Activation of initiation factor 2 by ligands and mutations for rapid docking of ribosomal subunits

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We previously identified mutations in the GTPase initiation factor 2 (IF2), located outside its tRNA-binding domain, compensating strongly (A-type) or weakly (B-type) for initiator tRNA formylation deficiency. We show here that rapid docking of 30S with 50S subunits in initiation of translation depends on switching 30S subunit-bound IF2 from its inactive to active form. Activation of wild-type IF2 requires GTP and formylated initiator tRNA (fMet-tRNA_i). In contrast, extensive activation of A-type IF2 occurs with only GTP or with GDP and fMet-tRNA_i, implying a passive role for initiator tRNA as activator of IF2 in subunit docking. The theory of conditional switching of GTPases quantitatively accounts for all our experimental data. We find that GTP, GDP, fMet-tRNA_i and A-type mutations multiplicatively increase the equilibrium ratio, *K*, between active and inactive forms of IF2 from a value of 4×10^{-4} for wild-type apo-IF2 by factors of 300, 8, 80 and 20, respectively. Functional characterization of the A-type mutations provides keys to structural interpretation of conditional switching of IF2 and other multidomain GTPases.

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Introduction

The GTPase (Bourne, 1995; Sprang, 1997) initiation factor 2 (IF2) has essential roles in initiation of messenger RNA (mRNA) translation in bacteria (Hershey, 1987). In interplay with initiation factors 1 (IF1) and 3 (IF3), IF2 promotes rapid binding of formylated initiator tRNA (fMet-tRNA_i) to the 30S pre-initiation complex (30S PIC) (Fakunding and Hershey, 1973; Benne *et al.*, 1973a; Wintermeyer and Gualerzi, 1983; Gualerzi *et al.*, 2001; Antoun *et al.*, 2006b). Subsequently, IF2 promotes rapid docking of 50S subunits to 30S PICs containing fMet-tRNA_i but not un-formylated Met-tRNA_i or elongator tRNA (Antoun *et al.*, 2006a; Zorzet *et al.*, 2010). By this two-step mechanism, IF2 confers remarkably high accuracy to fMet-tRNA_i selection into the 70S initiation complex (Antoun

et al., 2006a), thereby ensuring correct P-site positioning of the initiation codon of mRNA and correct in-frame mRNA translation (Hartz *et al.*, 1989; Antoun *et al.*, 2006a). The GTP-bound form of IF2, but not GTP hydrolysis, is required for rapid docking of the 50S subunit to the fMet-tRNA_i-containing 30S PIC (Antoun *et al.*, 2003, 2004). GTP hydrolysis on IF2 promotes its rapid release from the 70S initiation complex and rapid entry of this complex into the protein elongation phase of mRNA translation (Benne *et al.*, 1973a; Luchin *et al.*, 1999; Antoun *et al.*, 2003, 2004).

The formyl group of fMet-tRNA_i is crucial for recognition of initiator tRNA by IF2 (Sundari *et al.*, 1976; Gualerzi *et al.*, 2001; Antoun *et al.*, 2006a). Although formylation deficiency is not lethal for the bacterial cell, it greatly decreases growth rate (Guillon *et al.*, 1992, 1996; Steiner-Mosonyi *et al.*, 2004). A novel class of IF2 mutations complementing formylation deficiency was recently identified in *Salmonella typhimurium* (Zorzet *et al.*, 2010). These mutations, all located outside the tRNA-binding domain IV of IF2, strongly (A-type IF2 mutations) or weakly (B-type IF2 mutations) reduce the requirement of a formylated methionine in initiator tRNA for its rapid binding to the 30S PIC and the subsequent rapid docking of the 50S subunit to the 30S PIC (Zorzet *et al.*, 2010).

Here, we show that A-type IF2 mutants not only bypass the formylation requirement, but also the requirement of a P-site bound tRNA in the 30S PIC, for rapid docking of ribosomal subunits. Moreover, in the presence of fMet-tRNA_i these A-type IF2 mutants promote rapid subunit docking not only with GTP but also with GDP. These findings imply that rapid subunit docking does not *per se* depend on the presence of initiator tRNA on the 30S subunit. Instead, initiator tRNA serves as a cofactor to drive the 30S subunit-bound IF2 from its inactive to its active 50S subunit-docking conformation. Applying the theory of conditional switching of GTPases (Hauryliuk *et al.*, 2008a,b), we quantify the contributions of methionylation and formylation of initiator tRNA_i, the presence of tRNA_i, as well as the presence of GTP or GDP for activation of IF2. We show that the phenotypes of these novel IF2 mutants can be accounted for by a 20-fold (A-type mutants) or two-fold (B-type mutants) higher propensity of their apo-form to acquire active conformation as compared with wild-type apo-IF2. We show also that the previously observed deleterious effects of A-type mutations on the accuracy of initiation and bacterial fitness in a formylation-proficient background (Zorzet *et al.*, 2010) can be accounted for by over-activation of the apo-form of IF2 by mutations. We conclude that the apo-form of wild-type IF2 has been optimized in evolution to precisely adjust the extent of its activation by the GTP and fMet-tRNA_i ligands.

Results

Experimental setup and modelling

We have used stopped flow with scattered light detection (Antoun *et al.*, 2004) to study the kinetics of docking of 50S

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Table I Fraction f_A of active, 50S-docking conformation of 30S PICs, the equilibrium ratio K of active to inactive 30S PICs and the effective subunit joining rate, k_c in the presence of GTP for different IF2s in the 30S PIC

	WT IF2	B1 IF2	B2 IF2	B3 IF2	A1 IF2	A2 IF2	A3 IF2
<i>30S PICs with fMet-tRNA_i</i>							
f_A	0.90 ± 0.01	0.94 ± 0.01	0.93 ± 0.01	0.92 ± 0.02	0.99 ± 0.02	0.99 ± 0.02	0.99 ± 0.02
K	9.0 ± 2.4	16.7 ± 3.4	13.8 ± 2.3	10.7 ± 2.6	ND	ND	ND
k_c	37.4 ± 0.4	38.9 ± 0.4	40.0 ± 0.4	40.3 ± 0.4	41.7 ± 0.7	40.2 ± 0.8	39.6 ± 0.8
<i>30S PICs with Met-tRNA_i</i>							
f_A	0.52 ± 0.01	0.74 ± 0.01	0.69 ± 0.02	0.60 ± 0.01	0.942 ± 0.01	0.956 ± 0.01	0.951 ± 0.01
K	1.07 ± 0.07	2.87 ± 0.17	2.15 ± 0.31	1.46 ± 0.11	16.5 ± 3.7	22.0 ± 4.8	19.5 ± 4.4
k_c	9.8 ± 0.6	21.5 ± 0.7	18.5 ± 0.7	14.0 ± 0.7	41.2 ± 0.8	38.7 ± 0.9	36.9 ± 0.7
<i>30S PICs with tRNA_i</i>							
f_A	0.13 ± 0.01	0.29 ± 0.01			0.82 ± 0.01		
K	0.155 ± 0.031	0.395 ± 0.054			4.39 ± 0.65		
k_c	2.51 ± 0.05	5.70 ± 0.07			31.2 ± 0.2		
<i>30S PICs without tRNA</i>							
f_A	0.10 ± 0.02	0.24 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	0.74 ± 0.01	0.79 ± 0.01	0.72 ± 0.01
K	0.11 ± 0.01	0.32 ± 0.02	0.26 ± 0.03	0.25 ± 0.11	2.89 ± 0.15	3.81 ± 0.18	2.61 ± 0.21
k_c	1.92 ± 0.04	3.41 ± 0.05	4.18 ± 0.05	3.46 ± 0.04	24.5 ± 0.4	25.6 ± 0.4	20.5 ± 0.4
<i>30S PICs without tRNA, IF1 and mRNA</i>							
f_A	0.098 ± 0.02	0.19 ± 0.02	0.14 ± 0.01	0.14 ± 0.01	0.60 ± 0.01	0.64 ± 0.01	0.61 ± 0.01
K	0.10 ± 0.02	0.24 ± 0.03	0.16 ± 0.02	0.17 ± 0.02	1.48 ± 0.08	1.81 ± 0.07	1.56 ± 0.07
k_c	3.43 ± 0.09	4.86 ± 0.15	5.08 ± 0.18	4.75 ± 0.17	19.8 ± 0.3	22.1 ± 0.3	23.4 ± 0.4

