Prokaryotic initiation factor 2 acts at the level of the 30 S ribosomal subunit

A fluorescence stopped-flow study

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The mechanism of action of initiation factor 2 (IF2) from *Escherichia coli* during initiation of translation was investigated by kinetic analysis of the binding of *N*-AcPhe-tRNA^{Phe} or Phe-tRNA^{Phe} to poly(U)-programmed 30 S ribosomal subunits. The reaction was studied by using the stopped-flow technique, monitoring the fluorescence signal of a proflavine inserted next to the anticodon (position 37) of yeast tRNA^{Phe}. Both the rate and extent of *N*-AcPhe-tRNA^{Phe} binding to 30 S subunits are strongly enhanced by IF2. The effect of IF2 was studied at different stoichiometric ratios between factor, ribosomes, and *N*-AcPhe-tRNA^{Phe}, in both the presence and absence of the other two factors. In all cases, the IF2 effect titrates with the 30 S ribosomes. This is also the case in the presence of an equimolar amount of 50 S ribosomal subunits. Furthermore, IF2 was found to stimulate strongly the binding of Phe-tRNA^{Phe}, in spite of the inability of the latter to form a detectable binary complex with IF2. The results are interpreted to mean that IF2 acts in a stoichiometric rather than a catalytic fashion at the level of the 30 S ribosomal subunit. They do not support a model in which IF2 acts as a carrier for the initiator tRNA.

Protein synthesis *Initiation factor* *Stopped-flow kinetics* *Fluorescence*

1. INTRODUCTION

Of the three protein factors necessary for translational initiation in *Escherichia coli*, IF2 is the largest (M_r about 100000) and the one commonly regarded as being responsible for the recognition and the ribosomal binding of the initiator tRNA in response to the start triplet on the mRNA [1,2]. The exact molecular mechanism by which IF2 functions has been the subject of intense debate for more than a decade. In fact, early experiments describing the isolation of a complex consisting of IF2 and fMet-tRNA, with or without GTP [3–5] and some functional resemblances between IF2 and elongation factor Tu (i.e. the presence of a GTP-binding site, ribosome-dependent GTPase activity, stimulation of tRNA binding to ribosomes) led to the hypothesis that IF2 might function as an fMet-tRNA carrier. Further support for this premise came from the finding that, in eukaryotes, translational initiation begins with a factor-mediated binding of Met-tRNA$_i^{Met}$ [6]. Since then, the fMet-tRNA carrier mechanism of IF2 function received additional experimental support [7,8], was endorsed by several textbooks (e.g. [9,10]) and extended to include an active role in the selection of the initiation triplet of the mRNA [7]. The hypothesis of an extraribosomal recognition between fMet-tRNA and IF2 also prompted several studies aimed at the biochemical characterization of the IF2·fMet-tRNA complex and at the identification of the molecular basis of the specificity of such an interaction. Thus, the K_a of the IF2·fMet-tRNA

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complex was estimated to be 10^6 M^{-1} at 37°C [11]. Concerning the specificity of the interaction, it was concluded that this is chiefly due to the presence of a blocked α-NH_2 group in the amino acid. In fact, structural modifications of the $tRNA_f^{Met}$ molecule at 20 different sites did not preclude the formation of the binary complex. Furthermore, complexes between IF2 and several aminoacyl-tRNAs (including Phe-tRNA) were also detected provided that the α-NH_2 group was blocked [12]. More recently, protection from spontaneous chemical hydrolysis and footprinting experiments indicated that the 3′-terminus as well as parts of the T, D and anticodon stem of fMet-$tRNA_f^{Met}$ are in contact with IF2 in the binary complex [11,13,14]. Also, from these studies it was concluded that the blocking at the α-NH_2 group of the amino acid is essential for the interaction.

