Initiation of Protein Synthesis in Eukaryotes

NATURE OF TERNARY COMPLEX DISSOCIATION FACTOR

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Excessive concentrations of the eukaryotic initiation factor 2 (eIF-2)-stimulating protein, a factor that catalyzes the formation of binary (GTP·eIF-2) and ternary (GTP·eIF-2-initiator methionyl-tRNA) initiation complexes at physiological Mg\(^{2+}\) concentrations, can cause ternary complex dissociation when the Mg\(^{2+}\) concentration is raised from 0.5 to 5.0 mM. Stimulation of ternary complex formation and dissociation have similar (a) pH optima, (b) metal ion specificity, and (c) sensitivity to phosphorylation of the eIF-2 \(\alpha\) subunit. The results suggest that ternary complex dissociation is an artifact with no physiological significance.

Earlier (1) we described the isolation from reticulocyte lysates of a complex of the chain eukaryotic initiation factor 2 with a protein termed stimulating protein in virtually homo- 

The abbreviations used are: eIF-2, eukaryotic initiation factor 2; SP, eIF-2-stimulating protein; Met-tRNA\(_i\), eukaryotic initiator methionyl transfer RNA; HCl, heme-controlled translational inhibitor (an eIF-2 \(\alpha\) kinase); AUG, the nucleotide diophosphate ApUpG; Hapes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

EXPERIMENTAL PROCEDURES

Materials—Three different preparations of eIF-2 were used (1): eIF-2 CM-350 (5), approximately 25% pure (eIF-2-SP; a protein obtained by Sepharose-heparin chromatography (6) of the CM-350 fraction, approximately 75% pure (eIF-2-SP; a greater than 90% pure preparation (eIF-2-SP) prepared by the procedure of Benne et al. (7). Amounts given in the legends correspond approximately to pure eIF-2. eIF-2-SP is the factor earlier referred to as IF-eIF-2(1). eIF-2-SP, prepared as described, was virtually homogeneous. Its specific activity in the ternary complex formation assay was 15,000 pmol/mg (=18 pmol of ternary complex/pmol of eIF-2-SP). It consists of eight subunits, three of which correspond to eIF-2 and five to SP. With an estimated \(M_r \approx 450,000\), 1 pmol corresponds to 0.45 \(\mu\)g of protein. Partially purified HCl used to phosphorylate the eIF-2-SP subunit was prepared as described (1). Ribosomal subunits were prepared from Artemia salina embryos by the procedure of Zasloff and Ochoa (8). AUG was from Miles Laboratories, Inc., Elkhart, IN; GDP-free GTP and Met-tRNA\(_i\) were prepared as described earlier (5). [\(^{3}H\)]Met-tRNA, (specific radioactivity, 40,000-50,000 cpm/pmol) was used throughout.

Initiation Complex Assays—Ternary complex (GTP·eIF-2·Met-tRNA\(_i\)) formation was measured (assay A) as previously described (5) except for the addition of 5 \(\mu\)g of bovine serum albumin as a stabilizer. The samples (50 \(\mu\)l) contained the following components added at 0°C in the order listed: 20 mM Hapes buffer, pH 7.6, 100 mM KCl, 0.5 mM Mg(0Ac)\(_2\), 1 mM dithiothreitol, and eIF-2, with or without eIF-2-SP, as specified in the legends. \(^{3}H\)-labeled Met-tRNA\(_i\) (in the amounts specified) and 22 \(\mu\)M GTP (GDP-free) were added last. After incubation for 6 min at 30 °C, the amount of ternary complex present was determined by Millipore filtration. Binary complex formation between GDP and eIF-2 (GDP·eIF-2) was used as a model for the physiologically relevant reaction with GTP·eIF-2 since a reassessment of the original assay (9) revealed \(^{2,3}\) in agreement with a recent report (10), that only GDP forms a stable complex with eIF-2. Samples (50 \(\mu\)l) contained 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM Mg(OAc)\(_2\), 2 mM \(\beta\)-mercaptoethanol, and the amounts of [\(^{3}H\)]GDP, eIF-2, and eIF-2-SP indicated in the legends (no bovine serum albumin). After incubation for 5 min at 30 °C, the amount of binary complex present was determined by Millipore filtration. Controls without eIF-2 were run to correct for GDP binding to eIF-2-SP alone.