subunits with 30S PICs containing wild-type IF2 or IF2 mutants that strongly (A-type) or weakly (B-type) compensate for initiator tRNA formylation deficiency (Zorzet *et al*, 2010). The experiments were performed in the presence of GTP, GDP, or in the absence of guanine nucleotide in combinations with fMet-tRNA_i, Met-tRNA_i, tRNA_i or no tRNA in the 30S PIC. To ensure that subunit association always proceeded to completion, the experiments were performed in the absence of IF3 in the 30S PIC (Antoun *et al*, 2006a,b). The subunit-joining kinetics displayed one fast and one slow phase and could in all cases be quantitatively described by a two-state kinetic model for subunit docking:

Here, 30S_i is an inactive state of the small ribosomal subunit unable to dock with the 50S subunit, while 30S_A is its active, docking-competent state (Milon *et al*, 2008). The model assumes that the equilibrium between the active and inactive states of the 30S subunit has already been established before the addition of 50S subunits (see Materials and methods for details). Accordingly, the fast phase of the subunit-docking kinetics reflects the rate of direct 50S subunit association to 30S_A and its amplitude corresponds to the equilibrium fraction, f_A , of 30S subunits in the *active* state. The slow phase mainly reflects the rate of conversion of 30S_i to 30S_A and its amplitude corresponds to the equilibrium fraction, $1-f_A$, of 30S subunits in the *inactive* state. The fraction f_A is determined by the equilibrium constant $K=k_1/q_1$ of the first step in Equation (1) as $f_A=K/(1+K)$, from which follows the inverse relation $K=f_A/(1-f_A)$. The model fittings of experimental light-scattering curves are in each figure shown as solid lines and the model parameters estimated for each fitting (k_1 , q_1 and k_a) are given in Supplementary Tables S1 and S2. To simplify comparison of subunit-docking kinetics, we have for each experimental curve also defined a

Table II Fraction f_A of active 50S-docking conformation of 30S PICs, the equilibrium ratio K of active to inactive 30S PIC and the effective subunit joining rate, k_c in the presence of GDP or without G nucleotide (no GXP) for wild-type IF2 and the A1 IF2 mutant

	WT IF2	A1 IF2
<i>fMet-tRNA, GDP</i>		
f_A	0.20 ± 0.02	0.83 ± 0.02
K	0.25 ± 0.02	4.95 ± 0.25
k_c	0.60 ± 0.05	25.9 ± 0.2
<i>fMet-tRNA, no GXP^a</i>		
f_A	0.02 ± 0.002	0.41 ± 0.04
K	0.02 ± 0.03	0.61 ± 0.06
k_c	0.5 ± 0.04	1.73 ± 0.03

^aIn the absence of guanine nucleotides, 1 mM CTP was added to the reaction mixture to ensure the same level of Mg²⁺ chelation as in the presence of guanine nucleotides.

single rate constant, k_c , as the inverse of the time at which the extent of subunit docking was 50% of its maximal value (see Materials and methods for details). For f_A -values close to one and 1:1 ratio of 30S and 50S subunits, this definition of k_c allows estimation of the second-order rate constant for subunit docking as $k_a=k_c/[50S]$ (see Materials and methods). The equilibrium fractions, f_A , of active subunits, the corresponding equilibrium constants K and effective rates, k_c , for subunit docking are compiled in Tables I and II.

Effects of tRNA_i, Met-tRNA_i and fMet-tRNA_i on subunit docking with different IF2 variants in the presence of GTP

The effective rate, k_c , of subunit docking in the presence of mRNA, IF1 and fMet-tRNA_i on the 30S subunit was large ($k_c \approx 40 \text{ s}^{-1}$) and similar for wild type, the three B-type and the three A-type variants of *S. typhimurium* IF2 (Figure 1A and B; Table I). The corresponding second-order

Figure 1 Kinetics of 70S IC formation after rapid mixing of 50S subunits with 30S PICs containing different IF2s and either fMet-tRNA_i or no tRNA. (A) 30S PIC contained mRNA, IF1, no IF3, indicated IF2 and fMet-tRNA_i. (B) The same as (A) but fMet-tRNA_i was omitted. (C) 30S PICs contained mRNA, IF1, no IF3, indicated A-type IF2 mutants and either fMet-tRNA_i or no tRNA. (D) Effective docking rates of subunits with or without fMet-tRNA_i for indicated IF2s in the 30S PICs.

association rate constants, k_a , of about $140 \mu\text{M}^{-1} \text{s}^{-1}$, agree well with k_a -values previously obtained for subunit joining with wild-type IF2 from *Escherichia coli* (Antoun *et al*, 2006b). The docking rates for A-type IF2 mutants were slightly larger (average $k_c = 40 \text{s}^{-1}$) than for wild-type IF2 ($k_c = 37.4 \text{s}^{-1}$). The subunit-joining kinetics was close to mono-phasic with $f_A \approx 0.9$ for wild-type IF2 and B-type mutants (Table I). For A-type IF2 mutants, the fraction of active subunits f_A was close to 1.0, precluding reliable estimation of the equilibrium constants $K = k_1/q_1$ for these mutants (Table I).

When non-formylated, Met-tRNA_i replaced fMet-tRNA_i in the 30S PIC, the effective docking rate k_c was only slightly reduced for the A-type mutants, while k_c was reduced by about a factor of two for the B-type mutants and by a factor of four for wild-type IF2 (Figure 2A and B; Table I). While the kinetics of subunit joining with non-formylated Met-tRNA_i was near mono-phasic for A-type IF2, it was clearly bi-phasic for B-type and wild-type IF2s (Figure 2A and B). Analysis of the scattering curves in the framework of the kinetic model in Equation (1) estimated the f_A -value as 0.94 for the A1-type mutant, 0.74 for the B1-type mutant and 0.52 for wild-type IF2 (Table I), implying that the equilibrium constant K between the active and inactive forms of IF2 was increased by about a factor of 2.8 by the B1 mutation and by a factor of 16 by the A1 mutation (Table I). On average, K increased by factors of 2 and 20 by the B- and A-type mutations, respectively (Table I).

