Mechanism of translational initiation in prokaryotes

Evidence for a direct effect of IF2 on the activity of the 30 S ribosomal subunit

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Initiation factor IF2 from either *Escherichia coli* or *Bacillus stearothermophilus* was found to possess the previously undetected property of stimulating the template-dependent ribosomal binding of aminoacyl-tRNAs with free α-NH_2 groups. IF1, which had no detectable activity alone, was found to stimulate the activity of *E. coli* IF2 and, to a lesser extent, that of *B. stearothermophilus* IF2. Since in the absence of ribosomes not even a weak interaction between the two IF2 molecules and the aminoacyl-tRNAs was detected, the present findings indicate that IF2 can act at the ribosomal level stimulating aminoacyl-tRNA binding without prior formation of a binary complex with the aminoacyl-tRNA. IF2 does not appear to open or strengthen a weak A-site binding, but rather to enhance aminoacyl-tRNA binding to a 30 S site equivalent to the P-site by slowing down the rate of aminoacyl-tRNA dissociation from ribosomes.

Protein synthesis *Initiation factor* *Aminoacyl-tRNA binding* *Ribosome*

1. INTRODUCTION

To explain the mechanism by which initiation factor IF2 stimulates the formation of the 30 S and 70 S translational initiation complexes, two alternative models have been proposed: (i) IF2, like EF-Tu, functions as an aminoacyl-tRNA carrier specifically recognizing the initiator fMet-tRNA; (ii) IF2 exerts its effect at the level of the 30 S ribosomal subunit to which the factor binds, together with IF1 and IF3, before the binding of fMet-tRNA [1–3].

Compatible with the first hypothesis is the finding that IF2 can form a binary complex with fMet-tRNA [4] as well as with other *N*-acetylated aminoacyl-tRNAs [7,8]. Stopped-flow kinetic analysis showed, however, that the IF2-aminoacyl-tRNA carrier mechanism is not tenable, at least in the case of the model system in which the fMet-tRNA analogue NAcPhe-$tRNA^{Phe}_{Prf37}$ is bound to 30 S ribosomal subunits in response to poly(U) [5]. In this system, the stimulatory activity of IF2 was found to titrate with the 30 S ribosomal subunits and to occur at concentrations of NAcPhe-tRNA two–three orders of magnitude lower than the K_d of the IF2-NAcPhe-tRNA binary complex [5]. Several additional considerations also favor the second model [3,5].

In the present paper, we demonstrate that IF2 from two different sources (*Escherichia coli* and *Bacillus stearothermophilus*) can stimulate, under a variety of experimental conditions, the binding to 30 S ribosomal subunits of aminoacyl-tRNAs with free α-NH_2 groups in response to various templates. Since we show, in agreement with previous reports [4,6–8], that IF2 is unable to form binary complexes with these aminoacyl-tRNAs, our present data indicate that IF2 has a direct influence on the function of the small ribosomal subunit.

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2. MATERIALS AND METHODS

All chemicals were from Merck-Darmstadt and fine biochemicals from Boehringer-Mannheim.

E. coli 30 S and 50 S ribosomal subunits, aminoacyl-tRNAs and purified initiation factors were obtained essentially as described [9–11]. *B. stearothermophilus* IF2 was purified, as it will be described in a forthcoming paper, following a procedure similar to that used for *E. coli* IF2.

Aminoacyl-tRNA binding to 30 S ribosomal subunits was measured by standard nitrocellulose (Millipore HA, 0.45 μm) filtration [11]. Unless otherwise specified, each incubation mixture (50 μl) contained: 50 mM Tris-HCl, pH 7.7; 100 mM NH_4Cl; 7 mM Mg acetate; 1 mM dithiothreitol; 1 mM GTP; 15 pmol 30 S; initiation factors IF1, IF2 and IF3, when present, 40 pmol each. The incubation mixtures also contained the radioactive aminoacyl-tRNAs and the appropriate synthetic polyribonucleotides as indicated in each experiment. Unless otherwise specified, the reaction mixtures were incubated for 15 min at 37°C. The reactions were stopped by addition of 3 ml of buffer having the same ionic composition as the corresponding incubation mixtures and passed through nitrocellulose filters. After washing with 3 ml of the same buffer, the filters were dried and the radioactivity determined.

