Protein synthesis in rabbit reticulocytes* : characteristics of Co-eIF-2 protein complex

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ABSTRACT

A high molecular weight reticulocyte protein factor, named Co-eIF-2, contains Co-eIF-2A, Co-eIF-2B, and Co-eIF-2C activities and stimulates Met-tRNA_f binding to eIF-2 both in the presence and absence of Mg²⁺. Some characteristics of this stimulation in the absence of Mg²⁺ are: (1) Stimulation is most pronounced at low eIF-2 levels. (2) Stimulation is partially resistant to heat and NEM treatment, and thus appears to be due to the combined action of both heat and NEM-insensitive Co-eIF-2A, and heat and NEM-sensitive Co-eIF-2C activities. (3) [³H]GDP bound in eIF-2•[³H]GDP complex is rapidly displaced by unlabelled GTP during ternary complex formation. Co-eIF-2 stimulates Met-tRNA_f binding to eIF-2 even when added after the [³H]GDP from eIF-2•[³H]GDP has been completely displaced. This indicates that Co-eIF-2-stimulation is not due to GDP displacement from eIF-2•GDP. We propose that eIF-2 molecules become inactive in the presence of Mg²⁺ and at high dilution, and Co-eIF-2 restores the inactive eIF-2 molecules into an active form.

INTRODUCTION

The first step in peptide chain initiation in mammalian cells is the formation of a ternary complex between an eukaryotic peptide chain initiation factor, eIF-2, Met-tRNA_f and GTP; Met-tRNA_f•eIF-2•GTP. Recent work done in our laboratory and elsewhere has indicated that this ternary complex formation by eIF-2 and its proper functioning during peptide chain initiation in reticulocyte lysates are regulated by several ancillary protein factors which we term Co-eIF-2A, Co-eIF-2B and Co-eIF-2C (for recent reviews, see Refs. 1-3).

The ternary complex formation in the presence of Mg²⁺ requires Co-eIF-2C.⁴ It has been reported that Mg²⁺ inhibits ternary complex formation by eIF-2 and Co-eIF-2C relieves this Mg²⁺ inhibition of ternary complex formation.⁵⁻⁷ However, the precise mechanisms of Mg²⁺ inhibition of ternary complex formation and the reversal of Mg²⁺ inhibition by Co-eIF-2C are not fully understood. According to a recent mechanism proposed by Clemens et al.⁸ and Siekierka et al.,⁹ eIF-2 is purified as

eIF-2·GDP. In the absence of Mg^{2+} , GDP is easily exchangeable with exogenously added GDP and also can be easily displaced by GTP to form the eIF-2·GTP needed for ternary complex formation. However, in the presence of Mg^{2+} , GDP remains tightly bound to eIF-2 and prevents eIF-2 binding to GTP and Met-tRNA_f. The function of Co-eIF-2C is to permit replacement of GDP by GTP.

Co-eIF-2A stimulates, by 2-3 fold, Met-tRNA_f binding to eIF-2.¹⁰ Co-eIF-2A also binds to preformed ternary complex and forms a stable quaternary complex; Met-tRNA_f·eIF-2·GTP·Co-eIF-2A.¹¹ The precise role of Co-eIF-2B in peptide chain initiation is not known. In partial reactions, Co-eIF-2B promotes dissociation of the ternary complex at high Mg^{2+} concentrations (5 mM).¹²

Co-eIF-2A is a heat-stable low molecular weight (mol. wt. 25,000 daltons) protein factor.¹⁰ Co-eIF-2A activity from rabbit reticulocytes has been purified to homogeneity.¹⁰ Co-eIF-2C activity has been purified as a high molecular weight (mol. wt. 500,000 daltons) protein complex which also contains significant amounts of Co-eIF-2A and Co-eIF-2B activities.^{1-3,6,13,14} We now term this protein complex Co-eIF-2.

In this paper, we will describe our recent studies on the characteristics of Co-eIF-2 stimulation of Met-tRNA_f binding to eIF-2. We will provide evidence that both Co-eIF-2A and Co-eIF-2C activities in Co-eIF-2 stimulate Met-tRNA_f binding to eIF-2 in the absence of Mg^{2+} specially at low eIF-2 concentrations. Our studies indicate that Co-eIF-2 stimulation of Met-tRNA_f binding to eIF-2 is possibly not related to reversal of GDP inhibition of eIF-2 activity, as such stimulation is also observed in the absence of Mg^{2+} and under conditions where eIF-2-bound GDP is freely exchangeable with exogenous GTP. We propose that Co-eIF-2 activity is possibly involved in the restoration of the inactive eIF-2 molecules into an active form, and that the inactivation of the eIF-2 molecules can be caused by agents such as Mg^{2+} and at high dilution.