Ternary Complex Dissociation Assay—Ternary complex dissociation was measured by a two-stage assay, following the procedure of Majumdar et al. (3) (legend to Fig. 1), based on the fact that ternary complex is formed, but not dissociated, in the absence of Mg\(^{2+}\). The 50-\(\mu\)l samples containing 20 \(\mu\)l Hapes buffer, pH 7.6, 100 mM KCl, 0.3 mM EDTA, 1 mM dithiothreitol, 22 \(\mu\)M GTP, 5 mg of bovine serum albumin, and the indicated amounts of [\(^{3}H\)]Met-tRNA, and eIF-2 were incubated for 5 min at 30 °C and cooled in ice. After adding eIF-2-SP in the amounts indicated and Mg(OAc)\(_2\) to make the Mg\(^{2+}\) concentration 5 mM, the samples were incubated for a further 15 min at 0 °C and assayed for ternary complex. In control samples an equal volume of water replaced the Mg(OAc)\(_2\) at stage 2. Reactions were terminated by the addition of 1 ml of ice-cold washing buffer (20 mM Tris-HCl, pH 7.6, 100 mM KCl, with or without 5 mM Mg(OAc)\(_2\), and the samples were filtered through Millipore membranes. After washing, the membranes were dissolved in 1 ml of 2-methoxyethanol and the radioactivity retained was determined by scintillation counting in 10 ml of Hydrofluor (National Diagnostics, Inc., Somerville, NJ). When the eIF-2-SP subunit was phosphorylated by pretreatment with

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\(^{2}\) J. Sickierka and S. Ochoa, unpublished observations.
When the formation of binary and ternary initiation complexes is determined as a function of the concentration of added eIF-2-SP, the binary complex reaches a plateau at a certain eIF-2-SP level but the ternary complex, highest at this point, decreases as the eIF-2-SP concentration is further increased (Fig. 1). This decrease appears to be due to ternary complex dissociation.

**Ternary Complex Dissociation**—Fig. 2 illustrates the effect of 5 mM Mg\(^{2+}\) and increasing concentrations of homogenous eIF-2-SP on ternary complex stability. High levels of eIF-2-SP caused extensive (80% or higher) loss of ternary complex. Two experiments, run with eIF-2 preparations of widely different purities (~25% and ~90%), gave essentially the same results. Under similar conditions the level of binary complex remained essentially unchanged (not shown). Loss of methionine radioactivity from the filter could be caused by dissociation of the ternary complex, by deacylation of complexed Met-tRNA\(_i\), or both. The occurrence of a Met-tRNA, deacylated in reticulocytes has been reported (see, for example, Ref. 11). However, there was no loss of trichloroacetic acid-insoluble radioactivity after incubation under dissociation conditions, thus eliminating deacylation.

The time course of ternary complex dissociation at 0 °C and 30 °C is shown in Fig. 3. At the higher temperature the loss of ternary complex reached a maximum in about 2 min. Levels of ternary complex found after incubation at various pH values under conditions of ternary complex formation (○) or dissociation (●) are shown in Fig. 4. It may be seen that at pH 7.4, optimum for ternary complex formation, there was a very low ternary complex level under dissociation conditions, suggesting that eIF-2-SP-promoted complex formation and dissociation have similar pH optima. That eIF-2-SP is responsible for stimulation of both ternary complex formation and dissociation is further suggested by the experiments shown in Fig. 5. We have previously shown (2) that whereas a number of metal ions, including Mg, Mn, Ca, Co, Cu, Cd, and Zn, inhibit ternary complex formation, only the inhibition caused by the first three metal ions is prevented by eIF-2-SP. As seen in Fig. 5A, Mg, Mn, and Ca, but not Cu, promoted the dissociation of ternary complex by eIF-2-SP. Further proof that eIF-2-SP promotes both ternary complex formation and dissociation may be found in the fact that phosphorylation of the eIF-2 α subunit inhibits the catalytic effect of eIF-2-SP on ternary complex formation (1, 2) as well as the stimulation of ternary complex dissociation. The effect of α phosphorylation of eIF-2 on ternary complex dissociation is illustrated in Fig. 5B. The eIF-2 α subunit was phosphorylated by incubation with ATP and increasing amounts of HCl before assaying for ternary complex dissociation. It may be seen that dissociation
tion was completely blocked at the higher concentrations of HCl. This effect had already been noted by Ramu et al. (4), who attributed the dissociation to a hypothetical ternary complex dissociation factor.

40 S Complex Formation—The effect of eIF-2-SP on polypeptide chain initiation appears to be due solely to its promotion of binary and ternary complex formation in the presence of inhibitory concentrations of Mg$^{2+}$. At these concentrations Mg$^{2+}$ inhibits complex formation in the absence but not in the presence of eIF-2-SP. The effect of eIF-2-SP on 40 S complex formation is not direct but secondary to its effect on dissociation of the ternary complex.

The dissociation in the presence of eIF-2-SP. Ternary complex formation was completely blocked at the higher concentrations of Mg$^{2+}$. At these concentrations Mg$^{2+}$ inhibits complex formation in the absence but not in the presence of eIF-2-SP. The modified assay contained increasing concentrations of metal ions as indicated.

The amounts of eIF-2-SP (unless omitted) was 1.9 pg. Stage 2 samples were collected and their radioactivity was measured in Beckman Ready-solv HP.