In contrast to the fMet-tRNA carrier mechanism, an alternative model in which IF2 is bound to the 30 S ribosomal subunits (together with IF1 and IF3) prior to the initiator tRNA and acts at the ribosomal level, is suggested by other experimental results. In this connection particularly relevant are: (i) the finding that it is not obligatory for the ribosomes to bind fMet-tRNA first [15], (ii) the finding that IF2 does not affect the affinity of the 30 S subunit for fMet-tRNA [2], (iii) the effect of GTP on the rate and extent of initiation complex formation [16] which is not paralleled by a corresponding effect on the interaction between IF2 and fMet-tRNA [11,12,17] and, finally, (iv) the finding of mutual interactions and cooperative ribosome binding among the three initiation factors in the absence of initiator tRNA [18,19].

The influence of the initiation factors on the formation of the 30 S initiation complex can be studied using a model system in which the binding of *N*-AcPhe-$tRNA^{Phe}$ to poly(U)-programmed 30 S ribosomal subunits is followed kinetically by using the fluorescence stopped-flow technique, employing a fluorescent tRNA derivative (*N*-AcPhe-$tRNA^{Phe}_{Prf37}$) carrying proflavine in the anticodon loop. The choice of such a model system is fully justified by the well documented similarity between this ternary complex and the physiological 30 S initiation complex as far as factor requirements and mechanistic aspects are concerned [15,20–22]. Working with this system, we have been able to resolve the kinetics of the reaction and have shown previously that the three initiation factors considerably accelerate the formation of the ternary complex [23]. Here, we used the same experimental approach to determine whether the observed acceleration of ternary complex formation is due to the interaction of IF2 with the 30 S ribosomal subunits or with the *N*-AcPhe-$tRNA^{Phe}$.

2. MATERIALS AND METHODS

Ribosomes (tight couples) and ribosomal subunits (30 S and 50 S) were prepared from *E. coli* MRE 600 and characterized as described [23]. Initiation factors IF1, IF2 and IF3 were purified to electrophoretic homogeneity according to [24]. The fluorescent derivative of yeast *N*-AcPhe-$tRNA^{Phe}$ carrying proflavine 3′ to the anticodon (*N*-AcPhe-$tRNA^{Phe}_{Prf37}$; 1.5 nmol *N*-AcPhe/A_{260} unit) was prepared as described [25]. The aminoacylation of $tRNA^{Phe}_{Prf37}$ was performed with purified phenylalanyl-tRNA synthetase as described [25]; after aminoacylation, the mixture containing the Phe-$tRNA^{Phe}_{Prf37}$ (0.9 nmol Phe/A_{260} unit) was diluted 10-fold with buffer and used for the stopped-flow experiments without further purification.

The concentrations of ribosomal subunits, and tRNA were determined from their absorptions at 260 nm, using extinction coefficients ($\mu M^{-1} \cdot cm^{-1}$) of 14.3 and 0.595, respectively. The concentrations of the initiation factors were measured both colorimetrically [26] and spectrophotometrically at 280 nm using the published extinction coefficients [27].

Unless otherwise specified, the stopped-flow experiments were carried out as in [23] at 20°C in a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 7 mM magnesium acetate, 3 mM 2-mercaptoethanol, and 1 mM GTP. The concentrations of 30 S ribosomal subunits and initiation factors were kept at 3×10^{-7} M, and that of *N*-AcPhe-$tRNA^{Phe}_{Prf37}$ at 2×10^{-8} M. Thus, pseudo-first-order conditions with respect to the concentration of ribosomes were established.

The reaction progress curves obtained by the stopped-flow experiments were evaluated in terms of apparent first-order rate constants (k_{app}) and the respective amplitudes by two-exponential least-squares parameter fitting [23]. From the variation

observed in different experiments, the accuracy of the rate constants is estimated to ± 15%.