MATERIALS AND METHODS

The sources of the materials used in these studies, preparation of initiation factors eIF-2, Co-eIF-2A, and Co-eIF-2 complex and their assay methods have been described previously.^{6,13,14} Unlabelled GTP and radio-labelled [³H]GDP (sp. activity, 10 Ci/mmol) were obtained from Sigma Chemical and New England Nuclear, respectively. Partially purified eIF-2 and Co-eIF-2 preparations were obtained after the CM-Sephadex chromato-

graphic step.^{13,14} In some experiments, Co-eIF-2 preparation was further purified using DEAE-cellulose chromatography.^{13,14}

Millipore filtration methods were used for assays of eIF-2 and eIF-2-ancillary factor activities as described previously.^{6,13,14}

RESULTS

Previous reports have indicated that Co-eIF-2C activity stimulated Met-tRNA_f binding to eIF-2 in the presence of Mg²⁺.⁵⁻⁷ Mg²⁺ inhibited ternary complex formation by eIF-2 and Co-eIF-2C activity relieved this Mg²⁺ inhibition of ternary complex formation. We have now critically analyzed the characteristics of stimulation of the Met-tRNA_f binding to eIF-2 by the Co-eIF-2 preparation both in the presence and absence of Mg²⁺ (Fig. 1). As shown in Fig. 1, left panel, the Co-eIF-2 preparation significantly stimulated Met-tRNA_f binding to eIF-2 even in the absence of Mg²⁺. This stimulation was most pronounced (3-4 fold) at low eIF-2 concentrations and became less significant at higher eIF-2 levels. Apparently, the lack of Co-eIF-2C stimulation in the absence of Mg²⁺ observed in previous work⁵⁻⁷ is due to the use of high concentrations of eIF-2. In the presence of 1 mM Mg²⁺ (Fig. 1, right panel), ternary complex formation was strongly

Figure 1: Effects of Co-eIF-2 addition on Met-tRNA_f binding to eIF-2 at different eIF-2 concentrations and in the absence (left hand panel) and presence (right hand panel) of Mg²⁺. Standard Millipore filtration assay conditions were used. The experiments described in the right hand figure also contained 1 mM Mg²⁺. Approximately, 15 μg Co-eIF-2 were used where indicated.

inhibited and again this inhibition was most pronounced at low eIF-2 concentrations. The inhibitory effect of Mg^{2+} was not fully relieved and significant Co-eIF-2 stimulation of Met-tRNA_f binding to eIF-2 was observed even at the highest concentration of eIF-2 used in these experiments.

The above results thus demonstrate that the Co-eIF-2 preparation stimulates Met-tRNA_f binding to eIF-2 at low eIF-2 concentrations and also in the presence of Mg^{2+} . A likely explanation for this Co-eIF-2 effect is that eIF-2 changes into an inactive form at high dilution and also in the presence of Mg^{2+} , and Co-eIF-2 converts the inactive eIF-2 molecules into an active form.

To determine the component(s) in Co-eIF-2 preparation responsible for stimulation of Met-tRNA_f binding to eIF-2 in the absence of Mg^{2+} , we critically analyzed the characteristics of Co-eIF-2 stimulation of eIF-2 activity. Both Co-eIF-2A and Co-eIF-2C activities are known to stimulate Met-tRNA_f binding to eIF-2. However, these two activities can be easily distinguished by their heat stabilities and NEM sensitivities; Co-eIF-2A activity is heat-stable and NEM-insensitive,¹⁰ whereas Co-eIF-2C activity is heat-labile and NEM-sensitive.⁴ The results presented in Table 1, Exp. 1 show that approximately 50% of the Co-eIF-2 stimulation was lost after NEM treatment. These results indicate that Co-eIF-2 stimulation in the absence of Mg^{2+} is due to the combined action of both Co-eIF-2A and Co-eIF-2C activities. However, the results obtained with heated Co-eIF-2 preparation was less clear. Approximately 70% of the eIF-2 stimulatory activity was lost after heat treatment. Under similar conditions, a partially purified Co-eIF-2A preparation lost only 10% of the original activity. The reason for these differential heat stabilities of Co-eIF-2A activities in Co-eIF-2 complex and in partially purified Co-eIF-2A preparation is not apparent. As shown in Table 1, Exp. 3, the ternary complex formed in the presence of Co-eIF-2 is significantly resistant to aurintricarboxylic acid, again indicating that the Co-eIF-2 complex contains Co-eIF-2A activity.¹⁰