3. RESULTS

In the course of studying the role of IF2 in initiation complex formation, we observed that this factor stimulated the binding of Phe-tRNA to poly(U)-programmed 30 S ribosomal subunits. In view of the popular interpretation of the role of IF2 in initiation, which attributes to this factor the selection of the initiator tRNA [1], this result was quite unexpected.

The effect of IF2 can be seen from the results of a series of experiments summarized in fig.1. In these experiments, the binding of Phe-tRNA was studied as a function of incubation time (fig.1A), input of [^{14}C]Phe-tRNA (fig.1B), input of poly(U) (fig.1C) and Mg^{2+} concentration (fig.1D). In all cases, the amount of Phe-tRNA bound is clearly higher in the presence of the factor than in its absence. In the experiment shown in table 1, we compared the ribosomal binding of 4 aminoacyl-

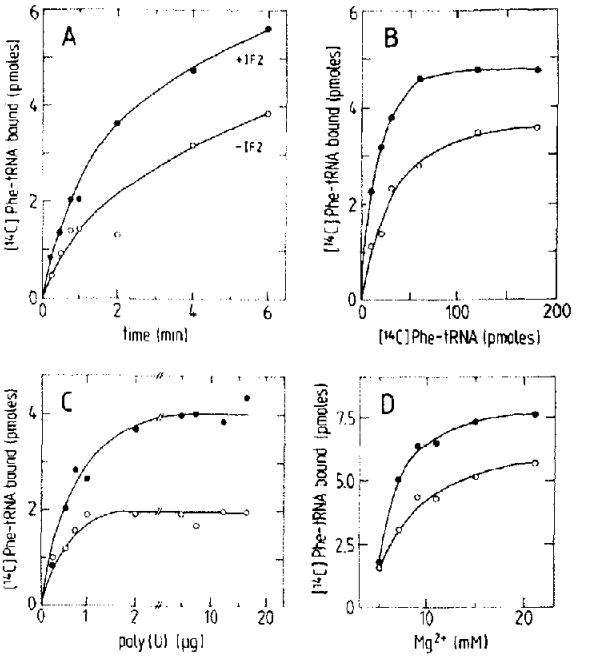

Fig.1. Effect of IF2 on the poly(U)-dependent binding of Phe-tRNA to 30 S ribosomal subunits. All experiments were carried out as described in section 2 varying only the parameters indicated in the abscissa. Open symbols: no initiation factors present; closed symbols: IF2 and IF1 present. [^{14}C]Phe-tRNA binding as a function of: (A) time; (B) input of [^{14}C]Phe-tRNA; (C) input of poly(U); (D) concentration of Mg acetate. A background count represented by the radioactivity remaining on the filters following incubation in the absence of template has been subtracted from all values.

tRNAs (Phe-tRNA, Lys-tRNA, Asp-tRNA and Val-tRNA) in response to 3 different polynucleotides (poly(U), poly(AG), poly(UG)) containing their cognate codons. It can be seen that the total amount of bound aminoacyl-tRNA varies greatly from one system to another, possibly reflecting differences in the stability constants of the individual ternary complexes [12,13]. In all cases, however, binding is substantially higher in the presence than in the absence of IF2 (table 1). To rule out the possibility that these results might stem from preparation artefacts, we repeated the experiments with different preparations of 30 S ribosomal subunits, aminoacyl-tRNAs and IF2. Thus, we compared the activity of 30 S subunits prepared from both high-salt washed ribosomes and tight-couples and 30 S subunits preincubated

