Mechanism of protein biosynthesis in prokaryotic cells

Effect of initiation factor IF1 on the initial rate of 30 S initiation complex formation

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To define the step at which translational initiation factor IF1 excercises its stimulation, initial rate kinetic analyses of 30 S initiation complex formation were carried out in the presence and absence of this factor. It was shown that, without affecting the affinity of the ribosomes either for the initiator tRNA or for the poly(AUG) used as template, IF1 increases approximately 2.5-fold the limiting V_{max} of the 'pre-ternary complex' → ternary complex transition which represents the rate-limiting step in 30 S initiation complex formation. This kinetic effect titrates with the 30 S ribosomal subunit which must therefore represent the target of IF1 action.

Translational initiation *Initiation factor* *30 S initiation complex* *Kinetics* *Ribosome*

1. INTRODUCTION

Translational initiation factor IF1, the smallest of the three initiation factors, has been found to stimulate several partial reactions pertaining to the initiation process (reviews, see [1–3]) and is required for optimal in vitro protein synthesis [4]. However, no autonomous role has been found for IF1, whose overall mechanism of action remains rather elusive.

IF1 stimulates the ribosome dissociation activity of IF3 [5–7], the recycling of IF2 from the 70 S ribosomes [8,9], as well as the codon-dependent binding of fMet-tRNA (or of its analogue NAcPhe-tRNA) to 30 S ribosomal subunits [9–11]. In turn, any one of these activities could account for the IF1 stimulation of 70 S initiation complex formation [9,12,13]. The basis for the stimulation of the 30 S initiation complex formation, on the other hand, could be attributed, in principle, to an IF1-induced enhancement of the affinity of the ribosomes for the IF2-GTP-fMet-tRNA complex [13,14] or for the initiation triplet [15]; neither explanation, however, seems to be fully satisfactory [3].

Here, to determine the step(s) at which IF1 exercises its stimulation of 30 S ternary complex formation, initial rates of poly(AUG)-dependent fMet-tRNA binding to 30 S ribosomal subunits were measured in complete systems or in systems from which IF1 was omitted. The results obtained, interpreted in light of the proposed mechanistic model of 30 S initiation complex formation [16,17], indicate that IF1 acts at the level of the 30 S ribosomal subunit and behaves as a kinetic effector of the rate-limiting step of 30 S initiation complex formation (i.e., a conformational rearrangement leading from the pre-ternary complex to the ternary complex).

2. MATERIALS AND METHODS

Escherichia coli MRE600 initiation factor-free 30 S ribosomal subunits, purified initiation factors IF1, IF2 and IF3 and f[^{3}H]Met-tRNA were

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prepared as previously described [18]. The formation of the 30 S initiation complexes was followed by rapid filtration through Millipore filters essentially as described [16]. The reaction mixtures (0.08 ml) contained 1.25×10^{-7} M each of 30 S ribosomal subunits, IF2, IF3 and 1 mM GTP in 10 mM Tris–HCl, pH 7.5, 7.5 mM Mg acetate, 50 mM NH_4Cl and 5 mM βMetOH. Furthermore, each incubation mixture contained the amounts of f[^{3}H]Met-tRNA and poly(AUG) indicated in each figure and, unless otherwise specified, 1.25×10^{-7} M IF1. The reaction mixtures, started by the addition of 30 S ribosomal subunits, were incubated at10 ± 0.5°C for 4 s, stopped by addition of 2.5 ml of ice-cold buffer (10 mM Tris–HCl, pH 7.5, 7.5 mM Mg acetate, 50 mM NH_4Cl, 5 mM βMetOH) and passed through Millipore filters (HA 0.45 μm) within 1–2 s under a vacuum of 25–30 inch Hg.

3. RESULTS

Results of previous experiments applying initial rate kinetics to the analysis of the mechanism of 30 S initiation complex formation are compatible with and suggestive of the mechanistic scheme depicted in fig.1 [16,17]. In this model, the 30 S ribosomal subunit contains separate and independent binding sites for fMet-tRNA and for the template, and a rapid equilibrium exists between 30 S with both sites vacant, with either one or the other site occupied, and with both sites occupied but non-interacting (pre-ternary complex).

