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THE ROLE OF eIF-4C IN PROTEIN SYNTHESIS INITIATION COMPLEX FORMATION

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Summary

eIF-4C has a pronounced stimulatory effect on initiation complex formation with native 80-S ribosomes (80-S_n) as the only source of ribosomal subunits, but only a small effect when washed 40-S subunits are used.

eIF-4C is accessary to eIF-3 in dissociating 80-S_n ribosomes.

eIF-4C is present on 40-S_n but absent on 40-S_n dimers, which occur in preparations of native ribosomes and are as such inactive in protein synthesis.

eIF-4C dissociates 40-S_n dimers into active monomers. These results can be explained by assuming that the presence of eIF-4C on 40-S_n prevents:

(a) premature association with 60-S ribosomal subunits and

(b) dimerisation, thus increasing the rate and extent of initiation complex formation.

Introduction

Initiation of protein synthesis in cell-free systems derived from eukaryotic cells proceeds via a complex sequence of events in which at least eight initiation factors participate [2—4]. In one of the first steps a ternary complex of initiation factor 2 (eIF-2), methionyl-$tRNA_f$ and GTP binds to the small ribosomal subunit [5,6]. This reaction is stimulated by two other factors eIF-3 (M_r 750 000) and eIF-4C (M_r 17 500) [2,3,4].

The interaction between eIF-3 and the 40 S ribosomal subunit in a stoichiometric fashion has been demonstrated [2,4,7], which seems to indicate that the

Abbreviations: 40 S_n, native 40 S ribosomal subunit; 40 S_{nd}, dimers of the native 40 S ribosomal subunit; 60 S_n, native 60 S ribosomal subunit; 80 S_n, native 80 S ribosome.

presence of eIF-3 on the 40 S subunit is required either to prevent its premature association with 60 S or to stabilize the nascent 40 S initiation complex [8].

The role of eIF-4C is much less clear, particularly because the observed stimulatory effects are small (1.5-fold) and attempts to demonstrate the presence of eIF-4C on the complex at any stage of the initiation process have failed [2–4,8].

The present study deals with the role of eIF-4C in initiation of protein synthesis. We find that the stimulatory activity of eIF-4C in the initiation of protein synthesis can be explained by its effect on the dissociation of 80-S_n ribosomes and the subsequent prevention of the formation of 40-S_n dimers, which are inactive in protein synthesis.

Materials and Methods

Biological compounds. Highly purified initiation factors from rabbit reticulocytes and rabbit liver tRNA were prepared according to Voorma et al. [9] and to Benne et al. [10]. [^{3}H]Methionyl-tRNA$_f$ (Met-tRNA$_f$), spec. act. 4500 cpm/pmol, was prepared according to Bose et al. [11] as modified by Stanley [12]. Washed ribosomal subunits were prepared according to Schreier and Staehelin [13].

Binding of [^{3}H]Met-tRNA$_f$ to 40 S. Binding of [^{3}H]Met-tRNA$_f$ to 40 S and analysis of the assay mixtures was performed as described by Van der Mast et al. [14]. A typical assay mixture contained in a final volume of 25 µl: 20 mM Hepes-KOH (pH 7.6)/1 mM ATP/0.4 mM GTP/5 mM creatine phosphate/1 mM dithiotreitol/120 mM $K(Ac)_2$/2 mM $Mg(Ac)_2$/0.1 mM spermine/10 pmol [^{3}H]-Met-tRNA$_f$/0.05 units of creatine kinase and ribosomes and initiation factors as indicated in the figure legends.

After incubating at 37° C for 15 min, 75 µl ice-cold buffer containing 20 mM Tris-HCl (pH 7.6)/3 mM $Mg(Ac)_2$/100 mM KCl and 7 mM 2-mercaptoethanol (Buffer A) was added.

The samples were then layered on isokinetic gradients of 10% to 30% sucrose in buffer A. After centrifugation, fractions of 250-µl were collected and counted.