Table 1

Effect of *E. coli* IF2 on the ribosomal binding of aminoacyl-tRNAs

Additions			Aminoacyl-tRNA bound			
Template	Aminoacyl-tRNA	pmol	No factors		+IF2,IF1	
			cpm	pmol	cpm	pmol
Poly(U)	[^{14}C]Phe-tRNA	15	685	0.98	1620	2.31
Poly(AG)	[^{3}H]Lys-tRNA	82	1777	0.89	2594	1.30
Poly(AUG)	[^{3}H]Asp-tRNA	150	101	0.08	661	0.50
Poly(UG)	[^{3}H]Val-tRNA	20	287	0.13	578	0.26
Poly(UG)	[^{3}H]Val-tRNA	40	317	0.14	679	0.30

The binding assays were carried out as described in section 2 using *E. coli* 30 S ribosomal subunits, the indicated polynucleotide templates (10 μg) and the indicated amounts of radioactive aminoacyl-tRNAs. A background count represented by the radioactivity remaining on the filters following incubation in the absence of template has been subtracted from all values

with total proteins derived from 30 S subunits; we also used either mixtures of total *E. coli* tRNAs charged with phenylalanine or pure *E. coli* Phe-tRNA^{Phe}. In all cases, we obtained results (not shown) similar to those presented in fig.1. Similar results were also obtained with both homologous and heterologous 30 S ribosomal subunits when IF2 from a different bacterial source (i.e. the thermophilic gram positive *B. stearothermophilus*) was used (table 2). With *E. coli* IF2, the maximum stimulation of Phe-tRNA binding (as well as that of the other aminoacyl-tRNAs) is obtained when IF1 is also present (table 3). IF1 alone, however, has no detectable effect. With *B. stearothermophilus* IF2 and *E. coli* 30 S subunits the activity of the factor is less dependent upon IF1 (table 3).

To determine the ribosomal site to which the IF2-dependent binding of Phe-tRNA takes place, the 30 S-poly(U)-[^{14}C]Phe-tRNA complexes made in the presence or absence of IF2 were subjected to the puromycin reaction. In good agreement with earlier data [14], our results show that a large por-

Table 2

Effect of *B. stearothermophilus* IF2 on the ribosomal binding of aminoacyl-tRNAs

Source of 30 S subunits	Additions			Aminoacyl-tRNA bound			
	Template	Aminoacyl-tRNA	pmol	No IF2		+IF2 (*B. stearo.*)	
				cpm	pmol	cpm	pmol
B. stearo.	poly(U)	[^{14}C]Phe-tRNA	60	815	1.16	1582	2.26
E. coli	poly(U)	[^{14}C]Phe-tRNA	8	780	1.11	1908	2.71
E. coli	poly(U)	[^{14}C]Phe-tRNA	42	1744	2.49	3650	5.21
E. coli	poly(AG)	[^{3}H]Lys-tRNA	20	2095	1.04	2990	1.50
E. coli	poly(AG)	[^{3}H]Lys-tRNA	110	2618	1.31	5924	3.00
E. coli	poly(UG)	[^{3}H]Val-tRNA	14	397	0.18	606	0.27
E. coli	poly(UG)	[^{3}H]Val-tRNA	60	1063	0.47	1225	0.55

The conditions of this experiment were identical to those described in table 1 with the exception that the samples did not contain IF1

Table 3

Effect of IF1 on the IF2-dependent stimulation of Phe-tRNA binding to 30 S ribosomal subunits

Expt	Additions	[^{14}C]Phe-tRNA bound	
		cpm	pmol
1	None	685	0.98
	IF1	631	0.90
	IF2 (*E. coli*)	1050	1.50
	IF2 (*E. coli*) + IF1	1623	2.31
2	None	896	1.28
	IF1	780	1.11
	IF2 (*B. stearo.*)	2312	3.30
	IF2 (*B. stearo.*) + IF1	2977	4.25
3	None	815	1.16
	IF1	644	0.92
	IF2 (*B. stearo.*)	1582	2.26
	IF2 (*B. stearo.*) + IF1	3071	4.39