When deacylated tRNA_i replaced fMet-tRNA_i in the 30S PIC, the effective docking rate was reduced by 25% for the A1 IF2 mutant ($k_c = 31.2 \text{s}^{-1}$), by a factor of 7 for the B1 IF2 mutant ($k_c = 5.7 \text{s}^{-1}$) and by a factor of 15 for the wild-type IF2 ($k_c = 2.5 \text{s}^{-1}$) (Figure 2C and D; Table I). From the subunit-docking curves in Figure 2, we estimated f_A as 0.82 for the A1 IF2 mutant, 0.29 for the B1 IF2 mutant and 0.13 for wild-type IF2 (Table I), implying that in this context, the equilibrium constant K was increased by about a factor of 2.5 by the B1 mutation and a factor of 27 by the A1 mutation (Table I).

Complete removal of tRNA from the 30S PIC reduced the docking rate about two-fold for the A-type mutants (average $k_c = 23.5 \text{s}^{-1}$), about 10-fold for the B-type mutants (average $k_c = 3.7 \text{s}^{-1}$) and about 20-fold for wild-type IF2 ($k_c = 1.9 \text{s}^{-1}$) (Figure 1A–D; Table I). The kinetics of subunit docking in the absence of tRNA exhibited a clear bi-phasic behaviour (Figure 1A–D) for the A-type IF2 mutants. Table I shows that in the absence of tRNA, the equilibrium constant K was again increased by factors 2 and 20 by the B- and A-type mutations, respectively.

In summary, these data indicate that the equilibrium constant, K , for wild-type IF2 was increased roughly by a factor of 2 by B- and by a factor of 20 by A-type mutations irrespective of whether there was fMet-tRNA_i, Met-tRNA_i, tRNA_i or no tRNA in the P-site of the 30S subunit (Table I).

Figure 2 Kinetics of 70S IC formation after rapid mixing of 50S subunits with 30S PICs containing different IF2s and either non-formylated Met-tRNA_i or deacylated tRNA_i. (A) 30S PICs contained mRNA, IF1, no IF3, indicated IF2 and Met-tRNA_i. (B) Effective rates of subunit joining for the experiments in panel (A). (C) 30S PICs contained mRNA, IF1, no IF3, indicated IF2s and deacylated tRNA_i. (D) Comparison of effective docking rates of 50S subunits to 30S PICs assembled with indicated IF2s for different tRNA_i types (fMet-tRNA_i, Met-tRNA_i, deacylated tRNA_i or no tRNA).

Figure 3 Kinetics of 70S ribosome formation in the absence of IF1, IF3, tRNA and mRNA after rapid mixing of 50S subunits with 30S subunits containing different IF2s. (A) 30S subunits contained only indicated IF2. (B) Comparison of effective docking rates of 50S subunits to 30S subunits containing only IF2s or IF2s supplemented with also IF1 and mRNA.

In the experiments described above, the 30S PICs contained IF1 and an mRNA with strong SD sequence: two features with putative effects on the conformation of the 30S subunit (Milon *et al*, 2008). However, similar k_c -values for subunit docking without tRNA were obtained in the absence and presence of IF1 and mRNA (compare Figures 1 and 3). Furthermore, alterations in the equilibrium constant, K , by the B- and A-type mutations were similar in

the absence and presence of mRNA and IF1: in the absence of IF1 and mRNA, the B- and A-type mutations increased K by factors of 1.8 and 16, respectively (Table I).

Comparison of the values of the equilibrium constant K for wild-type IF2 in the absence of tRNA, in the presence of deacylated tRNA_i or methionylated Met-tRNA_i (see Table I) shows that tRNA_i binding to the 30S PIC increased K by less than a factor of 1.5, while tRNA_i methionylation increased K

further by about a factor of 7 (Table I). Formylation of Met-tRNA_i led to a further nine-fold increase in K (Table I), thereby driving wild-type IF2 almost completely (90%) into its active form. Similar increases in K -values are also seen for B- and A-type IF2 mutants (Table I) showing that the binding of any particular tRNA ligand shifted the K -value by roughly the same factor for wild-type and mutated forms of IF2.

We note that removal of fMet-tRNA_i from the 30S PIC resulted in drastic reductions in subunit-joining rates with wild-type IF2 and B-type mutants but only to minor rate reductions with the A-type mutants (Table I). We conclude, therefore, that rapid subunit docking does not require the presence of initiator tRNA *per se*, but mainly depends on IF2 switching to its active 50S-docking conformation on the 30S subunit. Thus, the active conformation of IF2 could be induced either by the presence of fMet-tRNA_i in the 30S PIC or by an A-type mutation.

Effects of different guanine nucleotides on subunit joining with wild-type IF2 and an A-type IF2 mutant

In the experiments described so far, 1 mM GTP was always present in the reaction mixture. We showed previously that replacing GTP with GDP in a 30S PIC containing IF1, IF3,

mRNA, fMet-tRNA_i and the naturally truncated β -form of IF2 from *E. coli* resulted in a 20-fold reduction in the docking rate of ribosomal subunits (Antoun *et al*, 2003). The GTP to GDP swap resulted in an even larger, about 60-fold, reduction in the docking rate for the full-length α -form of *E. coli* IF2 (Antoun *et al*, 2004).

Figure 4 shows that a similar reduction in subunit-joining rate (60-fold from 37.4 to 0.6 s⁻¹) occurred also in the absence of IF3 when GDP replaced GTP on wild-type IF2 in a 30S PIC assembled with fMet-tRNA_i, mRNA, IF1 and IF2 (Table II). Surprisingly, when the A1 IF2 mutant replaced wild-type IF2, the rate of subunit docking decreased by only 40% from 41.7 to 25.9 s⁻¹ upon GTP to GDP swapping demonstrating that, in contrast to the wild-type IF2, the A-type IF2, *did not require* GTP for rapid subunit docking. Importantly, the A-type IF2 did not promote rapid subunit docking in the absence of guanine nucleotide: without guanine nucleotide, k_c was only 1.73 s⁻¹ compared with 25.9 s⁻¹ in the presence of GDP and 41.7 s⁻¹ in the presence of GTP (Table II). These results suggest that not only GTP but also GDP drives apo-IF2 towards its active form (see Discussion). In a parallel experiment with wild-type IF2, we obtained $k_c = 0.5$, 0.6 or 37.4 s⁻¹ in the absence of guanine nucleotide, in the presence of GDP or in the presence of GTP, respectively (Tables I and II).

Figure 4 Dependence of the kinetics of subunit joining on the presence of fMet-tRNA_i and G-nucleotide in the 30S PICs containing wild-type IF2 or A1 IF2 mutant. (A) Kinetics of 50S docking to 30S PICs containing mRNA, IF1, no IF3, wild-type IF2, indicated nucleotide and either fMet-tRNA_i or no tRNA. (B) The same as (A) but the 30S PICs contained A1 IF2 instead of wild-type IF2. (C) Comparison of the effective rates of 50S subunit docking to 30S PICs containing different G-nucleotides, fMet-tRNA_i or no tRNA and wild-type IF2 or A1 IF2 mutant.

The subunit-docking kinetics in the presence of GDP was also clearly bi-phasic (Figure 4A and B). Its analysis in the framework of the kinetic Equation (1) gave the fraction of the active IF2 form in the 30S PIC and, hence, the equilibrium constant K between the active and inactive forms of IF2·GDP. In the presence of fMet-tRNA_i, 21% of wild-type IF2·GDP and 83% of A1-type IF2·GDP were in the active conformation, corresponding to an about 19-fold increase in equilibrium constant K for IF2·GDP by the A-type mutation. This mutation-induced shift in K for transition from the inactive to the active form of IF2·GDP corresponds very well to the ~20-fold mutational shift in K observed for IF2·GTP (Table I). Importantly, comparison of K -values for wild-type IF2 with GTP and GDP in the presence of fMet-tRNA_i (Table I) shows that the GTP for GDP substitution on IF2 resulted in a 36-fold increase in the equilibrium constant K in favour of the active form of IF2, explaining the importance of GTP for IF2 activation.