Preparation of 80 S_n, 60 S_n + 40 S_{nd} and 40 S_n. Reticulocyte lysate was prepared as described by Schreier and Staehelin [15]. 250 ml lysate was centrifuged for 35 min at 60 000 rev./min in a 60-Ti rotor to remove the polyribosomes.

The supernatant was centrifuged for 16 h at 45 000 rev./min in the same rotor. The pellets from the second centrifugation were resuspended in buffer B (20 mM Tris-HCl (pH 7.6)/100 mM KCl/4 mM $Mg(Ac)_2$/14 mM 2-mercaptoethanol) containing 0.25 M sucrose. The ribosome suspension was loaded on a 10% to 40% isokinetic gradient in buffer B in a B XIV zonal rotor. After centrifugation for 4 h at 48 000 rev./min, 50 fractions of 10 ml were collected, from which the absorbance at 260 nm was measured. The ribosomes from the 80-S, 60-S and 40-S regions were pelleted in a 60-Ti rotor for 16 h at 40 000 rev./min. The pellets were resuspended in buffer A with 0.25 M sucrose. The purity of the preparations was determined on analytical gradients of 15 to 32%

sucrose in buffer B containing 100 or 500 mM KCl. The profiles are shown in Fig. 1.

Panels A, B and C represent the analysis of 40 S_n, 60 S_n and 80 S_n on low salt gradients, whereas panels D, E and F give the results of the high salt gradients.

From panel D it is obvious that the 40 S_n preparation is over 90% pure. Panel A, however, reveals a strong tendency of this particle to dimerise in low salt gradients. Panel C shows that the 80 S_n preparation is over 90% pure, since equimolar amounts of 40 S and 60 S arise from high salt induced dissociation (panel F). The occurence of 40-S_n dimers (40 S_{nd}) in otherwise homogeneous 60-S preparations, which explains the contamination of 60-S ribosomal subunit preparations with 40-S subunits, has been observed by others [16,17]. This is clear when panel B (60 S, low salt) is compared to panel E (60 S, high salt): the preparation of seemingly homogeneous 60-S_n subunits appeared to be contaminated for at least 50% with 40-S_n dimers. A typical preparation yielded: 50 A_{260} units of 40 S_n, 30 A_{260} units of 60 S_n (including 40 S_{nd}) and 550 A_{260} units of 80 S_n.

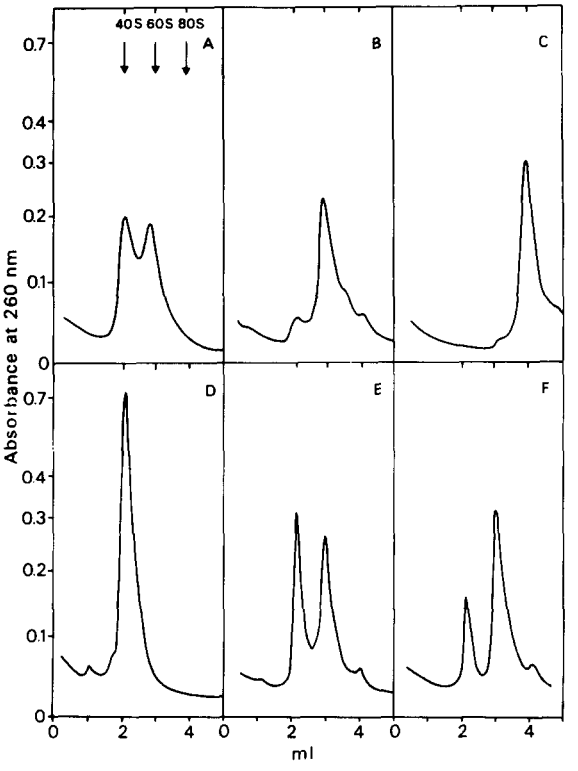


Fig. 1. Analytical sucrose gradients of 40 S_n, 60 S_n and 80 S_n. 0.75 A_{260} unit of each preparation was layered on a 15% to 32% isokinetic gradient and centrifuged for 90 min at 50 000 rev./min at 4°C in the SW 50.1 rotor. Panel A and D, 40 S_n; panel B and E, 60 S_n; panel C and F, 80 S_n. Panel A, B and C, 100 mM KCl; Panel D, E and F, 500 mM KCl.