The experimental conditions are essentially those described in section 2. The source of 30 S ribosomal subunits is *E. coli* in expts 1 and 2 and *B. stearothermophilus* in expt 3. [^{14}C]Phe-tRNA added is 15 pmol in expt 1 and 60 pmol in expts 2 and 3

Table 4

Puromycin reactivity of Phe-tRNA bound to the *E. coli* 30 S ribosomal subunits in the presence or absence of IF2 and IF1

Expt	Initiation factors present	[^{14}C]Phe-tRNA bound (cpm)	[^{14}C]Phe-puromycin formed	
			cpm	%
1	None	3513	1951	55.5
	IF1 + IF2	4796	4126	86.0
2	None	3108	1750	56.3
	IF1 + IF2	4442	3736	84.1

The binding of [^{14}C]Phe-tRNA to poly(U)-programmed 30 S ribosomal subunits was carried out as described in section 2 in the presence or absence of the initiation factors as indicated. Following the binding reaction, 50 S ribosomal subunits, in an amount stoichiometrically equivalent to that of the 30 S subunits and puromycin (final concentration, 1 mM) were added. To prevent possible translocation events, a 10-fold excess of cold Phe-tRNA (expt 1) or 0.1 mM fusidic acid (expt 2) was added together with the 50 S ribosomal subunits. The puromycin reaction was carried out for 30 min at 37°C essentially as described by Leder and Bursztyn [15]

tion (≥50%) of the Phe-tRNA bound in the absence of IF2 is puromycin-reactive (table 4). Moreover, it was found that the Phe-tRNA bound in the presence of IF2 is also, for the most part (≥80%), in the P-site (table 4). Furthermore, the addition of increasing amounts of non-radioactive NAcPhe-tRNA was found to chase completely from its site on the 30 S ribosomal subunit the radioactive Phe-tRNA bound in the presence of IF2 (not shown). Taken together, these data indicate that IF2 stimulates the binding of Phe-tRNA to a ribosomal site equivalent to the P-site rather than 'opening' or stabilizing a weak A-site binding.

To determine whether the stimulation by IF2 could be explained by the existence of a binary interaction between the factor and Phe-tRNA in the absence of the ribosome, we looked for an IF2-dependent protection of Phe-tRNA [16]. Similar experiments had allowed Petersen et al. [4] to assess the existence of an IF2-fMet-tRNA interaction. Our results clearly show that the rate of Phe-tRNA hydrolysis is identical in the presence and absence of *E. coli* IF2 (fig.2A). The primary and presumably the secondary and tertiary structures of *E. coli* and *B. stearothermophilus* IF2 are rather similar [17]; in the absence of ribosomes, however, *B. stearothermophilus* IF2 displays a substantially greater affinity for fMet-tRNA and NAcPhe-tRNA than *E. coli* IF2 (unpublished). Nevertheless, in protection experiments similar to those described above, *B. stearothermophilus* IF2 showed no measurable affinity for Phe-tRNA (fig.2A). Thus, our data demonstrate the inability of IF2 to protect Phe-tRNA from hydrolysis under conditions where nearly complete protection of both fMet-tRNA and NAcPhe-tRNA are observed. Furthermore, addition of cold Phe-tRNA^{Phe}, even in large (100-fold) excess, was found not to diminish the IF2-dependent protection of these N-blocked aminoacyl-tRNAs (fig.2B). From this experiment, we can rule out that IF2 might interact with Phe-tRNA without protecting it from hydrolysis because, unlike the case with the α-NH_2-blocked aminoacyl-tRNAs, the ester bond within the complex remains accessi-