For a 30S complex with mRNA, IF1 and wild-type IF2, but lacking fMet-tRNA_i, the rate of subunit docking decreased about four-fold from 1.9 to 0.46 s⁻¹ by the GTP to GDP swapping (Figure 4A and C). With an A-type IF2 replacing wild-type IF2 in the otherwise similar 30S complex, the rate of docking decreased almost 40-fold from 24.5 to 0.52 s⁻¹ (Figure 4B and C), indicating that fMet-tRNA_i was required to drive A1 IF2 into its active conformation in the absence of GTP. Thus, rapid subunit docking was promoted by A-type IF2 either in the presence of fMet-tRNA_i and GDP or in the

presence of GTP without tRNA. In contrast, wild-type IF2 required both GTP and fMet-tRNA_i to promote rapid subunit docking. In the absence of tRNA and presence of GDP, subunit joining with A1 IF2, with wild-type IF2 or without IF2 in the 30S PIC was similarly slow (Figure 4A and B).

Effect of the GTP/GDP ratio on subunit joining with wild-type IF2 and an A-type IF2 mutant

In the presence of fMet-tRNA_i in the 30S PIC, the rate of subunit docking promoted by wild-type and A1-type IF2 responded very differently to a varying fraction of GTP in the reaction mixture (Figure 5). For wild-type IF2, the effective rate of subunit docking, k_c , increased monotonically with increasing GTP content reaching half its maximal value of 37.2 s⁻¹ at about 40% GTP and continued to increase significantly all the way up to 100% GTP (Figure 5A and C). Moreover, the kinetics of subunit docking with wild-type IF2 was clearly bi-phasic when the reaction mixture contained GDP (Figure 5A). A very similar result was obtained for wild-type IF2 from *E. coli* (Supplementary Figure S1). In contrast, with A1 IF2, k_c was already larger than half its maximal value of 40.5 s⁻¹ in the presence of only GDP and was close to its maximum when the GTP content exceeded 20% (Figure 5B and C). Analysis of these kinetics in the framework of Equation (1) gives the fractions, f_A , of active IF2 and the equilibrium constant K between the active and inactive forms of IF2 as a function of the GTP fraction in the reaction mixture (Supplementary Figure S1D). These data were used to estimate the ratio,

Figure 5 Dependence of the kinetics of subunit joining on the fraction of GTP in the reaction mixture for wild-type IF2 and A1 IF2 mutant. (A) Kinetics of 50S docking to 30S PICs containing mRNA, IF1, no IF3, fMet-tRNA_i and wild-type IF2 for indicated GTP fractions in the reaction mixture. (B) The same as (A) but 30S PICs contained A1 IF2 instead of wild-type IF2. (C) Comparison of the effective rates of subunit joining with wild-type IF2 and A1 IF2 mutant for different GTP fractions [GTP]/([GTP] + [GDP]) in the reaction mixture.

Figure 6 Dependence of subunit-joining rate with wild-type IF2 and A1 IF2 mutant in the presence of IF3 on the fraction of GTP in the reaction mixture. (A) Kinetics of 50S docking to 30S PICs containing mRNA, IF1, IF3, fMet-tRNA_i and wild-type IF2 for indicated GTP fractions in the reaction mixture. (B) The same as (A) but 30S PICs contained A1 IF2 instead of wild-type IF2. (C) Comparison of the effective rates of subunit joining with wild-type IF2 and A1 IF2 mutant for different GTP fractions $[GTP]/([GTP] + [GDP])$ in the reaction mixture.

β , between the equilibrium dissociation constants of GDP and GTP to the active form of wild-type IF2 bound to the 30S subunit as 10 (see Materials and methods and Supplementary data for details). This value of β indicates, as expected, that GTP has a much higher affinity than GDP to the active form of IF2.

The dependence of the rate of subunit docking on the fraction of GTP with also IF3 present in the 30S PIC (Figure 6) was qualitatively similar to that in its absence (Figure 5). Under these physiologically more relevant conditions, the effective rate of subunit joining, k_c , increased monotonically with the GTP content for wild-type IF2 reaching half its maximal value of 8.3 s^{-1} at about 60% GTP (Figure 6C). For A1 IF2, k_c was 4.2 s^{-1} and close to half of the maximal k_c (10.5 s^{-1}) already at 0% GTP, and approached the maximal k_c at a GTP content just above 20% (Figure 6C). This suggests that under physiological conditions, the subunit-docking rate is much less sensitive to GTP content for A-type than for wild-type IF2.

Discussion

We recently identified novel mutations in *S. typhimurium* IF2 that compensate for the lack of initiator tRNA formylation *in vivo* and *in vitro* (Zorzet *et al*, 2010). These mutations, located well outside the tRNA-binding domain IV of IF2 (see Supplementary Figure S2), increase both the rate of non-formylated Met-tRNA_i binding to the 30S PIC and the

rate of subsequent docking of 50S subunits to the Met-tRNA_i-containing 30S PIC. They fall into two groups: one compensating strongly (A-type) and the other weakly (B-type) for formylation deficiency *in vivo* and *in vitro* (Zorzet *et al*, 2010). Here, we have studied the effects of these IF2 mutations on the kinetics of docking of 50S subunits to 30S PICs assembled with fMet-tRNA_i, Met-tRNA_i, tRNA_i or no tRNA and different G-nucleotides. In the absence of IF3, the subunit docking proceeded to completion with all IF2 variants also in the complete absence of tRNA in the 30S PIC. This permitted direct estimation of the effects of formylation, methionylation and omission of tRNA_i on the kinetics of subunit joining. We found the effective rate, k_c , of 50S subunit docking to the 30S PICs containing fMet-tRNA_i to be largely similar for the different IF2 variants (Figure 1). However, removal of tRNA from the 30S PIC led to a very different reduction of the subunit-docking rate for the different IF2 variants (Figure 1). For the A-type, B-type and wild-type IF2 variants, the reduction was about 2-, 10- and 20-fold (Figure 1D). Furthermore, exclusion of IF1 and mRNA from the 30S PIC did not significantly alter k_c in the absence of tRNA (Figure 3). From these findings we conclude that the A-type IF2 mutants, but not wild-type IF2, bypass the requirement of an initiator tRNA in the P site, implying that fMet-tRNA_i *per se* does not have an active role in subunit joining. It is, however, essential for activation of wild-type IF2 in the 30S PIC.