3. RESULTS

Previously, we have reported that the three initiation factors strongly accelerate the binding of a fluorescent derivative of *N*-AcPhe-tRNA^{Phe} to poly(U)-programmed 30 S ribosomal subunits [23]. IF2 is particularly efficient in this respect, in that, at Mg^{2+} concentrations about 10 mM, it accelerates tRNA binding to the ribosomes nearly 10-fold in the presence of the other two initiation factors and GTP [23]. At 7 mM Mg^{2+}, the formation of the ternary complex depends upon the presence of IF2 (fig.1). Under the same conditions, an only 2-fold acceleration is brought about by IF3. As seen in fig.1, in the presence of IF2, in addition to the fast step, a slow step is observed. The slow step, which has been attributed to a rearrangement of an initially formed complex [23], will not be considered in the present report, which concentrates exclusively on the fast step which, by its linear concentration dependence, has been characterized as the binding step [23]. According to the two alternative models of IF2 function described in section 1, IF2 could promote the binding of *N*-AcPhe-tRNA to 30 S ribosomes by forming an IF2·*N*-AcPhe-tRNA complex with or without GTP, which binds to the ribosomes with a higher rate and efficiency than *N*-AcPhe-tRNA alone. Indeed, complexes between IF2 and *N*-AcPhe-tRNA having stability and properties comparable to those consisting of IF2 and fMet-tRNA have been described [12]. Alternatively, a direct action of IF2 on the 30 S ribosomal subunit, which improves the tRNA-binding capacity of the ribosome, could be envisaged. Experiments designed to distinguish between the two possibilities are described below.

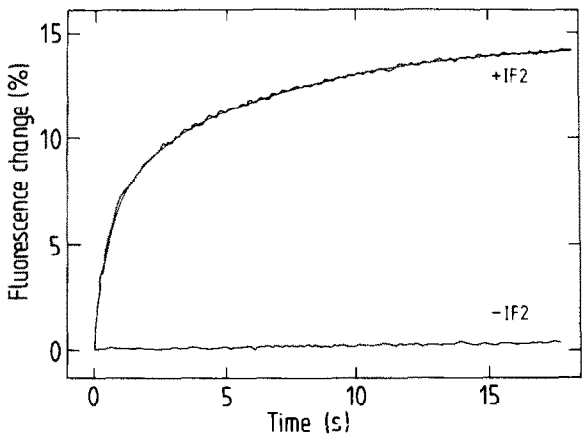

Fig.1. Stimulation of *N*-AcPhe-tRNA$^{Phe}_{Prf37}$ binding to poly(U)-programmed 30 S ribosomal subunits by IF2. The formation of the complex was measured without (lower curve) or with the addition (upper curve) of IF2 in the presence of both IF1 and IF3. The stopped-flow experiments were performed as described in section 2. See table 1 for the parameters obtained by two-exponential least-squares fitting.

First of all, the order of addition of IF2 was varied. As seen in table 1, the complex forms slightly faster, when, prior to the stopped-flow experiment, IF2 is incubated with the ribosomal subunits rather than with the *N*-AcPhe-tRNA^{Phe}. However, the effect is too small to allow a definite conclusion.

To correlate quantitatively the effect of IF2 with the ribosomal subunits, the rate of *N*-AcPhe-tRNA$^{Phe}_{Prf37}$ binding was measured as a function of the ratio of IF2 to 30 S ribosomal subunits. To ensure the stoichiometric uptake of IF2 by the ribosomal subunits, the concentration of the latter was fixed at values above the dissociation constant of the IF2·30 S complex. In the absence of IF1 and IF3, a nearly stoichiometric titration was obtained (fig.2A). The same result was obtained when the IF2 titration was performed in the

Table 1

Effect of the order of addition of IF2 on the rate of *N*-AcPhe-tRNA$^{Phe}_{Prf37}$ binding to poly(U)-programmed 30 S ribosomal subunits

Expt	IF2 with	k_{app1} (s^{-1})	k_{app2} (s^{-1})	A_1 (%)	A_2 (%)
1	ribosomes	1.7 ± 0.2	0.16 ± 0.02	7 ± 1	7 ± 1
2	tRNA	1.2 ± 0.2	0.15 ± 0.03	3 ± 1	5 ± 1

Stopped-flow experiments (cf. fig.1) were performed in the presence of equimolar amounts of IF1, IF3, and IF2 (see section 2). In expt 1, IF2 was preincubated with the ribosomes, as usual; in expt 2, IF2 was preincubated with *N*-AcPhe-tRNA$^{Phe}_{Prf37}$, prior to the stopped-flow experiment. Apparent first-order rate constants and amplitudes were obtained by two-exponential least-squares fitting. *A*, amplitude

