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Protein Synthesis in Rabbit Reticulocytes: Mg^{2+} - Inhibition of Ternary Complex ($Met\text{-}tRNA_f \cdot eIF\text{-}2 \cdot GTP$) Formation by Reticulocyte eIF-2

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SUMMARY: There are conflicting reports regarding Mg^{2+}-inhibition of ternary complex formation by reticulocyte eIF-2. Several laboratories have reported that eIF-2 is isolated as eIF-2·GDP and Mg^{2+} inhibits ternary complex formation, as in the presence of Mg^{2+}, GDP remains tightly bound to eIF-2 and prevents ternary complex formation. A protein factor, GEF is necessary for GDP displacement and subsequent ternary complex formation. Other laboratories have reported that Mg^{2+} has no effect on eIF-2 activity and eIF-2 forms near stoichiometric amount of ternary complex in the presence of Mg^{2+}. In this paper, we provide evidence which suggests that the Mg^{2+}-insensitive eIF-2 activity as reported by several laboratories might have been the result of the use of high $Met\text{-}tRNA_f$ concentrations in their assays as the nucleotides in excess tRNA bound Mg^{2+} in the reaction mixture and there was no free Mg^{2+} available to inhibit eIF-2 activity. Our data will show that the addition of excess tRNA promotes non-enzymatic GDP displacement from eIF-2·GDP and relieves Mg^{2+} inhibition.

A controversy in mammalian peptide chain initiation relates to Mg^{2+} sensitivity of reticulocyte eIF-2 and this controversy has led to postulation of different mechanisms for peptide chain initiation (For recent reviews, see refs. 1-4). According to one view (2,4), eIF-2 is isolated as eIF-2·GDP (5-6). In the presence of Mg^{2+}, GDP remains tightly bound to eIF-2 and prevents ternary complex formation (5-7). A protein factor, present in reticulocyte ribosomal salt wash (6-10), in reticulocyte cell supernatant (11-17) and in ascites ribosomal salt wash (18-19), and widely termed as GEF, promotes GDP displacement from eIF-2·GDP and thus facilitates ternary complex formation. Protein synthesis inhibitors, HRI and dsI phosphorylate eIF-2 α-subunit and thus inhibit protein synthesis as GEF does not promote GDP displacement from eIF-2α(P)·GDP and ternary complex is not formed.

Abbreviations: eIF-2, eukaryotic initiation factor 2, which forms a ternary complex, $Met\text{-}tRNA_f \cdot eIF\text{-}2 \cdot GTP$; GEF, guanine nucleotide exchange factor; HRI, heme-regulated protein synthesis inhibitor; dSI, double-stranded RNA activated protein synthesis inhibitor.

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According to the other view (1,3,14,20-23) eIF-2 is isolated as free protein (GDP-free). In the presence of Mg^{2+}, both eIF-2 and eIF-2α(P) (formed by phosphorylation using HRI or dsI) form near stoichiometric amount of ternary complex in the absence of additional protein factor(s) and both participate equally effectively in one cycle of peptide chain initiation.

We have recently analyzed the experimental results and also the experimental conditions supporting each view (2). Based on these analyses, we have suggested that these differences might have been the result of the use of widely different Met-tRNA$_f$ concentrations in the assays for ternary complex formation. Safer et.al. (14,21) and Benne et. al. (22) have used unusually high Met-tRNA$_f$ concentrations in their experiments (See Table 1, Ref. 2) and have thus missed the inhibitory effect of Mg^{2+} on eIF-2 activity since the excess nucleotides bound the Mg^{2+} in the reaction mixture and there was no free Mg^{2+} available to inhibit the eIF-2 activity.

In this paper, we provide experimental evidence in support of our above analyses.

MATERIALS AND METHODS

All the materials used in these experiments were the same as described previously (6,7,24,25). Purified eIF-2 preparation (Fraction VI) was obtained after phosphocellulose chromatographic step following the procedure described previously (24). This prepartion was approximately 90 percent pure as determined by ternary complex formation in the absence of Mg^{2+} and in the presence of excess Co-eIF-2 (6). eIF-2·[^{3}H]GDP was prepared by incubating partially purified eIF-2 with [^{3}H]GDP following the procedure described previously (6-7). eIF-2·[^{3}H]GDP was freed from excess [^{3}H]GDP by CM-Sephadex chromatography (6-7). Phosphorylated eIF-2 was prepared by phosphorylation of purified eIF-2 using HRI as described before (10).

RESULTS

In the absence of Mg^{2+}, GDP in purified eIF-2·GDP is non-enzymatically displaced and ternary complex is formed. In the presence of Mg^{2+}, GDP in eIF-2·GDP remains tightly bound to eIF-2 and prevents ternary complex formation. We have suggested that Mg^{2+}-insensitive eIF-2 activity reported by other laboratories (14, 23) is due to the use of excess Met-tRNA$_f$ in the reaction for ternary complex formation. We present below three sets of experiments in support of this suggestion.