Results and Discussion

The effect of eIF-4C on dissociation

A prerequisite for the utilization of 80-S_n ribosomes in protein synthesis is their dissociation into subunits. Therefore, any factor inducing dissociation will stimulate in an assay for initiation of protein synthesis with 80 S_n as the only source of ribosomal subunits. In order to exclusively determine the effect of eIF-4C on the dissociation, we have compared the dependency on eIF-4C of the methionyl-puromycin formation with 80-S_n and with salt-derived ribosomal subunits. The results are shown in Fig. 2. With 80-S_n ribosomes (Fig. 2A) the effect of eIF-4C on the methionyl-puromycin synthesis is pronounced. Almost 4-fold stimulation in the absence of eIF-3, 2-fold in its presence. On the other hand, when salt-derived subunits were used and 40-S initiation complexes were allowed to form prior to the addition of 60 S and puromycin, only a small stimulatory effect of eIF-4C was observed, 1.6-fold in the absence of eIF-3 and 1.1-fold in its presence (Fig. 2B), although the overall level of methionyl-puromycin synthesis with both classes of ribosomes was the same. From these results it is clear that in a system in which dissociation of 80-S ribosomes prior to initiation of protein synthesis does not need to occur, the effect of eIF-4C is small.

Furthermore, upon the addition of eIF-3, the effect of eIF-4C decreases both with 80-S_n and with salt-derived ribosomal subunits, suggesting that eIF-3 also partcipates in the dissociation of 80-S ribosomes, in agreement with the results of other groups [2,3].

The participations of eIF-4C in the dissociation was tested in an assay system directly measuring the extent of dissociation of 80-S_n ribosomes under the

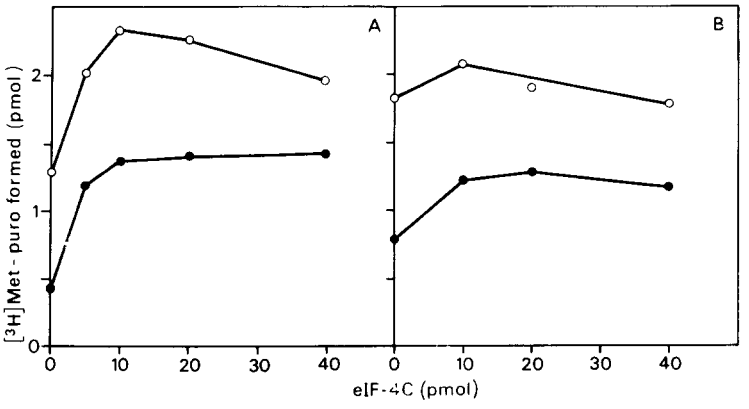


Fig. 2. Titration of eIF-4C in the assay for methionyl-puromycin synthesis. Panel A: Methionyl-puromycin formation with 80 S_n. The assay for the synthesis of methionyl-puromycin was performed according to Thomas et al. [18]. Formed methionyl-puromycin was extracted according to Leder and Bursztyn [19]; background synthesis, obtained in the absence of ribosomes was subtracted. Indicated amounts of eIF-4C were added to reaction mixtures containing 10 pmol of 80 S_n, both in the absence (●———●) and presence (○———○) of 10 pmol of eIF-3. Panel B: Methionyl-puromycin formation with salt-washed ribosomal subunits. The same as for panel A, but instead of 80 S_n 10 pmol of salt-washed 40-S and 60-S ribosomal subunits were used. Prior to the addition of 60 S and puromycin, 40 S initiation complexes were allowed to form by incubating for 5 min at 37°C.

ionic conditions used in the experiments described in Fig. 2. The results are shown in Fig. 3.