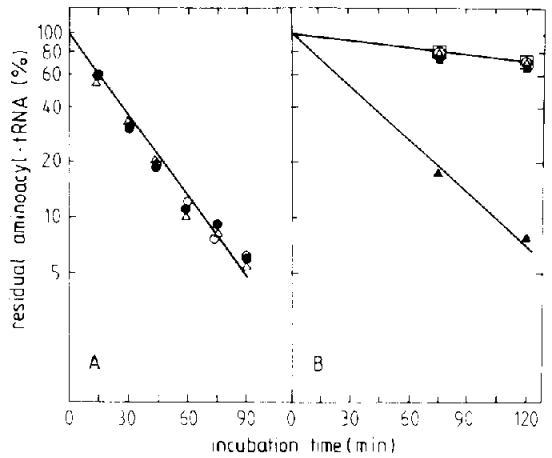

Fig.2. Lack of binary interaction between Phe-tRNA and IF2. (A) Spontaneous hydrolysis of [^{14}C]Phe-tRNA in the presence and absence of IF2 from *E. coli* or *B. stearothermophilus*. Three 0.1 ml reaction mixtures were prepared each containing 100 mM Tris-HCl, pH 8, 100 mM NH_4Cl, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, 1 mM GTP and 3.4 × 10^{-7} M [^{14}C]Phe-tRNA. One incubation mixture contained no IF2 (○), the others contained 4.3 × 10^{-6} M IF2 from either *E. coli* (●) or *B. stearothermophilus* (△). The tubes were incubated at 37°C and 10 μl aliquots were withdrawn from each sample at the indicated times and spotted onto Whatman 3MM paper discs for determination of the residual acid-insoluble radioactivity. (B) Lack of competition between Phe-tRNA^{Phe} and fMet-tRNA$_f^{Met}$ for binding to *B. stearothermophilus* IF2. The experiment was carried out as described above with 3.1 × 10^{-7} M f[^{3}H]Met-tRNA$_f^{Met}$ and with (△) and without (▲) 2.4 × 10^{-6} M IF2 from *B. stearothermophilus*. In addition to IF2 and f[^{3}H]Met-tRNA$_f^{Met}$, some samples contained non-labelled Phe-tRNA^{Phe} in a 25-fold (□), 50-fold (■), and 100-fold (○) molar excess over the fMet-tRNA$_f^{Met}$.

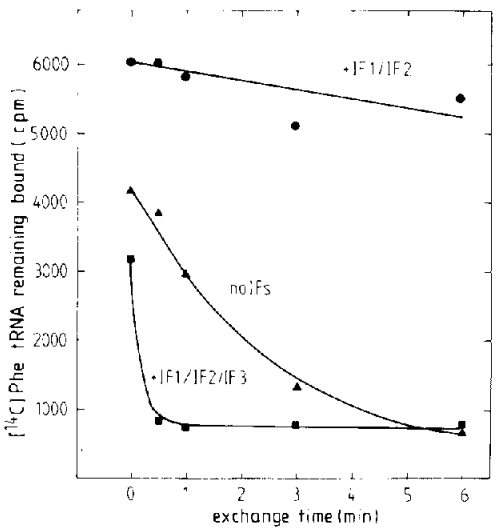

Fig.3. Effect of initiation factors on the rate of exchange of 30 S-bound Phe-tRNA. Reaction mixtures for the binding of [^{14}C]Phe-tRNA to *E. coli* 30 S ribosomal subunits (see section 2) were scaled up five times. The binding reaction was carried out either in the absence of initiation factors (▲) or in the presence of IF1 and IF2 (●) or of IF1, IF2 and IF3 (■). The values for binding at time 0 min represents the amount of complex formed in 1/5 of each reaction mixture after the standard 15 min incubation at 37°C. The incubation was continued with the rest of the samples after adding to each of them non-labelled Phe-tRNA in a 5-fold molar excess over [^{14}C]Phe-tRNA. Samples were withdrawn after 0.5, 1, 3 and 6 min to determine the residual amount of [^{14}C]Phe-tRNA retained in the ternary complex.

ble to the solvent. Thus, since no binary interaction with $K_a \geq 10^4$ M^{-1} between IF2 and Phe-tRNA takes place, we can conclude that the stimulation of the ribosomal Phe-tRNA binding by IF2 is not due to a tRNA-carrier mechanism.