The results presented in Fig. 1 shows the effects of addition of uncharged reticulocyte tRNA on GDP exchange between exogeneously added GDP and eIF-2·[^{3}H]GDP at different Mg^{2+} concentrations. As expected, in the absence of Mg^{2+} and added tRNA, in excess of 80% GDP in eIF-2·[^{3}H]GDP exchanged with exogeneous GDP during 2 minute incubation period. Addition of Mg^{2+} alone in increasing concentrations inhibited this exchange reaction; less than 20% GDP in

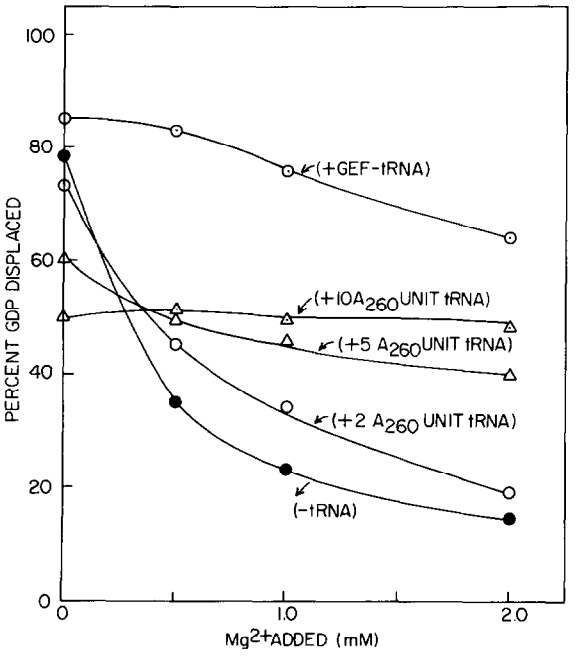

Fig. 1. Effects of addition of increasing concentrations of uncharged reticulocyte tRNA on [^{3}H] GDP displacement from eIF-2·[^{3}H]GDP at varying Mg^{2+} concentrations.

Standard reaction mixtures contained (in a total volue of 0.075 ml): 20mM Tris-HCl, pH 7.8, 100mM potassium chloride, 10 µg bovine serum albumin, 2mM dithiothreitol, 5 µM GDP, 1.2 µg eIF-2·[^{3}H] GDP and as indicated uncharged rabbit reticulocyte tRNA, magnesium acetate and 2 µg GEF (RF preparation, Fraction VI (15)). The reaction mixtures were incubated at 37^0 for 2 mins. and eIF-2 bound [^{3}H]GDP was determined by standard Millipore filtration assay (4).

eIF-2·[^{3}H]GDP exchanged with exogeneous GDP in the presence of Mg^{2+}. Upon addition of uncharged tRNA in increasing concentrations, GDP exchange in the absence of Mg^{2+} decreased considerably; 80% in the absence of tRNA and 50% in the presence of 10 A_{260} unit tRNA. The reason for this inhibition of the exchange reaction by excess tRNA is not obvious. However, in the presence of Mg^{2+}, addition of increasing tRNA concentrations progressively relieved Mg^{2+} inhibition of GDP exchange reaction. In the presence of 10 A_{260} unit tRNA per ml reaction, there was no difference in GDP exchange reaction in the absence or in the presence of up to 2mM Mg^{2+} concentration; approximately 50% GDP in eIF-2·[^{3}H]GDP was exchanged at all Mg^{2+} concentration tested. As expected addition of GEF enhanced GDP exchange reaction at all Mg^{2+} concentrations, although at a slightly lower rate at higher Mg^{2+}. These results thus provide direct evidence that addition of excess tRNA (5 to 10 A_{260} unit per ml) causes

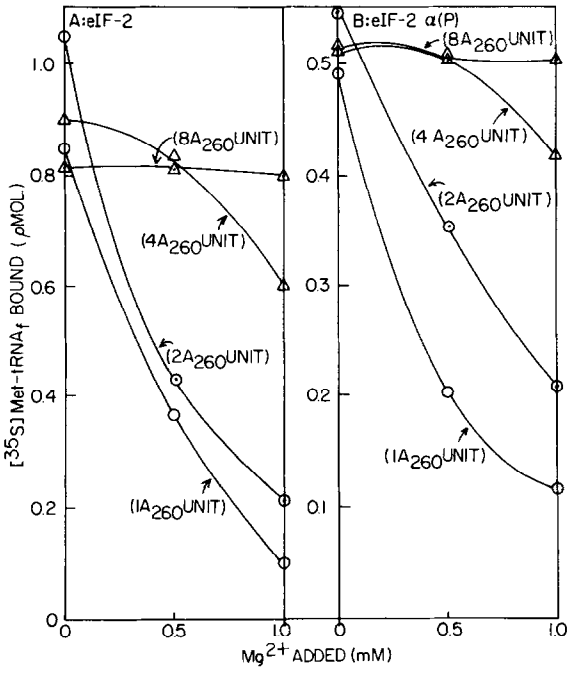

Fig. 2. Effects of addition of increasing [^{35}S] Met-tRNA$_f$ concentrations on ternary complex formation by eIF-2 and eIF-2α(P) at different Mg^{2+} concentrations.