A rate-limiting transition transforms the pre-ternary complex into a more stable ternary complex in which codon-anticodon interaction takes place. Fluorescence stopped-flow studies [11] supported the above scheme and indicated that step C in fig.1 consists of at least two separate steps.

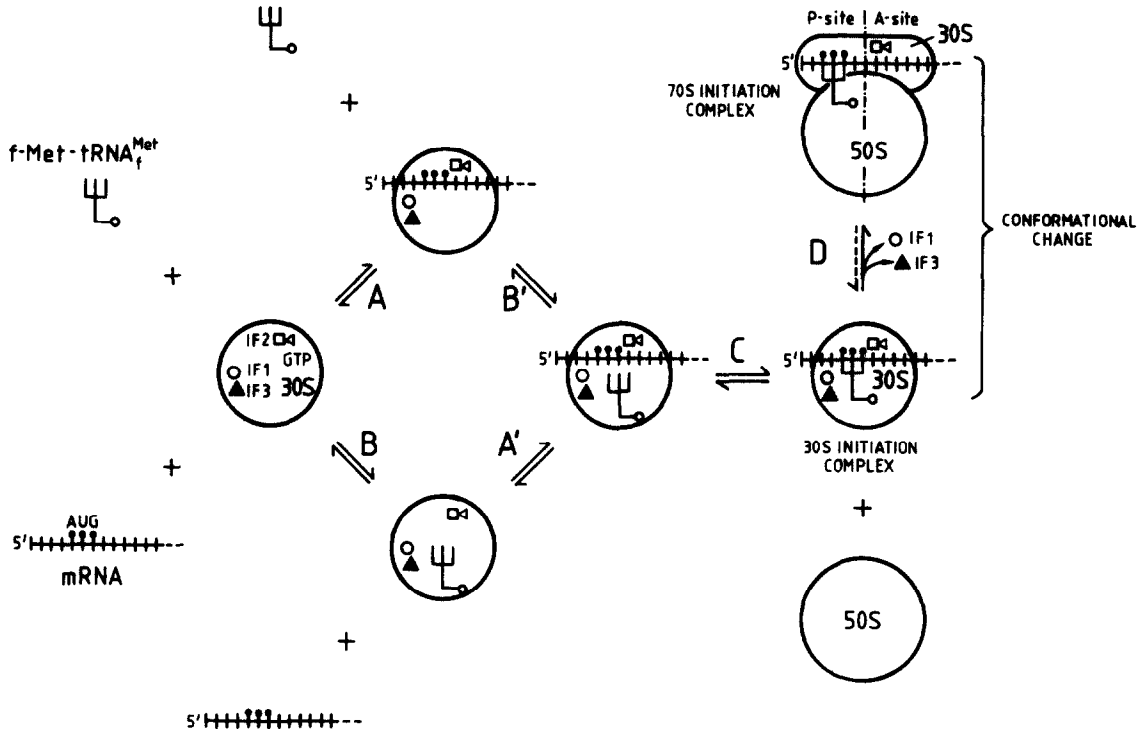

Fig.1. Schematic representation of initiation complex formation, showing the proposed random order mechanism of initiation complex formation [3,16,17]. Steps A, B, B′, A′ are in rapid equilibrium. The 'on' rate of step C is the rate-limiting step and probably consists of at least two first-order rearrangements of the initially formed pre-ternary complex [11]. Step D (i.e., the joining of the 50 S ribosomal subunits to the 30 S initiation complex) which encompasses the recycling of IF1 and IF3, is probably accompanied by a conformational change of ribosomes and is virtually irreversible [20].

The initial rate of ternary complex formation in the presence or absence of IF1 was determined by varying the concentration of poly(AUG) (fig.2A) or of fMet-tRNA (fig.2B) while keeping a fixed concentration of either fMet-tRNA (fig.2A) or of poly(AUG) (fig.2B). As seen in the figure, each double reciprocal plot yields two straight lines, and in both cases, IF1 increases the V_{max} of the reaction without affecting the apparent K_m of the ribosomes, either for poly(AUG) (0.9×10^{-8} M) or for fMet-tRNA (5×10^{-8} M).