Indeed eIF-4C has a distinct, although small, effect on the dissociation of 80-80-S_n ribosomes. This effect could be measured both in the absence and presence of eIF-3. The effects of eIF-3 and eIF-4C were additive, in agreement with the results of Fig. 2.

Exchange experiments (not shown) with ^{3}H-labeled 40-S subunits and 80-S_n ribosomes revealed that eIF-4C enhanced the rate of the incorporation of ^{3}H-labeled 40-S subunits into 80 S, suggesting that eIF-4C increases both the

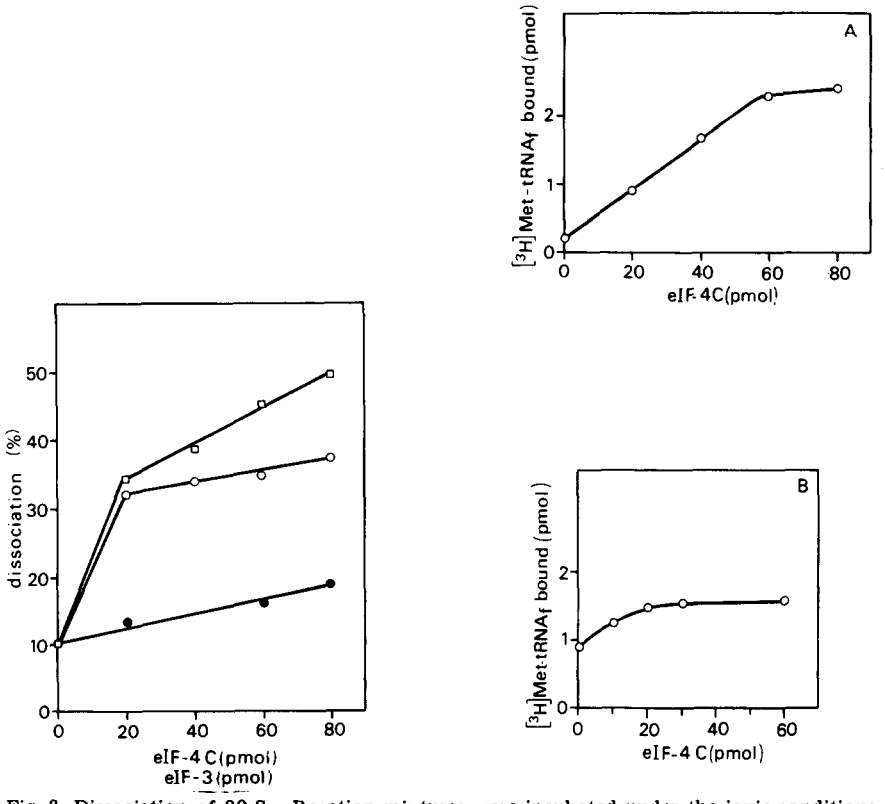


Fig. 3. Dissociation of 80 S_n. Reaction mixtures were incubated under the ionic conditions of the [^{3}H]-Met-tRNA$_f$ binding assay; the energy system was omitted, the $Mg^{2+}(Ac)_2$ concentration was 0.6 mM. Reaction mixtures containing 1 A_{260} unit of 80 S_n and indicated amounts of eIF-3 and eIF-4C, were incubated for 15 min at 37°C, whereafter gluteraldehyde was added to a final concentration of 0.1%. The mixtures were analyzed on sucrose gradients as described by Naaktgeboren et al. [20]. The level of dissociation is calculated from the absorbance profiles as:

$$\frac{40\text{ S} + 60\text{ S}}{40\text{ S} + 60\text{ S} + 80\text{ S}} \times 100\%$$

●——●, titration of eIF-4C, no eIF-3; ○——○, titration of eIF-3, no eIF-4C; □——□, titration of eIF-3, in the presence of 80 pmol eIF-4C.

