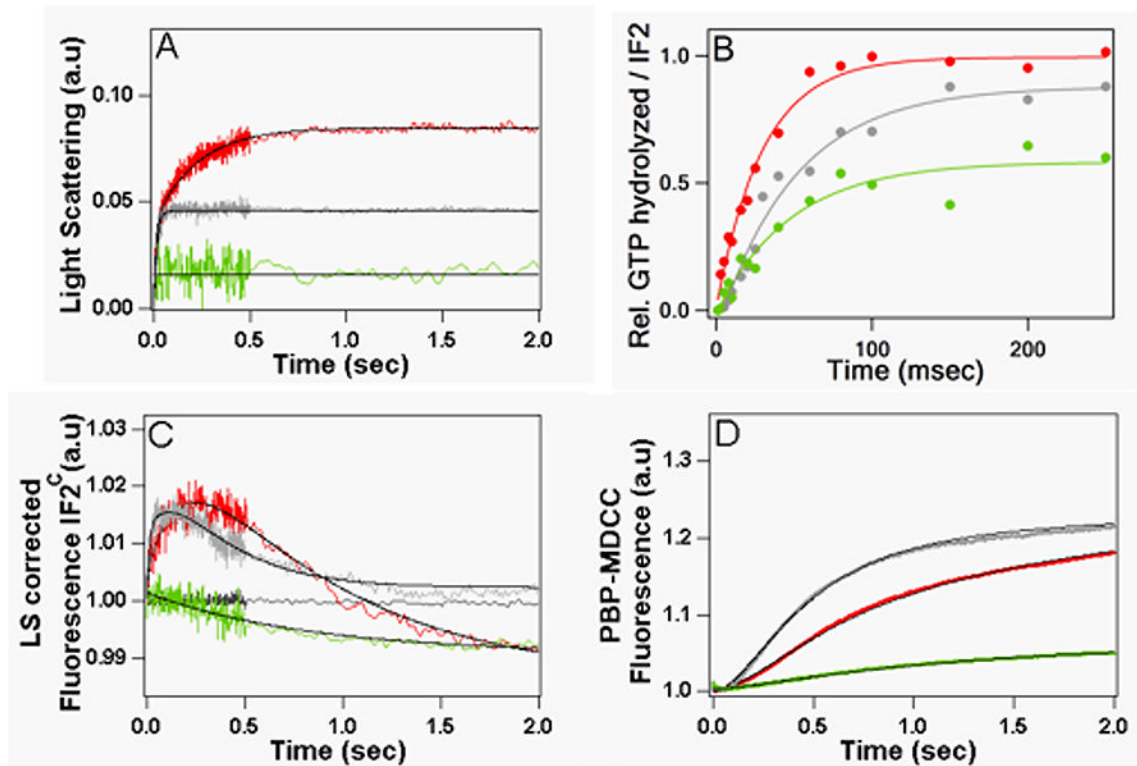
Figure 6. The effects of IF3 on 70S formation in the absence of fMet-tRNA^{fMet}
(A) GTPase. (B) Light Scattering. (C) IF2^C fluorescence change and (D) Pi Release. AUG022mRNA and GTP were used in all experiments. Green traces, +IF3. Grey traces, -IF3. Red traces, reproduced from Figure 3 for comparison, are in presence of fMet-tRNA^{fMet} and IF3.
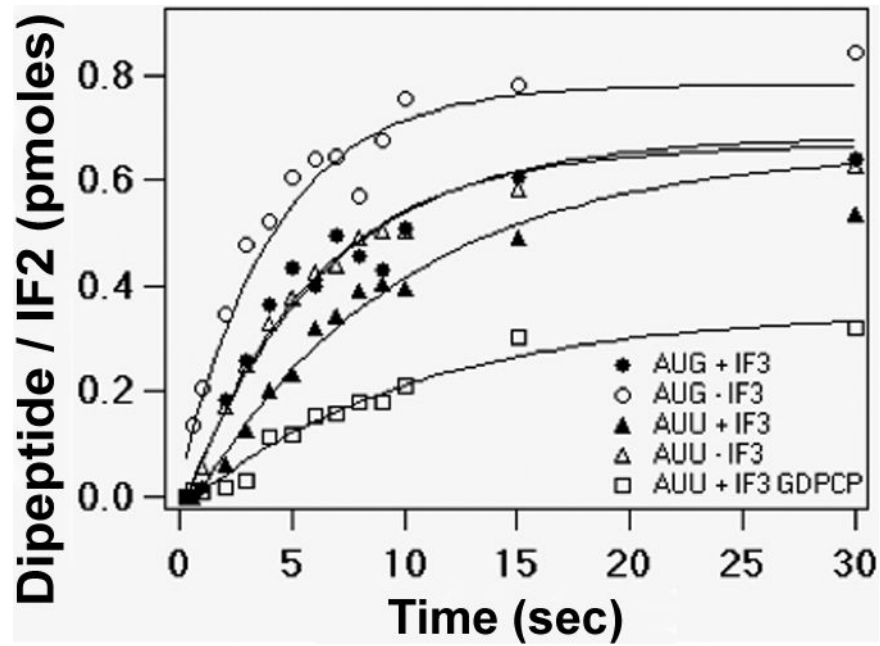
Figure 7. The effects of several substitutions on the rate of dipeptide formation from 70SIC