We studied the effects of Co-eIF-2 on Met-tRNA_f binding to eIF-2 and eIF-2 α (P) in the presence and absence of Mg^{2+} (Table 2). eIF-2 α (P) was prepared by phosphorylating a partially purified eIF-2 preparation using HRI and ATP, and was further purified using a phosphocellulose chromatography step.⁶ Previous results indicated that the ternary complexes formed using eIF-2 α (P) could be stimulated by Co-eIF-2A but not by Co-eIF-2C.⁶ Co-eIF-2C effect was studied in the presence of Mg^{2+} .⁶ As shown in Table

TABLE 1
Effects of NEM treatment, heat and aurintricarboxylic acid (ATA)
on Co-eIF-2A and Co-eIF-2 stimulation of Met-tRNA_f binding
to eIF-2 in the absence of Mg²⁺.

Additions	³⁵ S Met-tRNA _f bound (pmol)	
<u>Experiment No. 1</u>		
None	0.52	
Co-eIF-2	2.22	
NEM-treated Co-eIF-2	1.30	
Co-eIF-2A	1.26	
NEM-treated Co-eIF-2A	1.00	
<u>Experiment No. 2</u>		
None	0.60	
Co-eIF-2	1.74	
Heated Co-eIF-2	0.94	
Co-eIF-2A	1.42	
Heated Co-eIF-2A	1.29	
<u>Experiment No. 3</u>		
	-ATA	+ATA
None	0.60	0.03
Co-eIF-2	1.74	1.26
Co-eIF-2A	1.42	1.00

Standard Millipore filtration assay conditions were used.^{6,10,13,14}
Experiment 1: The reactions were carried out in two stages. In Stage I, Co-eIF-2 or Co-eIF-2A was preincubated with 5 mM N-ethylmaleimide (NEM) for 10 minutes at 37°C and the unreacted NEM was then neutralized using excess dithiothreitol (20 mM). In the control tubes, excess dithiothreitol was present during incubation of the factors with NEM. The factors were then used in the standard ternary complex formation assay in the presence of eIF-2, [³⁵S]Met-tRNA_f, GTP and salt components.
Experiment 2: Co-eIF-2 and Co-eIF-2A preparations were heated at 55°C for 4 minutes as described previously¹⁰ and were then used as in Exp. 1.
Experiment 3: The complex formation was assayed in the presence of 3 X 10⁻⁵ M aurintricarboxylic acid. Approximate concentrations of the factors used were: eIF-2, 2 µg; Co-eIF-2, 15 µg; Co-eIF-2A, 15 µg.

2, Met-tRNA_f binding to eIF-2 in the absence of Mg²⁺ was stimulated two-fold by untreated Co-eIF-2 and only one-fold by NEM-treated Co-eIF-2. Under similar conditions, Met-tRNA_f binding to eIF-2α(P) was stimulated only one-fold by Co-eIF-2, and such stimulation remained unchanged after NEM treatment. These results are consistent with the postulation that Co-eIF-2 stimulation in the absence of Mg²⁺ is due to the combined action

TABLE 2

Characteristics of Co-eIF-2 stimulation on Met-tRNA_f binding to eIF-2 and eIF-2 α (P) in the presence and absence of Mg²⁺.

