

Mode of action of the hemin-controlled inhibitor of protein synthesis

(protein phosphorylation/initiation factor eIF-2 kinase/initiation factor eIF-2 stimulating protein/ternary complex formation/
40S ribosomal complex formation)

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ABSTRACT Despite the finding that the hemin-controlled translational inhibitor in reticulocyte lysates is a cyclic AMP-independent protein kinase that phosphorylates the small subunit of the initiation factor eIF-2, the mechanism of inhibition of translation remained unexplained. Whereas treatment of hemin-containing lysates with inhibitor in the presence of ATP inhibited translation, the same treatment of highly purified eIF-2 did not affect its ability to form a ternary complex with initiator Met-tRNA and GTP or a 40S initiation complex. We have isolated from ribosomal salt washes a protein (eIF-2 stimulating protein) that enhances the capacity of unphosphorylated eIF-2 to form ternary or 40S initiation complexes but has no effect on the phosphorylated factor. At low concentrations, eIF-2 is virtually inactive without this stimulating protein. Therefore, the translational inhibitor acts by converting eIF-2 to a form that is not stimulated by the stimulating protein.

In reticulocytes and other eukaryotic cells the initiator species of eukaryotic Met-tRNA (Met-tRNA_i) binding factor eIF-2 is rendered inactive for polypeptide chain initiation by phosphorylation of its small subunit. This reaction is catalyzed (1–4), in the presence of ATP, by an adenosine 3',5'-cyclic monophosphate (cAMP)-independent protein kinase (active eIF-2 kinase, inhibitor) and there is evidence (5–7) that the active enzyme arises by phosphorylation of an inactive precursor (inactive eIF-2 kinase, proinhibitor) catalyzed by cAMP-dependent protein kinase. Hemin prevents the proinhibitor-inhibitor conversion (8) because it blocks the activation of cAMP-dependent protein kinase by cAMP (6, 7) through binding to the regulatory subunit of the enzyme (unpublished data). eIF-2 forms a ternary complex with Met-tRNA_i and GTP which, upon binding to a 40S ribosomal subunit, gives rise to a 40S initiation complex. This sets the stage for elongation of the polypeptide chain.

In the presence of inhibitor and ATP, the rate of protein synthesis in hemin-containing reticulocyte lysates soon shows a sharp decline (9). Although this effect would appear to be related to the phosphorylation of eIF-2, treatment of purified eIF-2 with inhibitor plus ATP does not interfere with its ability to form ternary or 40S initiation complexes (1, 5, 10). On the other hand, formation of the complex is impaired when partially purified eIF-2 is used (5, 10). This suggests that inhibition of translation involves another factor(s) present in lysates and in partially purified eIF-2 preparations. Ribosomal salt washes contain, along with eIF-2, a factor(s) necessary for inhibition of ternary complex formation when purified eIF-2 is treated with inhibitor and ATP. The new factor enhances the ability of unphosphorylated eIF-2 to form ternary or 40S initiation complexes but has no effect on phosphorylated eIF-2; it will be referred to as the eIF-2 stimulating protein (ESP). At the low concentrations present in reticulocyte lysates, eIF-2 is virtually

inactive without ESP. Thus, the hemin-controlled translational inhibitor acts by abolishing the stimulating effect of ESP.

MATERIALS AND METHODS

Assays. Ternary complex formation was assayed as described (11) with *A. salina* [³⁵S]Met-tRNA_i (specific radioactivity, 19,000–11,000 cpm/pmol), 1.5–3 pmol; GTP, 0.14 mM; and eIF-2 and/or ESP, as specified in the legends. The translational inhibitor, eIF-2 kinase (referred to throughout this paper as the inhibitor) was assayed routinely through its inhibition of ternary complex formation (5) by ESP-containing eIF-2, in the presence of ATP. Fig. 1 shows the inhibition as a function of either inhibitor or ATP. Note that there was no inhibition in the absence of added ATP and that 0.05–0.1 mM ATP saturated the system (Fig. 1B). For comparison with other preparations of inhibitor (12) we used the protein synthesis assay (5). ESP was determined with the ternary complex assay.