Canonical 70SIC, λ; -IF3, μ; AUU022mRNA, σ; AUU022mRNA, – IF3, Δ; AUU022mRNA, GDPCP, □.
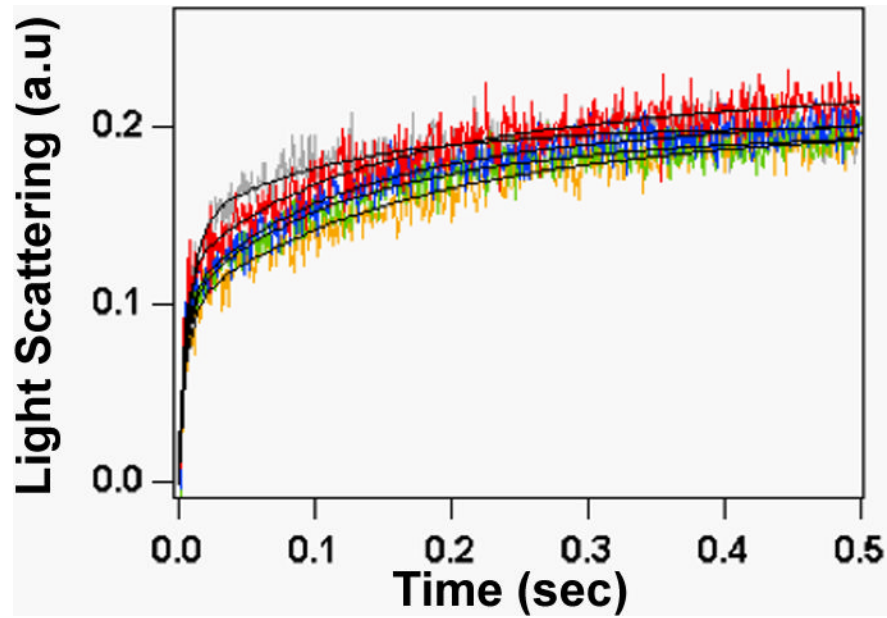
Figure 8. Effect of IF3 on 70SIC formation as monitored by light scattering
30SIC, containing increasing concentrations of IF3, was rapidly mixed with 3.0 μM 50S ribosomal subunits. IF3 concentrations were: 3.0 μM, red trace; 1.5 μM, blue trace; 0.45 μM, green trace; 0.225 μM brown trace; no IF3, grey trace. Other 30SIC components were employed at the standard final concentrations described in Materials and Methods.