The bi-phasic subunit-joining kinetics was in all cases described by the model in Equation (1), where the 30S

subunit was initially partitioned between an inactive form, 30S_I, and an active form, 30S_A. The fraction, f_A , of 30S subunits initially active in docking to 50S subunits depended on mutations in IF2 and the different forms of tRNA_i in the 30S PIC (Table I). For example, >90% of the 30S PICs with wild-type IF2 and about 100% of the 30S PICs with A-type IF2 mutants were active when fMet-tRNA_i was present in the 30S PIC. In contrast, in the absence of tRNA, the active fraction of the 30S PIC was only 11% for wild type but >74% for A-type IF2 (Table I). Accordingly, the equilibrium constant $K = k_1/q_1$ for the partitioning of the 30S subunit between inactive and active forms in the absence of tRNA was increased by factors of about 2.6 and 27 by the B- and A-type mutations, respectively (Table I). Moreover, these mutations induced similar K -value shifts in the presence of Met-tRNA_i, deacylated tRNA_i or in the absence of tRNA in the 30S PIC (Table I). Surprisingly, the mutations similarly increased the K -values not only of GTP- but also GDP-bound IF2 explaining why the A-type IF2 mutants, but not wild-type IF2, promoted fast subunit joining in the presence of GDP and fMet-tRNA_i in the 30S PIC (Table II). In the absence of guanine nucleotide, A-type IF2 did not promote rapid subunit docking even in the presence of fMet-tRNA_i, meaning that GDP binding activated apo-IF2 (Table II). Indeed, comparison of K -values for A1-type IF2 with GDP and without G-nucleotide shows that GDP binding increased K by a factor of 8 in relation to G-nucleotide-free IF2 (Table II).

General properties of GTPases and their roles in bacterial mRNA translation

How do the surprising findings described in the previous section relate to what is known about GTPase action? Most members of the GTPase super family function as molecular switches (Bourne, 1995; Sprang, 1997). When GTP bound, they acquire their active (A) and when GDP bound their inactive (I) conformation. The GTPases share a common core structure, the G-domain, in which the GDP and GTP effector molecules have a common binding site. Structural analyses have revealed large conformational changes in the positioning of the switch-I and switch-II loops in the G-domain from flexible, solvent exposed states in the GDP-bound form to fixed, solvent protected, states in the GTP-bound form (Sprang, 1997; Haurlyuk *et al*, 2009). Rearrangements of these loops in response to guanine nucleotide swapping may result in large domain rearrangements of multidomain GTPases (Bourne, 1995; Sprang, 1997). It is commonly thought that global rearrangements, essential for GTPase function, are strictly correlated with guanine nucleotide exchange in the G-domain (Kong *et al*, 2004).

Four main GTPases function in bacterial protein synthesis (reviewed in Hershey, 1987; Ramakrishnan, 2002): (i) IF2 in initiation of mRNA translation (Fakunding and Hershey, 1973; Benne *et al*, 1973a, b; Dever *et al*, 2001; Antoun *et al*, 2003), (ii) elongation factor Tu (EF-Tu) in aminoacyl-tRNA delivery to the ribosomal A site (Nissen *et al*, 1995; Ramakrishnan, 2002), (iii) elongation factor G (EF-G) in tRNA and mRNA translocation (Rodnina *et al*, 1997; Ramakrishnan, 2002; Frank *et al*, 2007) as well as in ribosomal recycling back to a new round of initiation after termination of protein elongation (Janosi *et al*, 1996; Karimi *et al*, 1999) and finally (iv) release factor 3 (RF3) in recycling of class-1 release factors after termination of protein synthesis

(Freistoffer *et al*, 1997; Pavlov *et al*, 1997; Zavialov *et al*, 2001; Gao *et al*, 2007). To date, no crystal structure of bacterial IF2 is available, but the crystal structures of the GTP-bound, GDP-bound and guanine nucleotide free (apo) forms of its aIF5B archaeal orthologue display a similar global shape (Roll-Mecak *et al*, 2000). Crystal structures of EF-Tu show large domain movements between its GDP- and GTP-bound forms (Berchtold *et al*, 1993; Kjeldgaard *et al*, 1993; Nissen *et al*, 1999). In contrast, the crystal structures of GTP (GDPNP)-bound, GDP-bound and apo-forms of EF-G are very similar (AEvarsson *et al*, 1994; al-Karadaghi *et al*, 1996; Ramakrishnan, 2002; Hansson *et al*, 2005). However, a different conformation of GDPNP-bound EF-G has been observed in ribosomal complexes by cryo-electron microscopy (cryo-EM) (Frank *et al*, 2007). Concerning RF3, its crystal structure is only available for the GDP-bound form (Gao *et al*, 2007), but another global conformation of the GDPNP-bound form of RF3 has been observed by cryo-EM (Gao *et al*, 2007).

Conditional GTPase switching explains the effects of the A- and B-type mutations of IF2

To account for the paradox that some GTPases in solution remain in their inactive conformation even when GDP is replaced with GTP, while adopting their active conformation in complexes with the ribosome or other ligands (co-factors), we developed the concept of conditional conformational switching of GTPases (Haurlyuk *et al*, 2008a). In essence, the theory shows that when the equilibrium between the inactive (I) and active (A) conformation of the apo-form of the GTPase is greatly shifted towards the I-conformation, swapping of GDP to GTP may fail to significantly drive the GTPase to its A-conformation. However, in the presence of an auxiliary co-factor, such as tRNA, GDP to GTP swapping may readily switch the GTPase into its active A-form.

We analysed the entire data set in Tables I and II in terms of co-factor dependent switching from the 30S_I to the 30S_A form of the 30S PIC, and found that all results could be accounted for by conditional changes of the equilibrium constant, K , defining the ratio between the active and inactive forms of IF2 in complex with the 30S subunit, when written as (Materials and methods)

$$K = K_0 l_x m_G n_T \quad (2)$$

Here, K_0 is the equilibrium constant for the partitioning of the active and inactive apo-forms of 30S-bound wild-type IF2 in the absence of guanine nucleotide and tRNA. The l_x factor accounts for changes in K by mutations, m_G accounts for the increase in K by addition of GTP or GDP and n_T accounts for the increase in K due to addition of fMet-tRNA_i, Met-tRNA_i or tRNA_i. From these definitions follow that $l_x = 1$ for wild-type IF2, $m_G = 1$ in the absence of guanine nucleotide and $n_T = 1$ in the absence of P-site bound tRNA. The validity of Equation (2) requires that 30S-bound IF2 is saturated by GTP or GDP. This assumption is supported by previous observations on the affinities of GDP and GTP to free as well as 30S-bound IF2 as discussed below in the section 'Effect of IF2 activation on its guanine nucleotide binding affinity'.

We have found that l_x , m_G and n_T were largely independent of each other, meaning that the contributions to the standard free energy difference between the active and inactive forms of IF2 on the 30S subunit from mutations, guanine nucleotide and P-site tRNA were additive. Accordingly, the whole data

set of these experiments is described by eight parameters: $K_0 = 3.8 \times 10^{-4}$ for the apo-form of 30S-bound wild-type IF2, $l_A = 20$ for the A-type mutant, $l_B = 2$ for the B-type mutant, $m_{\text{GTP}} = 300$, $m_{\text{GDP}} = 8$, $n_{\text{fMet-tRNA}_i} = 80$, $n_{\text{Met-tRNA}_i} = 10$ and $n_{\text{tRNA}_i} = 1.5$. This result implies that the whole effect of an A-type or B-type mutation is a 20-fold or two-fold increase, respectively, of the equilibrium constant K_0 for wild-type apo-IF2 from its value of $\sim 4 \times 10^{-4}$. We note, further, that the fMet group on fMet-tRNA_i increased K by a factor of 60, that the methionine group on Met-tRNA_i increased K by a factor of 7, while addition of tRNA_i itself did not significantly increase K (Table I). Binding of GTP to the apo-form of IF2 had the largest activation effect, increasing K by a factor of 300. Remarkably, binding of GDP to the apo-form of IF2 increased K by almost an order of magnitude, showing that not only GTP but also GDP binding activated IF2. This also implies that GDP has a larger affinity to the active than to the inactive form of IF2.