We suspected that ribosomal washes contained a factor(s) necessary for inhibition of eIF-2 activity, after incubation with inhibitor and ATP, because such incubation decreased the activity of crude but not of purified preparations of eIF-2 (5, 10). Using the inhibitor assay as a guide, we separated eIF-2 from X, a factor that enhanced the ability of eIF-2 to form a ternary complex. However, X did not enhance eIF-2 activity when inhibitor and ATP were also present. X (ESP) was assayed by measuring ternary complex formation in each of four samples, all containing ESP-free eIF-2 and ATP, with the following reactants: (i) no ESP, no inhibitor; (ii) ESP, no inhibitor; (iii) no ESP, inhibitor; (iv) ESP, inhibitor. In a typical assay the amount of ternary complex formed (pmol/50 μl) was as follows: (i) 0.38; (ii) 0.78; (iii) 0.37; (iv) 0.39. Thus, ESP doubled the amount of complex formed in the absence of inhibitor (ii – i) and had no effect in the presence of inhibitor (iv – iii). Comparing the data in a different way, inhibitor depressed complex formation (by 50%) in the presence (ii – iv) but not in the absence (i – iii) of ESP. Therefore, ESP can be assayed in two ways. Assay A: inhibition of ternary complex formation by excess inhibitor plus ATP in samples containing both eIF-2 and ESP (ii – iv). Assay B: enhancement of ternary complex formation by ESP in samples containing eIF-2 but no inhibitor plus ATP (ii – i).

To study the effect of the inhibitor on ternary complex formation after allowing it to act separately, rather than simultaneously, on eIF-2 and ESP (see legend to Fig. 3B), the ATP was

Abbreviations: Met-tRNA_i, initiator species of eukaryotic methionyl-transfer RNA; cAMP, adenosine 3',5'-cyclic monophosphate; ESP, eIF-2 stimulating protein; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-1-ethanesulfonic acid.

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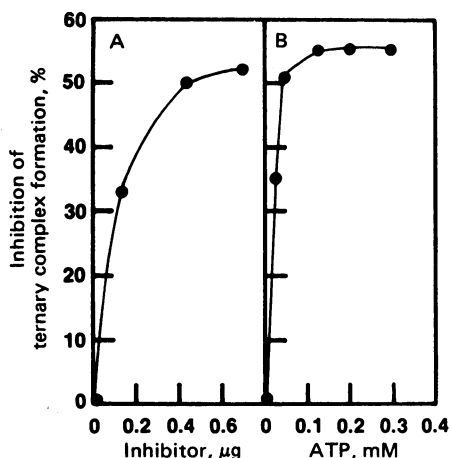


FIG. 1. Inhibition of ternary complex formation by reticulocyte inhibitor (eIF-2 kinase) plus ATP as a function of the concentration of inhibitor (A) or of ATP (B). (A) ESP-containing eIF-2 DE-180, 34 μg ; ATP, 0.4 mM; and various amounts of step 5 inhibitor. (B). eIF-2 CM-400, 3 μg ; ESP SE-200, 3.2 μg ; step 5 inhibitor, 0.7 μg ; and various amounts of ATP. Preincubation with inhibitor and ATP, 2 min; final incubation, 5 min. Temperature, 30°.

removed after preincubation of one or the other factor with ATP and inhibitor. The samples (175 μl) were shaken with 20 μl of a 100 mg/ml suspension of charcoal (charcoal adsorbent, Radiochemical Centre, Amersham, England) for 30 sec at room temperature. The charcoal was sedimented by centrifugation and the samples were processed further as described in the legend to Fig. 3B.

40S complex formation was analyzed by sucrose density gradient centrifugation essentially as in the one-step procedure of Nombela *et al.* (13) with 40S subunits from undeveloped *A. salina* embryos (14). Samples (30 μl) containing N-2-hydroxyethylpiperazine-*N'*-1-ethanesulfonate (Hepes) buffer, pH 7.6, 40 μM ; KCl, 100 mM; Mg(OAc)₂, 10 mM; dithiothreitol, 4 mM; ATP, 0.67 mM; and inhibitor, eIF-2, and ESP as specified in the legend to Fig. 3C were preincubated for 2 min at 30°. They were then supplemented with GTP, 0.12 mM final concentration; [³⁵S]Met-tRNA_i (12,000–15,000 cpm/pmol), 8 pmol; and 40S subunit, 1.0 A₂₆₀ unit, and brought to a final volume of 60 μl by addition of enough KCl to make its final concentration 80 mM. After incubation for 20 min at 30° and 5 min at 0°, the samples were processed as described (13). Protein was determined by the method of Lowry *et al.* (15) with bovine serum albumin as the standard.