To understand better the mechanism of the IF2-dependent stimulation of aminoacyl-tRNA binding, we investigated whether IF2 might influence the rate of exchange between free and ribosome-bound aminoacyl-tRNA. Thus, 30 S-poly(U)-[^{14}C]Phe-tRNA complexes were formed in the presence or absence of IF2 and IF1 after which an excess of unlabelled Phe-tRNA was added. The amount of radioactive Phe-tRNA remaining in the complexes was then determined as a function of the time allowed for the exchange. Since it is well established that IF3 strongly accelerates the rate of this exchange [18], we included, as a positive control, a 30 S-poly(U)-[^{14}C]Phe-tRNA complex formed in the presence of all three factors. As seen from fig.3, compared to the case where no factors are present, the rate of Phe-tRNA exchange is drastically reduced in the complex containing IF1 and IF2 while, as expected, the presence of IF3 resulted in a much faster exchange.

4. DISCUSSION

We have demonstrated that IF2 can stimulate the binding to the 30 S ribosomal subunits of 4 dif-

ferent aminoacyl-tRNAs with free α-NH_2 groups. Even though the extent of the stimulation and the total amount of ternary complex formed is different with each aminoacyl-tRNA and with each type of template used, stimulation by IF2 was observed under all conditions tested. Initiation factor IF1, which alone had no effect, was required for maximum activity with *E. coli* and, to a lesser extent, with *B. stearothermophilus* IF2. The effect of IF1 can be taken as an additional indication that the activity described here bears close resemblance to that by which IF2 stimulates the formation of 30 S initiation complexes.

We have also shown that IF2 affects a tRNA binding site of the 30 S ribosomal subunit equivalent to the P-site and slows down considerably the rate of exchange between free and 30 S-bound aminoacyl-tRNA. This can be explained by a decrease in the off-rate of the codon-dependent aminoacyl-tRNA-ribosome interaction. Since IF2 was also found to produce a strong increase in the apparent first order rate constant of $Phe\text{-}tRNA^{Phe}_{Ptf37}$ binding to poly(U)-programmed ribosomes [5], it seems that IF2 affects both on- and off-rates of the (presumably numerous) transitions leading to the formation of a ternary complex from a pre-ternary complex and vice versa [2,3,5]. The final consequence of these changes is a more or less marked shift to the right (i.e. towards association) of the overall equilibrium regardless of the type of template or aminoacyl-tRNA (e.g. fMet-tRNA or NAcPhe-tRNA in the widely studied cases or, as in the present report, aminoacyl-tRNAs with free α-NH_2 groups).

The present data show that IF2 has no measurable affinity for the aminoacyl-tRNAs whose ribosomal binding it stimulates. The most restrictive interpretation of these results is that IF2 alters somehow the properties of the 30 S ribosomal subunits to which it binds. On the other hand, if the stimulation of the ribosomal binding of the aminoacyl-tRNAs is taken as a model for the mechanism of action of IF2, such a mechanism must operate without the need for the formation of an IF2- (initiator) aminoacyl-tRNA complex off the ribosome.

So far, it has been assumed that the selection of the initiator tRNA is due to the discrimination of IF2 against aminoacyl-tRNAs bearing free α-NH_2 groups. Since the present data show, however, that IF2 can stimulate the ribosomal binding of these aminoacyl-tRNAs, the issue of the mechanism of fMet-tRNA selection should be reconsidered. While we do not yet have a satisfactory answer to this problem, it should be mentioned that, though stimulated several-fold by IF2, the binding of Phe-tRNA was found to be substantially slower than that of the corresponding *N*-acetylated form [5,9] and that the off-rate from ribosomes of non-initiator aminoacyl-tRNAs is increased by IF3 ([18] and see also fig.3) much more than that of the initiator fMet-tRNA [13]. Thus, the available data seem to be in line with the hypothesis of a kinetic selection of the correct initiation complex mediated by the initiation factors [2,3].

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