The latter result seems counterintuitive, since it is commonly believed that GTP stabilizes the active form of a G-protein and GDP stabilizes its inactive form. However, the partial activation of IF2 by GDP is in line with recent isothermal titration calorimetry (ITC) measurements of the enthalpies and entropies of guanine nucleotide binding to IF2 (Hauryliuk *et al*, 2009). Indeed, the ITC data suggest a conformational change in IF2 in response to GDP binding, which may account for the partial IF2 activation by GDP observed here. They further suggest that a similar conformational change occurs in response to GTP binding, but now accompanied by another conformational change, identified as solvent protection of the switch loops of IF2 (Hauryliuk *et al*, 2009). At the same time, ITC observations for EF-G do not indicate a GDP-induced conformational change (Hauryliuk *et al*, 2008b), suggesting that partial GDP activation is idiosyncratic to IF2 and does not occur for GTPases in general.

Effect of IF2 activation on its guanine nucleotide binding affinity

The dependence of the equilibrium constant K between the active and inactive forms of 30S-bound IF2 on mutations and the presence of tRNA ligands (Equation 2) determines also how the dissociation constant, K_d , for guanine nucleotide binding to IF2 depends on mutations and tRNA ligands. As previously shown (Hauryliuk *et al*, 2008a,b), K_d is equivalent to an effective equilibrium constant, K_{eff}^G , averaged over the inactive and active forms of IF2 (see Supplementary data for details):

$$K_d = K_{\text{eff}}^G = K_I^G \frac{1 + K_0 l_X n_T}{1 + K_0 l_X n_T m_G} = K_I^G \frac{1 + K/m_G}{1 + K} \quad (3)$$

Here, G identifies the guanine nucleotide as GTP or GDP (i.e. for the m_G parameter: $m_{\text{GTP}} = 300$ and $m_{\text{GDP}} = 8$) and K_I^G is the dissociation constant for guanine nucleotide binding to the *inactive* form of IF2. Since the ratio, β , between the dissociation constants for GDP (K_A^{GDP}) and GTP (K_A^{GTP}) to the *active* form of IF2 was estimated as 10 (Supplementary Figure S1D), it follows that $K_I^{\text{GTP}} = (m_{\text{GTP}}/\beta \times m_{\text{GDP}}) \times K_I^{\text{GDP}} \approx 3.7 \times K_I^{\text{GDP}}$ (Supplementary data). Accordingly, Equation (3) predicts the affinity of 30S-bound IF2 to GDP and GTP for all combinations of mutations and tRNA ligands in Tables I and II, provided that K_I^{GDP} is obtained from another type of experiment. Equation (3) also predicts that the measured K_d -value should be close to K_I^G when $K \ll 1$.

The affinity of GDP to IF2 is not noticeably affected by IF2 binding to the 30S subunit or by the presence of fMet-tRNA_i in the 30S PIC (Antoun *et al*, 2003). A K_d -value of about 10 μM for GDP binding to 30S-bound IF2 in the absence of tRNA was estimated from GDP titration data in Antoun *et al* (2003). This K_d -value is in a good agreement with the K_d -value of 12 μM previously measured by equilibrium dialysis and NMR (Pon *et al*, 1985) and with an estimate $K_d = 4.5 \mu\text{M}$ obtained recently by ITC (Hauryliuk *et al*, 2009). Using the K_d -value of 10 μM as an approximation for K_I^{GDP} , the model (Equation 3) predicts the K_d -values for the complex between the 30S-bound wild-type IF2 and GTP in the absence and presence of fMet-tRNA_i as 33 and 3.6 μM , respectively. These values are reasonably close to the previously estimated K_d -values of 40 and 2 μM for GTP binding to the 30S-bound IF2 in the absence and presence of fMet-tRNA_i (Antoun *et al*, 2003). Moreover, the predicted K_d -value for GTP in the absence of tRNA is also similar to the K_d -value of about 30 μM for the binding of the fluorescent GTP analogue, mant-GTP, to free IF2 (Milon *et al*, 2006). This similarity is in line with the similar affinities of GTP to the free and 30S-bound IF2 in the absence of fMet-tRNA_i observed previously (Antoun *et al*, 2003). Further validation of the model may be provided by comparison of the predicted with as yet unknown K_d -values for binding of guanine nucleotides to A- and B-type IF2 mutants in the presence of different tRNA ligands in the 30S PIC. For instance, Equation (3) predicts a much larger effect of the presence of fMet-tRNA_i in the 30S PIC on the guanine nucleotide binding to the A-type than to wild-type IF2: it predicts the K_d -values for GDP binding to the 30S-bound A-type IF2 in the absence and presence of fMet-tRNA_i as 9.5 and 2.7 μM , respectively and the corresponding K_d -values for GTP binding as 11.4 and 0.32 μM , respectively. Finally, the measured K_d -values of GTP and GDP binding to the 30S-bound wild-type IF2 in complex with different tRNA ligands and the predicted K_d -values for the IF2 mutants imply that at the 1 mM concentration of guanine nucleotides used in our experiments, IF2 is always saturated with GTP or GDP. This justifies the use of Equation (2) instead of a more general relation valid at non-saturating concentrations of guanine nucleotide (see Equation (8) in Materials and methods).

Structural anatomy of the tunable GTPase IF2

As discussed above, addition of GTP and fMet-tRNA_i to 30S-bound apo-IF2 increases the equilibrium constant K by a factor of $m_{\text{GTP}} \times n_{\text{fMet-tRNA}_i} = 300 \times 80 = 24\,000$. What is the structural corollary to this remarkably large, $\sim 2 \times 10^4$ -fold, shift between the inactive and active forms of IF2, and what determines the evolutionary fine-tuning of the equilibrium constant K_0 of the apo-form of wild-type IF2 to such a low value as $K_0 \approx 4 \times 10^{-4}$?

To answer these questions, we first note that the interaction between domain IV (C2) of IF2 and the fMet group of fMet-tRNA_i, firmly anchored by codon-anticodon interactions in the P-site of the 30S subunit, results in a continuous bridge-like structure, mainly composed of the IF2 body, complementary to the 50S surface (Simonetti *et al*, 2008). This, we suggest, is the active form of IF2 in the 30S PIC, which promotes rapid subunit docking. The structure of the inactive form of IF2 is not known, but some of its features may be guessed from X-ray crystal structures of the archaeal

IF2 homologue, aIF5B (Roll-Mecak *et al*, 2000). When GDP replaces GTP in eIF5B, there is an 8° rotation of domains III and IV of the factor in relation to domain II and the G-domain. Such a rotation, expected to warp the IF2 surface on the 30S subunit, could be part of the conformational change that connects the active and inactive forms of the factor. In line with this, comparison of the three cryo-EM structures of IF2 in complex with the 70S ribosome and the 30S subunit shows IF2 to have different conformations in the 30S PIC and the 70S initiation complex (Allen *et al*, 2005; Myasnikov *et al*, 2005; Simonetti *et al*, 2008). In the 70S complex, the conformations of the GDP- and GTP- (GDPNP-) bound IF2 differ greatly (Myasnikov *et al*, 2005; Simonetti *et al*, 2008), due to different rotations of the C-terminal domains III and IV of IF2 in relation to domain II and the G-domain (Simonetti *et al*, 2008). From this, we propose that the A-type mutations in domain III of IF2 strongly favour rotation of the C-terminal domains of the 30S-bound IF2 relative to domain II and the G-domain towards the active conformation of the factor.