Results qualitatively identical to those presented in fig.2A,B were obtained when the effect of the linear variation of one substrate (either poly(AUG) or fMet-tRNA) was checked against three different fixed concentrations of the other (fMet-tRNA or

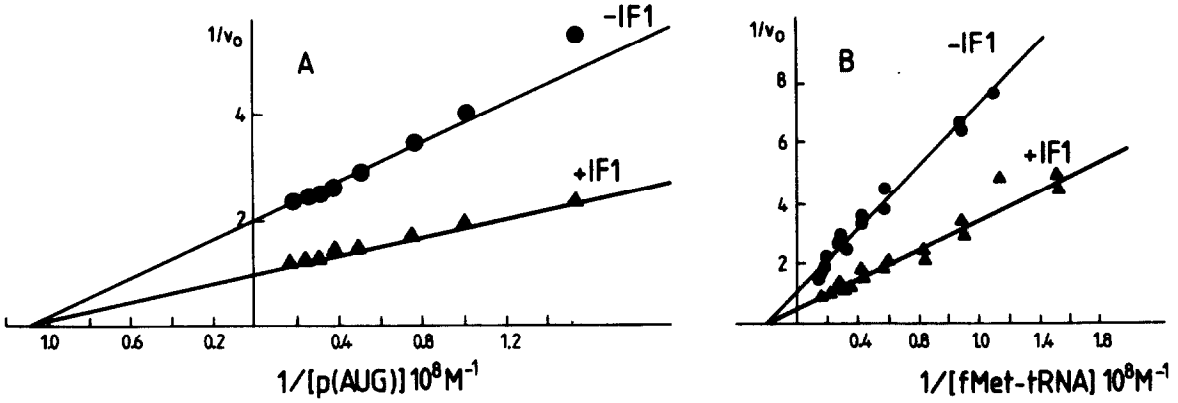

Fig.2. Dependence of the initial velocity of 30 S initiation complex formation on the substrate concentration in the presence and absence of IF1. The reaction conditions were as described in section 2. (A) Dependence on poly(AUG) concentration; (B) dependence on fMet-tRNA concentration. (▲) IF1 present; (●) IF1 absent.

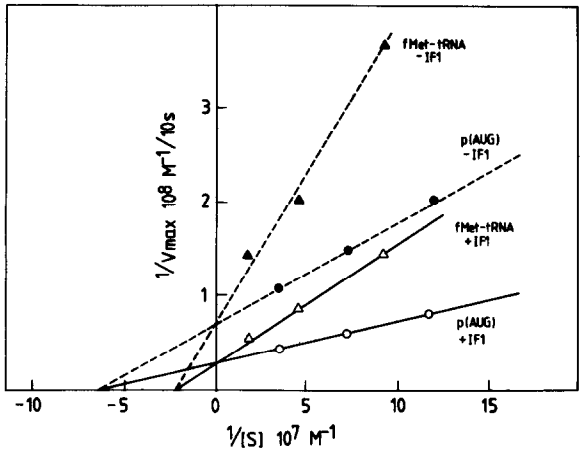

Fig.3. Dependence of the V_{max} of 30 S initiation complex formation on substrate concentration in the presence and absence of IF1. Secondary plots of primary Lineweaver–Burk plots similar to those shown in fig.2. (△, ○) IF1 present; (▲, ●) IF1 absent. (▲, △) variation of fMet-tRNA concentration; (●, ○) variation of poly(AUG) concentration.

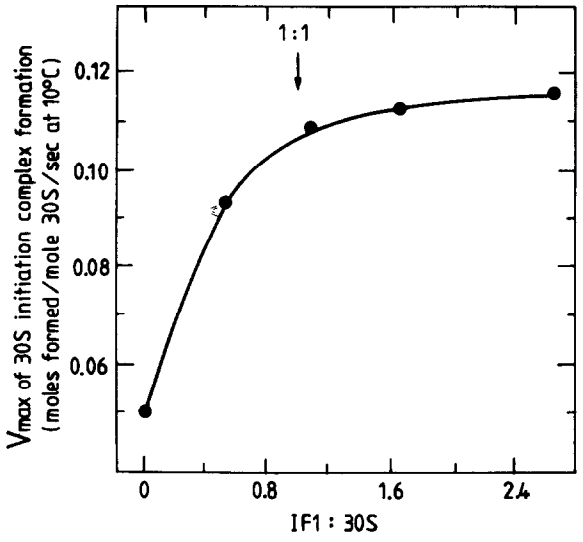

Fig.4. Dependence of the V_{max} of 30 S initiation complex formation on the IF1:30 S molar ratio. The reaction conditions were as described in section 2 except that the concentration of 30 S ribosomal subunits, IF2 and IF3 was 2.4×10^{-7} M and that the concentration of IF1 was varied as indicated in the figure.