Additions	[³⁵ S]Met-tRNA _f bound (pmol)	
	-Mg ²⁺	+1 mM Mg ²⁺
eIF-2	0.44	0.15
Co-eIF-2	1.31	1.00
NEM-treated Co-eIF-2	0.92	0.29
eIF-2 α (P)	0.42	0.12
Co-eIF-2	0.91	0.32
NEM-treated Co-eIF-2	0.92	0.23

Standard Millipore filtration assay conditions were used as described in Table 1. For details, see text.

of both Co-eIF-2A and Co-eIF-2C activities, and that the Co-eIF-2C activity does not recognize eIF-2 α (P). The effects of Co-eIF-2 on Met-tRNA_f binding to eIF-2 and eIF-2 α (P) in the presence of 1 mM Mg²⁺ were similar to those described previously.⁶

The above results demonstrate that Co-eIF-2C component in Co-eIF-2 can stimulate Met-tRNA_f binding to eIF-2 even in the absence of Mg²⁺. These results are not apparently consistent with a recently proposed mechanism of Co-eIF-2C action by Clemens et al.⁸ and Siekierka et al.⁹ According to this mechanism, Co-eIF-2C effect is related to the reversal of Mg²⁺ inhibition of ternary complex formation by displacement of GDP from eIF-2•GDP complex as GDP is more tightly bound to eIF-2 in the presence of Mg²⁺. According to Siekierka et al.,⁹ Co-eIF-2C does not stimulate ternary complex formation by eIF-2 in the absence of Mg²⁺, as GDP in eIF-2•GDP complex can be easily displaced during ternary complex formation in the absence of Mg²⁺. In our studies, we have attempted to correlate GDP displacement from eIF-2•GDP during ternary complex formation in the absence of Mg²⁺ and Co-eIF-2 stimulation of ternary complex formation (Fig. 2). For these studies we formed eIF-2•[³H]GDP by incubating a partially purified eIF-2 preparation (Fraction II)¹² with [³H]GDP and further purified eIF-2•[³H]GDP complex by phosphocellulose chromatography and CM-Sephadex chromatography.¹³ We studied the kinetics of [³H]GDP displacement from eIF-2•[³H]GDP complex during ternary complex formation in the absence of Mg²⁺ and without or with Co-eIF-2 added at different time

Figure 2: Kinetics of $[^3\text{H}]\text{GDP}$ release (upper panel) and $[^{35}\text{S}]\text{Met-tRNA}_f$ binding (lower panel) during ternary complex formation using eIF-2. $[^3\text{H}]\text{GDP}$ was prepared by incubating a partially purified eIF-2 preparation (Fraction II, Ref. 12) with $[^3\text{H}]\text{GDP}$ ($10\text{ }\mu\text{M}$) at 37° for 15 minutes. eIF-2. $[^3\text{H}]\text{GDP}$ complex was further purified using phosphocellulose and CM-Sephadex chromatography. Purified eIF-2. $[^3\text{H}]\text{GDP}$ was incubated under standard assay conditions in the presence of $[^{35}\text{S}]\text{Met-tRNA}_f$, GTP and salt components and, where indicated, Co-eIF-2. The reaction mixtures were then assayed using standard Millipore filtration conditions for $[^3\text{H}]\text{GDP}$ release (upper panel) and $[^{35}\text{S}]\text{Met-tRNA}_f$ bound (lower panel). In the experiments described in the lower panel, Co-eIF-2 was added at different time intervals as indicated by arrows. $\bigcirc-\bigcirc-\bigcirc$, eIF-2. $[^3\text{H}]\text{GDP}$ alone; $\square-\square-\square$, eIF-2. $[^3\text{H}]\text{GDP}$ + Co-eIF-2.

intervals. Under the experimental conditions, $[^3\text{H}]\text{GDP}$ in eIF-2. $[^3\text{H}]\text{GDP}$ complex was rapidly displaced in the absence of Mg^{2+} and the displacement was more than 85% complete in 5 minutes. This displacement rate was significantly increased in the presence of Co-eIF-2 as was the rate of the overall ternary complex formation. However, as can be seen in Fig. 2, bottom panel, Co-eIF-2 stimulation of ternary complex formation was also observed, even when this factor was added after the ternary complex formation by eIF-2 alone reached a plateau and $[^3\text{H}]\text{GDP}$ from the original eIF-2. $[^3\text{H}]\text{GDP}$ complex was almost completely displaced. It should be noted that in the above experiments we used approximately 2.5 pmol s eIF-2. $[^3\text{H}]\text{GDP}$, and under conditions of total displacement of $[^3\text{H}]\text{GDP}$, 0.7 pmol s ternary complex was formed in the absence of Co-eIF-2 and 1.8 pmol s ternary complex was formed in the presence of Co-eIF-2.