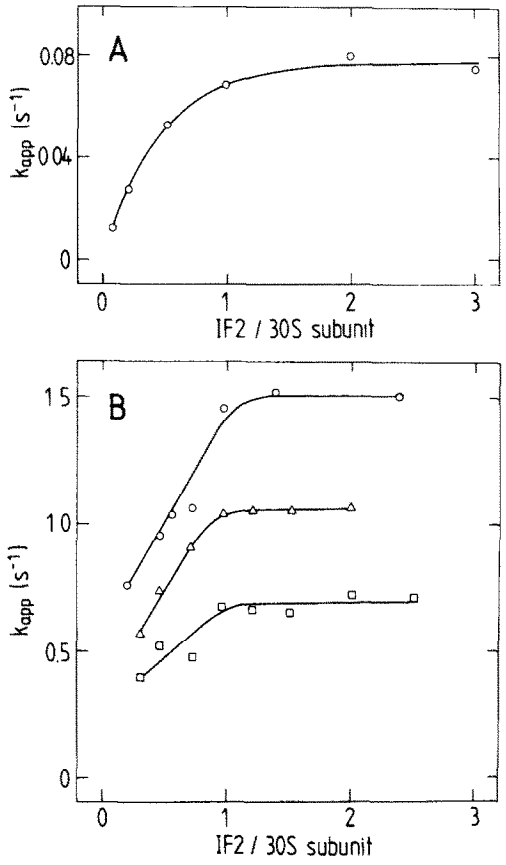

Fig.2. Stoichiometry of the effect of IF2 on ternary complex formation. The rate of binding of *N*-AcPhe-$tRNA^{Phe}_{Prf37}$ to 30 S ribosomal subunits in the presence of 1 mM GTP was measured (A) with increasing amounts of IF2 without IF1 and IF3 (3×10^{-7} M 30 S). (B) IF2 titrations in the presence of IF1 and IF3 with or without 50 S ribosomal subunits. The final concentrations of 30 S and of IF1 and IF3 were either 3×10^{-7} M (○) or 1.5×10^{-7} M (□, △). No 50 S ribosomal subunits (○, □); with 1.5×10^{-7} M 50 S ribosomal subunits (△). The lines fitting the experimental points are calculated on the basis of a model in which the observed acceleration is due to IF2 binding to 30 S ribosomal subunits, using a binding constant of 2×10^8 M^{-1}, which is comparable to the published value obtained under similar conditions [18].

presence of the other two factors (fig.2B). It can also be seen from the figure that at two ribosome concentrations, differing by a factor of two, the respective maximum rates of ternary complex formation were reached at about a 1 : 1 ratio of IF2 to ribosomes. The observed 2-fold difference of the maximum rates is to be expected from the linear dependence of the rate upon the concentration of ribosomes plus initiation factors [23]. In the presence of an equimolar amount of 50 S ribosomal subunits, the rate of *N*-AcPhe-$tRNA^{Phe}$ binding is increased by about 30% (fig.2B). The reason for this slight acceleration is not known at present. Nevertheless, in the presence of 50 S subunits, the 1 : 1 stoichiometry of IF2 to 30 S ribosomal subunits is maintained, indicating that the reaction under study is due to a stoichiometric rather than catalytic action of IF2 on the 30 S ribosomal subunit.

A binding pathway involving an IF2 · *N*-AcPhe-$tRNA^{Phe}$ complex cannot explain the titration curves of fig.2, which were performed at concentrations of tRNA and IF2 2–3 orders of magnitude below the estimated dissociation constant ($\geq 10^{-6}$ M) [11,12] of such a complex. In particular, the clear 1 : 1 stoichiometry of IF2 and 30 S ribosomes cannot be explained by IF2 binding to *N*-AcPhe-$tRNA^{Phe}$. Furthermore, in control experiments, performed without 30 S ribosomes, the