Fig. 4. Titration of eIF-4C in the Met-tRNA$_f$ binding assay. Panel A: Met-tRNA$_f$ binding to 40 S_{nd}. Reaction mixtures containing 19 pmol 40 S_{nd}, 16 pmol eIF-2 and the indicated amounts of eIF-4C were incubated as described in Materials and Methods. Panel B: Met-tRNA$_f$ binding to 40 S. Reaction mixtures containing 10 pmol of washed 40 S, 16 pmol eIF-2 and the indicated amounts of eIF-4C were incubated as described in Materials and Methods.

k_1 and k_2 (although not to the same extent) of the equilibrium reaction:

$$80\ S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} 40\ S + 60\ S \qquad (1)$$

Dependency on eIF-4C of 40 S-initiation complex formation

Part of the stimulation by eIF-4C of the initiation of protein synthesis may be explained by its effect on the dissociation equilibrium of 80-S ribosomes (Figs. 2 and 3). However, studies on the formation of 40-S initiation complexes which utilize 40-S ribosomal subunits as the sole source of ribosomes still show a small but consistent stimulatory effect with eIF-4C [2,3,6,8]. Such an effect of eIF-4C could not be explained by a stabilization of the Met-tRNA-binding [8] or the binding of other initiation factors (results not shown).

An indication as to what may be the cause of this stimulation comes from studies using native 40-S dimers (40-S_{nd}). The tendency of 40-S ribosomes to dimerize has long been recognized [15,16]. From the results of Fig. 1 it became clear that a considerable (50%) part of the native 40-S ribosomal subunits is isolated as a dimer sedimenting at approx. 60 S.

We have performed the assay on 40 S initiation complex formation with 40 S_{nd}, obtained as described in the legend to Fig. 1, and determined the effect of eIF-4C. The results are given in Fig. 4A. First of all, such dimers are quite stable upon centrifugation at low salt gradients (compare Fig. 1) and are, as such, virtually inactive in the assay system: hardly any Met-$tRNA_f$ could be detected in the 40 S position if no eIF-4C was added. The addition of eIF-4C resulted in a drastic increase (up to 10-fold) of the Met-$tRNA_f$ binding reaching normal values of Met-$tRNA_f$ bound per 40 S added.

On the other hand, only a small stimulation (1.4-fold) by eIF-4C was observed when the experiment was performed with washed ribosomal 40-S subunits (Fig. 4B), (in line with the results of Fig. 2B and completely in agreement with literature values [2,3,4]), although the level of Met-$tRNA_f$ binding to both classes of subunits was the same, provided that excess eIF-4C was present.

'Dedimerisation' of 40 S_{nd}

The possibility arises that eIF-4C converts inactive 40-S dimers into monomers, which are active in the initiation of polypeptide synthesis. Therefore, we investigated the effect of eIF-4C on the sedimentation behaviour of 40-S dimers. Fig. 5 shows that upon incubation of 40 S_{nd} with increasing amounts of eIF-4C under the ionic conditions of protein synthesis an increasing 'dedimerisation' can be observed. Such an increase in monomers is correlated with an increase in activity in the assays for initiation (see Fig. 4). Furthermore, a pronounced additional stimulation of 'dedimerisation' occurred in the presence of all the components required for 40 S initiation complex formation.

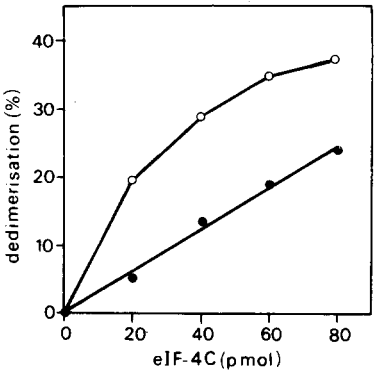
The process of 'dedimerisation' and subsequent formation of a 40 S initiation complex can be represented by the following equation:

$$40\ S_{nd} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} 40\ S \xrightarrow[\text{factors}\ \text{Met-tRNA}_f,\ \text{AUG}]{\text{initiation}} 40\ S\ \text{initiation complex} \qquad (2)$$

In this scheme the effect of eIF-4C is explained by an increase of the k_1, whereas the addition of other factors and Met-tRNA$_f$ will result in an overall decrease of the dimer-level.