DISCUSSION

All three eIF-2-ancillary factor activities Co-eIF-2A, Co-eIF-2B and Co-eIF-2C remain associated with a high molecular weight protein complex even after several purification steps. The significance of this tight association of the factors is not clear at present. As reported previously, a part of the Co-eIF-2A activity remains in the free form and this activity has been purified to homogeneity.¹⁰ The precise physical nature of Co-eIF-2A and also Co-eIF-2B and Co-eIF-2C activities in the Co-eIF-2 complex is not known. These activities are possibly associated with different polypeptide component(s) of the protein complex. An intriguing possibility is that these activities may reside in a single polypeptide and different domains of the polypeptide may be responsible for different activities. The isolation of the heat-stable low molecular weight factor, Co-eIF-2A, may be the result of degradation of the original polypeptide during isolation. It should be noted that although the Co-eIF-2A activity in Co-eIF-2 complex is similarly NEM-insensitive and forms an intracarboxylic acid resistant quaternary complex as the free Co-eIF-2A, this activity is significantly more sensitive to heat than free Co-eIF-2A. It is conceivable that Co-eIF-2A as it remains associated with Co-eIF-2 complex either as an individual polypeptide or as a part of a long polypeptide, is more active than free Co-eIF-2A and heat treatment leads to loss of the organized structure with concomitant loss of Co-eIF-2A activity in Co-eIF-2 complex.

An important observation reported in this paper relates to the basic mechanism of Co-eIF-2 action. Co-eIF-2 stimulates Met-tRNA_f binding to eIF-2 both in the presence and absence of Mg²⁺, and in both cases, this stimulation is most pronounced at low eIF-2 concentrations. The results indicate that both Co-eIF-2A and Co-eIF-2C components in Co-eIF-2 complex are responsible for this stimulated Met-tRNA_f binding to eIF-2. We propose that the Co-eIF-2C activity in Co-eIF-2 complex is involved in the activation of the eIF-2 molecules which may be rendered inactive by agents such as Mg²⁺ and at high dilution. This mechanism of Co-eIF-2C action apparently differs from a recently proposed mechanism by Clemens et al.⁸ and Siekierka et al.⁹ According to this mechanism, Co-eIF-2C activity stimulates Met-tRNA_f binding to eIF-2 only in the presence of Mg²⁺ as this activity is required to displace GDP from a tight eIF-2·GDP complex formed in the presence of Mg²⁺. The results presented in this paper demonstrate: (1) Co-eIF-2C activity stimulates Met-tRNA_f binding to eIF-2 even in the

absence of Mg^{2+} , and, therefore, may not be related to GDP displacement from eIF-2·GDP which is presumably necessary only in the presence of Mg^{2+} .⁹ (2) Co-eIF-2C stimulation both in the presence and absence of Mg^{2+} is most pronounced at low eIF-2 concentrations and this stimulation decreases progressively as eIF-2 concentration is increased. This observation is apparently inconsistent with the GDP displacement mechanism as it is not clear how increasing the substrate (eIF-2·GDP) concentration should decrease the factor (Co-eIF-2C) requirement. (3) Co-eIF-2C stimulation in the absence of Mg^{2+} can be observed even when this factor is added under conditions which promote rapid displacement of GDP from eIF-2·GDP complex.

The above observation, however, can be explained by a mechanism involving Co-eIF-2C activation of the inactivated eIF-2 molecule as described above. The inactivation of eIF-2 molecules may be caused by Mg^{2+} and at high dilution. However, the precise mechanisms of inactivation of eIF-2 molecules (Mg^{2+} - and dilution-induced) and the activation of the inactivated eIF-2 molecules by Co-eIF-2C are not clear. A striking difference in the characteristics of the Mg^{2+} - and dilution-induced inactivated eIF-2 molecules is that the Mg^{2+} -induced inactive eIF-2 molecules do not promote GDP exchange⁹ (includes GDP binding to eIF-2 and GDP displacement from eIF-2·GDP) whereas the dilution-induced inactive eIF-2 molecules freely promote GDP exchange. The reason for this difference is not apparent and may reflect different stages of inactivation. It is possible that the eIF-2 molecules at high dilution are only partially inactivated and in this state the eIF-2 molecules can promote GDP exchange. On the other hand, Mg^{2+} causes more complete inactivation of eIF-2 and in this state the inactivated eIF-2 molecules do not promote GDP exchange. Alternatively, the inactivation of the eIF-2 molecules by Mg^{2+} and at high dilution may follow independent pathways. In both cases, Co-eIF-2C restores the active form of eIF-2.

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