While the $\sim 2 \times 10^4$ -fold shift in favour of the active IF2 conformation due to fMet-tRNA_i and GTP binding to the apo-form of IF2 was the same for the different IF2 variants, the absolute value of *K* was roughly 20-fold higher for the A-type than for the wild-type IF2 for any combination of tRNA and guanine nucleotide. Assuming that the *K₀*-value for the apo-form of wild-type IF2 has been fine-tuned during evolution, one would expect the excessive IF2 activation by mutations to adversely affect bacterial growth. Indeed, while the excessive activation of IF2 by the A-type mutations increased the subunit-docking rate, the fitness of formylation-proficient cells harbouring A-type IF2 mutants was considerably reduced in relation to that of wild type (Zorzet *et al*, 2010). This effect has been explained by a higher propensity of A-type IF2 to promote formation of aberrant 70S ICs containing deacylated or un-formylated tRNAs (Zorzet *et al*, 2010). The reason underlying this behaviour can now be traced to the similar and large fractions, *f_A*, of activated A-type IF2 in the presence of deacylated (82%), non-formylated (94%) and formylated (99%) initiator tRNA (Table I). We note, however, that GDP activation of A-type IF2 may preserve its active conformation in the 70S complex after GTP hydrolysis, thereby delaying dissociation of IF2 and subsequent peptide bond formation. This scenario could provide yet another fitness cost of the A-type mutations, and is presently studied in our laboratory.

A-type IF2 also displayed excessive activation (83%) in the presence of GDP and fMet-tRNA_i (Table II), making initiation with A-type IF2 much less sensitive to variations in the GTP/GDP ratio than initiation with wild-type IF2 (Figure 6). This implies that bacterial strains with A-type IF2 could maintain excessive protein synthesis during conditions of energy shortage (reduced GTP/GDP ratio), with adverse effects on cell viability.

The present discussion about the structural background of IF2 activation by GTP and fMet-tRNA_i for rapid subunit docking is qualitative and tentative. Yet, one would rather like to have structure-based, quantitative explanations for the 300- or 8-fold activation of IF2 on the 30S subunit upon GTP or GDP binding to IF2, respectively. One would, furthermore, like to have structural explanations for the 60-fold activation of IF2 by the fMet group in tRNA_i, and its partitioning in about 9- and 7-fold contributions from the formyl and

Met groups, respectively. Moreover, we have ascribed the transition between 50S-docking active and inactive states of the 30S PIC to conformational changes in IF2 assuming the structure of the 30S subunit unaltered. However, the 30S subunit is flexible (Ramakrishnan, 2008; Zhang *et al*, 2009) and translation-factor promoted conformational changes of this subunit are well documented (Ogle *et al*, 2003; Ramakrishnan, 2008), so that the quantitative structure-based explanation of the 30S PIC activation should include the structural alteration both in IF2 and in the 30S subunit. The recent advances in ribosome crystallography at near-atomic resolution (Selmer *et al*, 2006; Laurberg *et al*, 2008; Weixlbaumer *et al*, 2008; Gao *et al*, 2009; Schmeing *et al*, 2009; Schuette *et al*, 2009; Voorhees *et al*, 2009) in combination with powerful molecular simulation techniques (Carlsson *et al*, 2008; Trobro and Aqvist, 2008; Wallin and Aqvist, 2010) may make such a quantitative structural analysis feasible in the near future. In fact, the power of such a combination has recently been demonstrated by the explanation (Trobro and Aqvist, 2009; Sund *et al*, 2010) of the accuracy of codon reading by class-1 release factors (Freistoffer *et al*, 2000).

In summary, our results demonstrate a stepwise activation of IF2 by its natural GTP and fMet-tRNA_i ligands. We have also shown that mutations in IF2 may compensate for the absence of one of these ligands. Such a stepwise IF2 activation provides a striking example of conditional activation of a GTP-binding protein by GTP and other ligands (Hauryliuk *et al*, 2008a).

Materials and methods

Chemicals and buffers

Phosphoenolpyruvate (PEP), myokinase (MK), pyruvate kinase (PK), inorganic pyrophosphatase (PPi), putrescine and spermidine were purchased from Sigma. Experiments were conducted in a polymix-like buffer, LS4, containing 95 mM KCl, 5 mM NH₄Cl, 0.5 mM CaCl₂, 8 mM putrescine, 1 mM spermidine, 30 mM HEPES pH 7.5, 1 mM DTE, 2 mM PEP, 1 mM GTP, 1 mM ATP and 6 mM Mg(OAc)₂, supplemented with 1 µg ml⁻¹ PK and 0.1 µg ml⁻¹ MK (Pavlov *et al*, 2008). Since each ATP or GTP molecule chelate one Mg²⁺ cation, the actual free Mg²⁺ concentration in the LS4 buffer was 4 mM.

Components of the *in vitro* translation system

70S ribosomes, 50S and 30S subunits, [³H]fMet-tRNA_i, initiation factors as well as Met and Phe animacyl-tRNA synthetases (MetRS and PheRS) were prepared from *E. coli* as described (Freistoffer *et al*, 1997; Antoun *et al*, 2004, 2006b). Initiation tRNA_i, tRNA^{Phe} were purchased from Sigma. mMFTI mRNA with a strong SD sequence was prepared as described in Pavlov *et al* (2008). Overproduced N-terminus-His-tagged wild-type and mutant *S. typhimurium* IF2s were isolated from *Salmonella enterica* Var. Typhimurium LT2 essentially as described in Antoun *et al* (2004). The following IF2 mutants were prepared: A1 (S755Y), A2 (S755F), A3 (E732K), B1 (A740V), B2 (A783V) and B3 (A393V) (Zorzet *et al*, 2010). The positions of the mutation in the IF2 structure are depicted in Supplementary Figure S2.

Comparison of the amino-acid sequences of wild-type IF2 from *S. typhimurium* and *E. coli* showed that they were >96% identical. The identity level was even higher, >98%, when the functionally dispensable N-terminal domain of IF2 (Caserta *et al*, 2010) was excluded. Accordingly, wild-type IF2s from *S. typhimurium* and *E. coli* behaved practically identically in all *in vitro* initiation experiments, which justifies the use of the well-characterized *E. coli* components in the biochemical experiments conducted in this study.