Preparations. Partially purified inhibitor (eIF-2 kinase) was prepared from the postribosomal supernatant of commercial (Gibco) rabbit reticulocyte lysate. The procedure involved precipitation with ammonium sulfate, chromatography on DEAE-cellulose [to give a fraction[†] equivalent to protein kinase DEAE peak III of Traugh and Traut (16), chromatography on phosphocellulose, and filtration on Sephadex G-200. (The details of this procedure will be described elsewhere.) The inhibitor solution (2 mg of protein per ml) was stored in small aliquots in liquid nitrogen. The amount of protein required to decrease protein synthesis in a hemin-containing lysate to the level of inhibition observed in the absence of hemin (taken as 100% inhibition) was assumed to contain 1 unit of inhibitor activity. An amount of inhibitor containing 1.4 μg of protein caused 100% inhibition in the protein synthesis assay. Its specific activity, 714 units/mg of protein, was similar to that of the step

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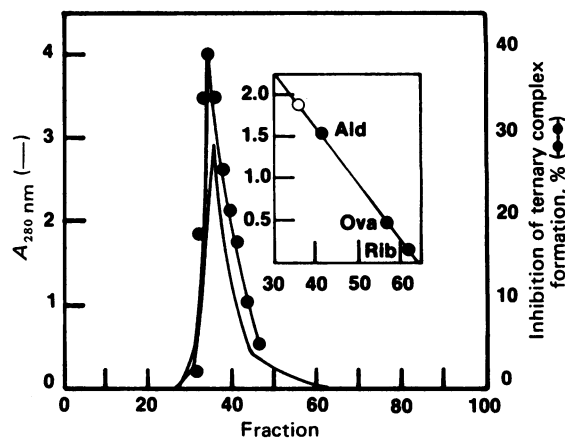


FIG. 2. ESP purification: chromatography on Sephadex G-200. (Inset) In a separate experiment, a mixture of muscle aldolase (Ald), ovalbumin (Ova), and ribonuclease A (Rib), molecular weights 152,000, 45,000, and 13,700, respectively, was run on the same column and the fraction numbers corresponding to the elution peaks of the three proteins (●) were plotted against molecular weight ($\times 10^{-5}$); ○ corresponds to the ESP activity peak in assay A (see Table 1).

4 inhibitor of Ranu and London (12). The preparation of ESP-free eIF-2 and ESP is described below. Other preparations and materials were as in previous work (5–7).

RESULTS

Isolation of ESP. ESP and eIF-2 were isolated from the ribosomal salt wash of developing *A. salina* embryos. **Step 1.** Ribosomal wash (11) dialyzed overnight against buffer A [20 mM Hepes, pH 7.6/0.1 mM EDTA/1 mM dithiothreitol/5% (vol/vol) glycerol] containing 80 mM KCl. **Step 2.** The dialyzed solution (7.5 ml, 21 mg of protein per ml) was applied to a DEAE-cellulose (Whatman DE-52, 1.5 \times 8 cm) equilibrated with buffer A containing 80 mM KCl. After the column was washed with the same buffer, eIF-2 and ESP were eluted with buffer A containing 180 mM KCl (DE-180 fraction). **Step 3.** The step 2 fraction was applied to a column (0.6 \times 8 cm) of carboxymethyl-Sephadex C-50 (Pharmacia) equilibrated with buffer A containing 180 mM KCl. This separates eIF-2, which is retained by the column, from ESP, which is not retained (ESP CM-180). eIF-2 free from ESP was eluted with buffer A containing 400 mM KCl, yielding 2 ml of solution containing 3 mg of protein (eIF-2 CM-400). It was stored in small aliquots in liquid nitrogen. The specific activity of this preparation (ternary complex assay with excess ESP) was 125 pmol/mg of protein. **Step 4.** The protein in the CM-180 effluent fraction was concentrated by precipitation with ammonium sulfate between 25 and 80% saturation. The precipitate was dissolved in 2.5 ml of buffer A, and the solution was dialyzed overnight against the same buffer. The dialyzed solution was passed through a column of carboxymethyl-Sephadex C-50 as above, previously equilibrated with buffer A. ESP (CM-0) is not retained. **Step 5.** The ESP fraction CM-0 was chromatographed on Sephadex G-200 (0.9 \times 57 cm column) with buffer A as eluent; 0.5-ml fractions were collected. The bulk of the ESP activity was eluted in fractions 32–46 (Fig. 2). Fractions 32–41 were pooled to give a solution containing 1.6 mg of protein per ml (ESP SE-200) and stored in small aliquots in liquid nitrogen.