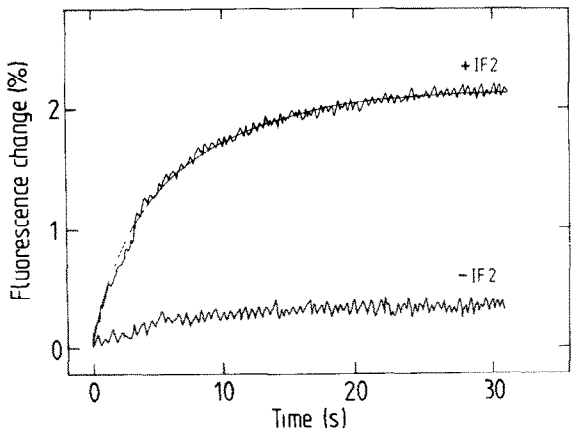

Fig.3. Effect of IF2 on the binding of Phe-$tRNA^{Phe}_{Prf37}$ to 30 S ribosomal subunits. The rate of binding of Phe-$tRNA^{Phe}_{Prf37}$ to poly(U)-programmed ribosomal subunits was measured without (lower curve) or with (upper curve) the addition of IF2 in the presence of GTP (1 mM) IF1 and IF3, and an Mg^{2+} concentration of 13 mM. By least-squares parameter fitting, k_{app} = 0.15 s^{-1} was determined for complex formation in the presence of IF2. The lower curve is due to 30 S binding of uncharged $tRNA^{Phe}_{Prf37}$ mainly. Separate experiments, not shown here, revealed practically no effect of IF2 on this reaction.

formation of a complex of IF2 with *N*-AcPhe-$tRNA^{Phe}_{Prf37}$ could not be detected by measuring fluorescence polarization. Thus, the present results strongly suggest that the stimulation of ternary complex formation by IF2 is due to an interaction of the factor with the 30 S ribosomal subunit.

As mentioned above, it is well established that a prerequisite for the formation of a binary complex between IF2 and an aminoacyl tRNA is the acylation of the α-amino group of the amino acid. The stopped-flow experiments, presented in fig.3, show that the binding of Phe-$tRNA^{Phe}_{Prf37}$ to poly(U)-programmed 30 S ribosomal subunits is strongly stimulated by IF2. Stimulation by IF2 of the rate and extent of Phe-tRNA binding to 30 S ribosomal subunits was also observed by the nitrocellulose filtration method under a variety of experimental conditions. Since in these experiments a Phe-tRNA·IF2 complex could not be found (Canonaco, M., Calogero, R. and Gualerzi, C., unpublished), these observations also support the premise that IF2 acts at the ribosomal level.

4. DISCUSSION

Taken together, the present results give a strong indication that the formation of an extraribosomal binary complex between IF2 and *N*-AcPhe-tRNA is not relevant for the stimulation of *N*-AcPhe-tRNA binding to the 30 S ribosomal subunits by IF2. Since all available evidence indicates that the assembly mechanism of 30 S·acylated aminoacyl-tRNA·template complexes and the stimulation of their formation by the initiation factors is the same regardless of whether the binding concerns the physiological initiator fMet-tRNA or its analogue *N*-AcPhe-tRNA, one can reasonably extend the above conclusion to include the extraribosomal binary complex between IF2 and fMet-tRNA. The data shown here provide evidence in favour of a model in which IF2 binds to 30 S ribosomal subunits prior to the aminoacyl-tRNA and thereby stimulates the binding of the latter.

The molecular mechanism by which the effect of IF2 on the 30 S subunit is brought about remains unknown. Insofar as there is no indication of any interaction between IF2 and non-acylated Phe-tRNA, the IF2-induced stimulation of Phe-tRNA binding reported here can be taken as an indication that IF2 affects some intrinsic property of the ribosomal subunit. This could result from an allosteric effect of the factor on the ribosome. The undoubtedly greater stimulation conferred by IF2 on the ribosomal binding of acylated aminoacyl-tRNA compared to non-acylated aminoacyl-tRNA suggests, on the other hand, that an interaction between IF2 and tRNA on the ribosome, dependent upon the presence of a blocked α-NH_2 group, may play an additional role.

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