Table 1

Ligand binding to 30S[a]

	Added ligand	IF3	fMet-tRNA	mRNA	k_1, $\mu M^{-1} s^{-1}$	k_{-1}, s^{-1}	k_2, s^{-1}	k_{-2}, s^{-1}	K_{ov}, nM
1	IF2^c.GTP	+	+	AUG	285±12	1.2±0.1	1±0.1	0.5±0.1	2.1
2	IF2^c.GTP	−	+	AUG	310±23	1.2±0.24	0.5±0.1	0.75±0.10	5.8
3	IF2^c.GTP	+	−	AUG	190±12	3.4±0.4	0.4±0.1	0.8±0.1	35
4	IF2^c.GTP	+	−	AUG	111±12	37±5	1.1±0.1	0.9±0.1	273
5	IF2^c.GTP	+	+	AUU	94±3	0.3±0.04	0.7±0.1	0.6±0.1	2.7
6	IF2^c.GTP	−	+	AUU	79±3	0.1±0.03	0.4±0.1	0.6±0.12	1.9
7	fMET	+	+	AUG	14.5[b]		0.45		
8	fMET	+	+	AUU	15.8[b]		0.43		

[a] All solutions contained IF1, mRNA, and 30S subunits in a molar ratio of 1.5:3:1. When present the ratio of IF3:30S was 1.5:1. GTP was 100 μM. For experiments 1 – 6, 30S concentration varied from 0.05 – 0.30 μM, IF2 was 0.15 μM. For experiments 7 and 8, concentrations were: 30S, 0.3 μM; IF2, 0.45 μM, fMet(Prf20) 0.18 μM. All concentrations are final.

[b] in s^{-1}

Table 2

Apparent rate constants in 70SIC formation

Expt		Reaction components						Apparent rate constants (s^{-1})					
#	mRNA	IF3	fMet-tRNA	GXP	LS1/IF2−1/fMet1[a]	GTP1	LS2	IF2−2	fMet2	Pi1	fMet3	Pi2	IF2−3
1	AUG	+	+	GTP	127±24	33±3	6.5±1	5.6±0.5	4.9±0.5	4.6±0.5	2.2±0.2	1.9±0.2	1.2±0.1
2	AUU	+	+	GTP	89±9[b]	32±5	0.36±0.04	4.0±0.4	−[d]	3.4±0.3	−[d]	1.1±0.1	1.3±0.1
3	AUG	−	+	GTP	108±16	18±2	6 ± 2	5.1±0.5	10 ± 3[e]	5.3±0.5	2.9±0.3	2.9±0.2	1.6±0.2
4	AUU	−	+	GTP	91±9	12±1	10±1	9±1	11 ± 3	8±2	3.3±0.3	2.5±0.3	1.2±0.1
5	AUG	+	+	GDPCP	129±13	na[c]	5.3±0.5	5.2±0.5	4.0±0.4	na	1.1±0.1	na	1.3±0.1
6	AUU	+	+	GDPCP	89±10[b]	na	0.47±0.05	3.2±0.6	−[d]	na	−[d]	na	1.0±0.1
7	AUU	−	+	GDPCP	84±8	na	6.3±0.6	7.6±0.7	5.9±0.8	na	1.7±0.2	na	1.2±0.1

[a] In general, these three processes occur with indistinguishable apparent rate constants. Accordingly, values obtained by fitting light scattering data were used for all three accept as otherwise noted for IF2−1 (footnote b).

[b] No rapid phase of IF2 fluorescence change was detected in these experiments,.

[c] na, not applicable

[d] lack of fluorescent change following initial fMet-tRNA^{fMet}(prf20) binding prevented rate constant calculation

[e] anomalously high value of low precision

Table 3

Apparent rate constants for dipeptide formation

Expt	mRNA	GXP	IF3	k_{app} (s^{-1})
1	AUG	GTP	+	0.18 ± 0.02
2	AUG	GTP	−	0.24 ± 0.04
3	AUU	GTP	+	0.10 ± 0.01
4	AUU	GTP	−	0.16 ± 0.01
5	AUU	GDPCP	+	0.10 ± 0.01