Standard Millipore filtration assay conditions for ternary complex formation was used. As indicated, different concentrations of precharged [^{35}S] Met-tRNA$_f$ and magnesium acetate were used. 2 A_{260} unit tRNA used in these experiments contained 130 pmol precharged [^{35}S]Met-tRNA$_f$. Approximately, 0.4 µg factor (eIF-2 or eIF-2α(P)) was used in each experiment.

non-enzymatic GDP-displacement from eIF-2·GDP. As noted elsewhere (2), Benne et al (20) used 50-70 A_{260} unit tRNA per ml reaction for ternary complex formation. Obviously, under their assay conditions eIF-2 bound GDP would be non-enzymatically displaced and the inhibitory effect of eIF-2 bound GDP would not be detected.

The results presented in Fig. 2 (A,B) show the effects of addition of increasing precharged [^{35}S] Met-tRNA$_f$ on ternary complex formation at different Mg^{2+} concentrations using both eIF-2 (Fig. 2A) and eIF-2α(P) Fig. 2B). In our standard experiments, we use 2 A_{260} unit [^{35}S] Met-tRNA$_f$ (retic.) per ml reaction mixture. As shown in Fig. 2 (A,B), at this [^{35}S] Met-tRNA$_f$ concentration range (1 to 2 A_{260} unit per ml) both eIF-2 and eIF-2α(P) formed ternary complexes in the absence of Mg^{2+} and such complex formation was progressivley inhibited in both cases upon addition of increasing Mg^{2+}.

TABLE I

Mg^{2+} requirement for inhibition of ternary complex formation at different Met-$tRNA_f$ concentration

Met-$tRNA_f$ used A_{260} unit/ml	Ternary complex formed (pmol)/0.075 ml reaction mixture				
	$-Mg^{2+}$	+0.5mM Mg^{2+}	+1mM Mg^{+}	+5mM Mg^{2+}	+10mM Mg^{2+}
2	1.3	0.3	0.2	0.1	0.1
8	1.2	1.2	1.1	0.2	0.1

Standard Millipore filtration assay conditions were used as in Fig. 2. Mg^{2+} and [^{35}S] Met-$tRNA_f$ concentrations were varied as indicated.

However, in the presence of increasing Met-$tRNA_f$ concentration, Mg^{2+} inhibition in both cases was progressivley relieved. In the presence of 4 A_{260} unit [^{35}S] Met-$tRNA_f$ per ml reaction, inhibition due to 0.5mM Mg^{2+} was almost completely reversed but significant inhibition was observed in the presence of 1mM Mg^{2+}. In the presence of 8 A_{260} unit per ml reaction, inhibition due to 1mM Mg^{2+} was almost completely reversed. The results presented in Table I demonstrate that there is a clear relationship between Mg^{2+} requirement and inhibition of ternary complex formation at a specific Met-$tRNA_f$ concentration. As shown here, 0.5mM Mg^{2+} inhibited ternary complex formation when 2 A_{260} units Met-$tRNA_f$ (per ml) were used and Mg^{2+} concentration in excess of 1mM was necessary to inhibit ternary complex formation in the presence of 8 A_{260} unit Met-$tRNA_f$.

DISCUSSION

Data presented in this paper provide evidence that the addition of excess tRNA (8-10 A_{260} unit per ml) in the reaction mixture containing 0.5 to 1mM Mg^{2+} causes non-enzymatic GDP displacement from eIF-2·GDP and reverses Mg^{2+}-inhibition of ternary complex formation by eIF-2. These results thus explain why Konieczny and Safer (14) and Benne et al (23) failed to observe Mg^{2+} - inhibition of eIF-2 activity.

Recently, Clemens et. al. (3) have suggested that Konieczny and Safer (14) and Benne et. al. (23) might have purified GDP-free eIF-2 and have thus observed Mg^{2+}-insensitive high eIF-2 activity. The results presented in this paper show that in the presence of high Met-$tRNA_f$ concentrations as used by Benne et al (23) and also possibly by Safer et al (14, 21), eIF-2 bound GDP would be non-enzymatically displaced and the inhibitory effect of GDP, even if it were bound to eIF-2, would not be detected.

We should point out, however, that it is possible to obtain GDP-free eIF-2 using certain purification conditions and such GDP-free eIF-2 will be Mg^{2+} insensitive. However, using standard purification procedures, a significant part of eIF-2 can be isolated as eIF-2.GDP (5,6) and it has been generally agreed that, in the presence of physiological GDP concentration (5μM), eIF-2 exists as eIF-2·GDP and in both cases ternary complex formation is strongly inhibited by Mg^{2+} and requires GEF for such complex formation. Also in both cases, eIF-2 phosphorylation (HRI or dsI catalyzed) leads to complete inactivation of eIF-2α(P)·GDP.

Finally, we wish to point out that the data presented in this paper are in agreement with our previously proposed mechanism for the early steps in mammalian peptide chain initiation (2).

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