40-S$_n$ dimers do not contain eIF-4C

The results of Figs. 4 and 5 are puzzling particularly because a physiological role of 40-S dimers is not immediately obvious. Isolation procedures of ribosomal subunits might remove eIF-4C from the 40 S subunit, which could result in dimerisation of the particle. In this respect it should be pointed out that



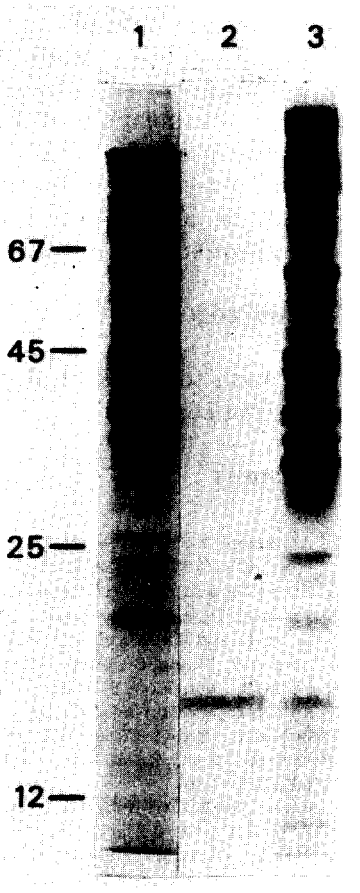


Fig. 5. 'Dedimerisation' of 40 S$_{nd}$. 0.7 A_{260} unit of 40 S$_{nd}$ was incubated with the indicated amounts of eIF-4C under the ionic conditions of the Met-tRNA$_f$ binding assay without any further addition (●———●), or in the complete system (○———○). 'Dedimerisation' was calculated from the absorbance profiles as:

$$\frac{40\ S_n}{40\ S_{nd} + 40\ S_n} \times 100\%$$

Fig. 6. Polyacrylamide gel electrophoresis of 0.5 M KCl washes of 40 S$_n$ and 40 S$_{nd}$. 0.5 M KCl wash was prepared from 40 S$_n$ and 40 S$_{nd}$ (6 A_{260} units each), following standard procedures. The washed were subjected to electrophoresis on polyacrylamide gels according to Laemmli [21]. Lane 1, 60 μg 40 S$_{nd}$ wash; lane 2, 1 μg eIF-4C; lane 3, 60 μg 40 S$_n$ wash.

40-S_n dimers, once isolated, remain dimers upon recentrifugation, whereas apparently homogenous 40 S monomers are converted into dimers for at least 50% when subjected to a second centrifugation step (see Fig. 1).

We prepared a 0.5 M KCl wash from 40-S_n dimers and monomers and checked on polyacrylamide gels for the presence of eIF-4C. The results, indeed, are in line with the hypothesis that removal of eIF-4C results in dimerisation of 40-S subunits (Fig. 6). No eIF-4C could be detected on 40-S_n dimers (lane 1) but almost stoichiometric amounts are present on 40-S monomers (lane 3), as can be deduced by comparison with lane 2, in which a known amount of eIF-4C was electroforesed.

The effect of eIF-4C in assay systems utilizing only 40-S ribosomal subunits, therefore may be explained by its effect on dimer formation, although the physiological relevance of 40-S dimers remain obscure. The significance, however, of the observed stimulatory effect by eIF-4C on the dissociation of 80 S_n is obvious: dissociation of 80 S_n is a prerequisite for the participation of the ribosome in a new round of initiation. The explanation for both effects on the molecular level might be the same: the presence of eIF-4C on the 40 S subunit reduces its affinity for both 60-S and other 40-S particles, thus ensuring a proper sequence of initiatory events (see also Eqns. 1 and 2).

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