poly(AUG)). The double reciprocal plots of these experiments yielded straight lines crossing the abscissa at the same point (same apparent K_m), but having different intercepts on the ordinate (different V_{max}). Replots (1/[S]) *vs* $1/V_{max}$) of these primary data are presented in fig.3. As seen in the figure, two straight lines yielding the same V_{max} (limiting V_{max}) and the two K_m values (for the template and for the initiator tRNA), identical to those obtained in the primary plot, are obtained in the presence of IF1. In the absence of IF1, similar results (and identical K_m values) are obtained. In this case, however, the curves meet on the ordinate at a different (higher) point yielding a 2.5-times lower limiting V_{max}.

To determine the nature of the target of the IF1-induced rate increase of complex formation we carried out an experimental identical to that shown in fig.2 but for the variation in the IF1 concentration to yield increasing IF1 : 30 S ratios. As seen in fig.4, the increase in V_{max} of initiation complex formation titrates with the amount of IF1 present in the system, reaching a plateau when IF1 and 30 S ribosomal subunits are present in roughly equimolar amounts.

4. DISCUSSION

The data presented here are consistent with the kinetic laws governing the formation of a ternary complex via a random pathway and, thus, also with the previously drawn conclusions concerning the mechanism by which the 30 S initiation complex is formed [16,17]. Furthermore, these data indicate that the presence of IF1 does not alter, from the mechanistic point of view, the way by which the 30 S initiation complex is formed and does not affect the K_m for either template or aminoacyl-tRNA. According to the random order rapid equilibrium rate equation [19] these K_m values can be regarded as being equivalent to the dissociation constants of the two intermediate binary complexes (30 S–fMet-tRNA and 30 S–poly(AUG)) or of fMet-tRNA and poly(AUG) from the pre-ternary complex [16,17]. Thus, it is clear that IF1, unlike IF3 [16], does not affect the affinity of the 30 S ribosomal subunit for either the initiator tRNA or the template. This is at variance with that reported by others, who found an increased affinity (lower K_m) of the ribosomes for the template in the presence of IF1 [15]. This discrepancy, however, is not surprising; in fact, in the earlier experiments initial rates were most likely not measured since, after 15 min at 25°C, the system had probably reached equilibrium.

The present data indicate that IF1 stimulates kinetically the formation of the initiation complex by increasing (approx. 2.5 times) the limiting V_{max} of the process which corresponds to the 'on' rate constant of the rate-limiting step (step C in fig.1) [16]. Accordingly, the extent of this stimulation agrees well with the extent to which IF1 increases the level of fMet-tRNA binding at equilibrium [9] or the amount of protein synthesized [4]. Thus, it would appear that the kinetic stimulation observed here is sufficient, by and large, to account for the overall effect of IF1 on protein synthesis, in accordance with the widely accepted premise that initiation complex formation is the rate-limiting step in translation.

As to the actual value of the limiting V_{max} for the 30 S initiation complex formation, our data indicate approx. 0.11 mol complex formed/mol 30 S per s at 10°C. As a rule of thumb, this rate should increase approx. 6-fold going from 10 to 37°C. If one also takes into account that no more than 50% of the 30 S ribosomal subunits are 'active', one comes to an estimation of better than one polypeptide chain started in vitro per s. Since the estimated rate of in vivo elongation is 15–20 amino acids/s, our value for in vitro initiation complex formation cannot be too far from the physiological one.

Since the effect of IF1 on the rate of ternary complex formation titrates with the 30 S ribosomal subunits, it is possible to conclude that these are the targets of IF1 action. The present experiments, however, do not allow us to differentiate between a direct action on the ribosomal particle or an effect mediated via the ribosome-bound IF2 and IF3.

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