In vitro kinetic experiments

Two mixtures, 1 and 2, were prepared in LS4 buffer. Mixture 1 contained 0.32 µM 30S subunits, 0.8 µM mMFTI mRNA with a

strong SD sequence (Pavlov *et al*, 2008), 1 μM IF1 and 0.6 μM IF2 unless specified otherwise. IF2 always refers to IF2 from *S. typhimurium* unless specified otherwise. Mixture 1 contained also 0.9 μM fMet-tRNA_i, or 1.6 μM Met-tRNA_i or 1.6 μM of deacylated tRNA_i or no tRNA as specified for each experiment. Mixture 2 contained 0.36 μM 50S subunits. Both mixtures (1 and 2) were pre-incubated for 20 min at 37°C. When the effects of GDP were studied, the LS4 buffer contained 1 mM of GTP + GDP instead of 1 mM GTP and MK/PK enzymes were excluded. Met-tRNA_i was methionylated *in situ* as follows. First, 200 μM initiator tRNA, 1 mM [³H]Met amino acid and 800 unit per ml MetRS in LS4 buffer supplemented with 1 $\mu\text{g ml}^{-1}$ PK, 0.1 $\mu\text{g ml}^{-1}$ PPi and 0.1 $\mu\text{g ml}^{-1}$ MK (Jelenc and Kurland, 1979) were combined and pre-incubated for 20 min at 37°C and then put on ice. Just before loading into a stopped flow instrument (see below), a portion of this mixture was added to mixture 1 containing 30S PICs to obtain the final Met-tRNA_i concentration of 1.6 μM . Pre-incubated mixtures 1 and 2 (0.6–0.8 ml volume of each) were loaded into the syringes of a stopped flow instrument (SX-20, Applied Photophysics, Leatherhead, UK). The kinetics of subunit joining was monitored at 37°C with light scattering after rapidly mixing equal volumes (usually 0.06 ml) of mixtures 1 and 2 as described (Antoun *et al*, 2006b). For each particular subunit association experiment, 6–8 high-quality light-scattering traces per 0.6–0.8 ml of mixes 1 and 2 were usually obtained. All concentrations in mixes 1 and 2 specified above are the final concentrations after the mixing.

Treatment of light-scattering data

Each particular subunit-joining experiment provided 6–8 light-scattering traces. These traces were used to obtain an average scattering trace and to estimate the average and standard deviation of the time, $t_{0.5}$, at which the amplitude of light scattering change reached 50% of its maximal value. The time $t_{0.5}$ obtained for the average scattering trace was always very close to the average $t_{0.5}$ time. The effective rate, k_c , of the initiation reaction was defined as the inverse of $t_{0.5}$ for the average light-scattering trace. The above definition of k_c is motivated by the relation:

$$\frac{I(t) - I(0)}{I(\infty) - I(0)} = \frac{k_c \times t}{1 + k_c \times t} = \frac{k_a[50S]_0 \times t}{1 + k_a[50S]_0 \times t} \quad (4)$$

between scattering intensity and time, valid for the irreversible mono-phasic formation of a binary complex, A:B, from particles A and B mixed at equal concentrations (Antoun *et al*, 2006b). The effective rate k_c of the A:B complex formation is equal to the product of the second-order rate constant k_a of the binding reaction by the initial concentration of B particles (50S subunits in our case). Numerical simulations show that the relation $k_a = k_c/[50S]$ holds also when B particles are added in a small excess over A particles.

Light-scattering traces describing the docking of 50S subunits to 30S PICs containing non-formylated Met-tRNA_i, deacylated tRNA_i or no tRNA and different IF2s were mainly bi-phasic and could not be fitted using relation (4) above. However, all light-scattering traces obtained in this study could be numerically fitted to a simple three-parameter kinetic model in Equation (1). This model describes the transitions between an active and an inactive states of the 30S PICs (with first-order rate constants k_1 and q_1) and the docking of 50S subunits to the active 30S PICs with the second-order rate constant k_a . The obtained values of the three parameters k_1 , q_1 and k_a are compiled in Supplementary Tables S1 and S2 along with the derived parameters $K = k_1/q_1$ and $f_A = K/(1 + K)$. The numerical fitting procedure takes into account that the equilibrium between the active and inactive states of the 30S PICs defined by k_1 and q_1 has already established before the addition of 50S subunits (see also 'Remarks on the fitting procedure' in the Supplementary data). This pre-existing equilibrium leads to a strong co-variation of the k_1 and q_1 parameters effectively reducing errors in K (see Supplementary Tables S1 and S2). For clarity of presentation, we used a digital filter to reduce the noise and the number of data points in the scattering traces shown in the figures. We have also attempted to fit our data using more complicated models involving additional intermediate states of the 30S PIC (between the fully active and inactive in the 50S binding) with reduced rate constants for the 50S binding. These more complicated models resulted only in a marginally better fit of the experimental data, which justifies the use of the simple kinetic model in Equation (1).

Conditional switching of IF2 from inactive to active form on the 30S subunit

The present analysis of how 30S-bound IF2 is partitioned between its inactive, 30S_I, and its docking-active, 30S_A, states under the influence of mutations, guanine nucleotides and P-site tRNA variants is adapted from previous work (Hauryliuk *et al*, 2008a).

The equilibrium partitioning of the inactive and active apo-forms of 30S-bound IF2, that is IF2 in the absence of guanine nucleotide and tRNA, have concentrations (or probabilities) related through an equilibrium constant $K_0 l_X$:

The parameter l_X , where X = wt, A or B, accounts for the effect of A-type and B-type mutations on the equilibrium constant, that is $l_{wt} = 1$. In the presence of various forms of initiator tRNA and guanine nucleotides under saturating conditions, the equilibrium relation (5) extends to (see Supplementary data for details):

In the absence of guanine nucleotide, the parameter $m_G = m_0 = 1$. In the presence of GDP or GTP, $m_G = m_{GDP}$ or $m_G = m_{GTP}$, respectively, and

$$m_G = K_I^G / K_A^G, \quad (7)$$

where K_I^G and K_A^G are the (equilibrium) dissociation constants for the binding of the guanine nucleotide variant G to the inactive or active forms of IF2, respectively. In the case of non-saturating concentrations of either GTP or GDP, the relation for K in (6) becomes (Supplementary data):

$$K = K_0 l_X n_T \times \frac{1 + m_G [G] / K_I^G}{1 + [G] / K_I^G}. \quad (8)$$

In the absence of P-site tRNA, the parameter $n_T = n_0 = 1$. In the presence of fMet-tRNA_i, Met-tRNA_i or tRNA_i, $n_T = n_{i\text{Met-tRNA}_i}$, $n_T = n_{\text{Met-tRNA}_i}$ OR $n_T = n_{\text{tRNA}_i}$, respectively, and

$$n_T = K_I^T / K_A^T, \quad (9)$$

where K_I^T and K_A^T are the (equilibrium) dissociation constants for the binding of the tRNA variant T to the inactive or active forms of IF2, respectively.

When there is a mixture of GTP and GDP in the solution, defined by the ratio $\alpha = [\text{GDP}]/[\text{GTP}]$, the fraction, f_A , of IF2 in the active form is given by (see Supplementary data for details):

$$f_A = \frac{1}{1 + 1/K} = \frac{1}{1 + \frac{(\alpha/m_{GDP} + \beta/m_{GTP})}{K_0 l_X n_T (\alpha + \beta)}} \quad (10)$$

Here, β is the ratio between the dissociation (equilibrium) constants for GDP (K_A^{GDP}) and GTP (K_A^{GTP}) binding to the A-form of IF2:

$$\beta = K_A^{GDP} / K_A^{GTP} \quad (11)$$

By setting $\alpha = 0$ or letting $\alpha \rightarrow \infty$ in Equation (10), we retrieve Equation (6) with G = GTP or GDP, respectively. The value of parameter β for wild-type IF2 was determined from Equation (10) by non-linear fitting of the data on the f_A dependence on GTP/(GTP + GDP) fraction (Supplementary Figure S1D) re-plotted as the α -dependence of f_A .

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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