**FIG. 4.** pH dependence of ternary complex formation and dissociation in the presence of eIF-2-SP. Ternary complex formation (C) was measured with 0.5 m mol of [3H]Met-tRNA$_i$, 3 pmol of eIF-2, and 0.1 m g of eIF-2-SP. Maximum (100% binding) was at pH 7.4 and amounted to 1.6 pmol. Ternary complex dissociation (B) was assayed with 50 m mol of Mg(OAc)$_2$, 3 pmol of [3H]Met-tRNA$_i$, and 0.5 m g of eIF-2-SP. Maximum (100% binding) was at pH 7.3 and amounted to 1.7 pmol. Buffers were as follows: 30 m M 2-[N-morpholino]ethanesulfonic acid for pH 5.0 to 6.5; 20 m M Tris-HCl for pH 7.0 to 9.0.

**FIG. 5.** Ternary complex dissociation. A, effect of divalent cations on ternary complex dissociation. Two kinds of controls were run (a) with no added metal ion and (b) with 5 m M metal ion but no eIF-2-SP. This was done in case cations other than Mg$^{2+}$ caused ternary complex dissociation in the absence of eIF-2-SP. The Met-tRNA$_i$ binding values were virtually the same; they were averaged and plotted at zero metal ion concentration. The amounts of [3H]Met-tRNA$_i$, and eIF-2 c were 3.0 and 4.4 pmol, respectively, and the amount of eIF-2-SP (unless omitted) was 1.9 m g. Stage 2 samples contained increasing concentrations of metal ions as indicated. Mg$^{2+}$; Δ, Mn$^{2+}$; Ca$^{2+}$; Cu$^{2+}$. B, phosphorylation of the eIF-2 a subunit inhibits ternary complex dissociation. The modified assay used for this experiment is fully described under "Experimental Procedures." The arrow signals the binding in a control sample without HCl or eIF-2-SP.

**TABLE I**

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| mm | pmol | pmol | pmol | pmol | fold |
| 1 | 0.5 | 5.0 | 0 | 2 | 3.3 | 0.05 | 9 | 14 |
| 2 | 0.5 | 5.0 | 1.1 | 0 | 3.3 | 0.7 | 3.7 | 2 |

**TABLE II**

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a Free (uncomplexed) SP substituted for eIF-2-SP.

Two-stage formation of ternary and 40 S complex in the presence of Mg$^{2+}$ without and with eIF-2-SP

Ternary complex was first formed in stage 1 incubation. Samples (50 m l) contained 20 m M Heps buffer, pH 7.6, 100 m M KCl, 0.5 m M Mg(OAc)$_2$, 1 m M dithiothreitol, 22 m M GTP, 5 m g of bovine serum albumin, 10 pmol of [3H]Met-tRNA$_i$, 5 pmol of eIF-2 b, and, where indicated, eIF-2-SP. Ternary complex formation was measured in one of two duplicate tubes after incubation for 8 min at 30 °C. The second tube was supplemented with 0.64 A$_{260}$ unit of A. salina 40 S ribosomal subunits, 0.1 A$_{260}$ unit of AUG, and MgCl$_2$ to a concentration of 3.3 m M. The final volume was 75 m l. Incubation at 30 °C (stage 2) was continued for a further 6 min. The samples were chilled, layered on 4.2 m l of a 5-20% (w/v) linear sucrose gradient containing 20 m M Hepes buffer, pH 7.6, 100 m M KCl, 5 m M MgCl$_2$, 2 m M dithiothreitol, and centrifuged for 100 min and 50,000 rpm in the SW-56 rotor of the Spinco ultracentrifuge. Fractions (0.15 m l) were collected and their radioactivity was measured in Beckman Ready-solv HP.

**DISCUSSION**

Majumdar et al. (3) and Ramu et al. (4) reported that eIF-2 copurifies with two other factors referred to as Co-eIF-2B and Co-eIF-2C (12). Whereas Co-eIF-2C corresponds to eIF-2-SP, Co-eIF-2B is described as a ternary complex dissociation factor which paradoxically is required for 40 S complex formation. The effect of eIF-2-SP on 40 S complex formation is not direct but secondary to its effect on ternary complex dissociation. Co-eIF-2B is described as a ternary complex dissociation factor which paradoxically is required for 40 S complex formation of the ternary complex. eIF-2-SP stimulates ternary and 40 S complex formation to a similar extent when the ternary complex is formed in the presence of 0.5 m M Mg$^{2+}$ (Table I), but has little further effect when the ternary complex is first formed in the absence of Mg$^{2+}$ (Table II).
formation (3). Our results suggest, however, that ternary complex dissociation is an artifact caused by high concentrations of eIF-2-SP and Mg$_2^+$, so that the existence of a dissociating factor (Co-eIF-2B) distinct from eIF-2-SP is doubtful. Since the preparations of Co-eIF-2B so far described are rather crude, they probably contained eIF-2-SP (Co-eIF-2C) which, as shown here, can cause ternary complex dissociation. Furthermore, our results concerning 40 S complex formation (Tables I and II) are consistent with the view that eIF-2-SP stimulates this reaction only indirectly as it enhances the formation of binary and ternary complexes in the presence of Mg$_2^+$. The mechanism of ternary complex dissociation is unknown.

REFERENCES
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