A summary of the preparation of ESP is given in Table 1. The molecular weight of ESP (see Fig. 2) is approximately 180,000. eIF-2 CM-400 and ESP SE-200 were used throughout the following studies unless otherwise specified.

Table 1. Purification of ESP from ribosomal salt wash of *A. salina* embryos

Step	Protein mg	Units*	Specific activity†
1. Salt wash	160		
2. DEAE-cellulose (DE-180)	62		
3. First CM-Sephadex (CM-180)	37	4100	111
4. Second CM-Sephadex (CM-0)	26	4300	165
5. Sephadex G-200 (SE-200)	8	2500	312

Assay at steps 1 and 2, prior to separation of ESP from eIF-2, was only qualitative. eIF-2 CM-400 (see text), 6 μ g per sample, was added for assay at steps 3–5. Assay A (see *Materials and Methods*) was used.

* One unit is the amount of protein required to obtain 50% inhibition of ternary complex formation with purified eIF-2 in the presence of excess inhibitor and ATP.

† Units/mg of protein.

Effect of ESP and Inhibitor on Initiation Complex Formation. Because ESP was isolated as a factor required for inhibition of ternary complex formation with purified eIF-2 upon incubation with inhibitor and ATP, we were surprised to find (Fig. 3A) that, in the absence of inhibitor, ESP actually stimulated complex formation (bars 1 and 2). In the presence of inhibitor and ATP (bars 3 and 4), ESP was without effect. Enhancement of the activity of eIF-2 by ESP probably involves interaction of the two factors. Hence, blocking of this interaction could be caused by (i) phosphorylation of the 38,000-dalton subunit of eIF-2 alone, (ii) phosphorylation of ESP alone, or (iii) phosphorylation of both eIF-2 and ESP. Fig. 3B shows that

explanation (i) is the correct one. Here, either eIF-2 or ESP was first preincubated with ATP, with or without inhibitor, and the ATP was then removed with charcoal. The samples were then supplemented with the missing factor (ESP or eIF-2) together with GTP and [³⁵S]Met-tRNA_i and incubated further for ternary complex formation. Exposure of eIF-2 alone to inhibitor and ATP blocked its interaction with ESP (Fig. 3B, bars A and B) but the same treatment of ESP alone did not affect its interaction with eIF-2 (Fig. 3B, bars C and D). Comparison of black bars in A and B and the corresponding ones in C and D show that ATP was effectively removed by the charcoal treatment, because inhibitor was without effect unless ATP was added again. Fig. 3C and D show that what is true of ternary complex is also true of 40S initiation complex formation. Thus, with ESP-containing eIF-2, formation of 40S complex was decreased when the samples contained inhibitor and ATP (Fig. 3C, bars 1 and 2). With ESP-free eIF-2, ESP stimulated 40S complex formation in the absence (Fig. 3C and D, bars and samples 3 and 4) but not in the presence (bars and samples 5 and 6) of inhibitor and ATP.

The enhancement of ternary complex formation by eIF-2 in the presence of increasing concentrations of ESP is shown in Fig. 4A. Note that no complex was formed when ESP was present without either GTP or eIF-2. The kinetics of ternary complex formation with and without ESP and/or inhibitor plus ATP are shown in Fig. 4B. Maximal complex formation required 5–7 min. ESP increased not only the extent but also the rate of complex formation. Again, inhibitor plus ATP had no effect in the absence of ESP but virtually abolished the ability of the latter to enhance complex formation. ESP enhanced

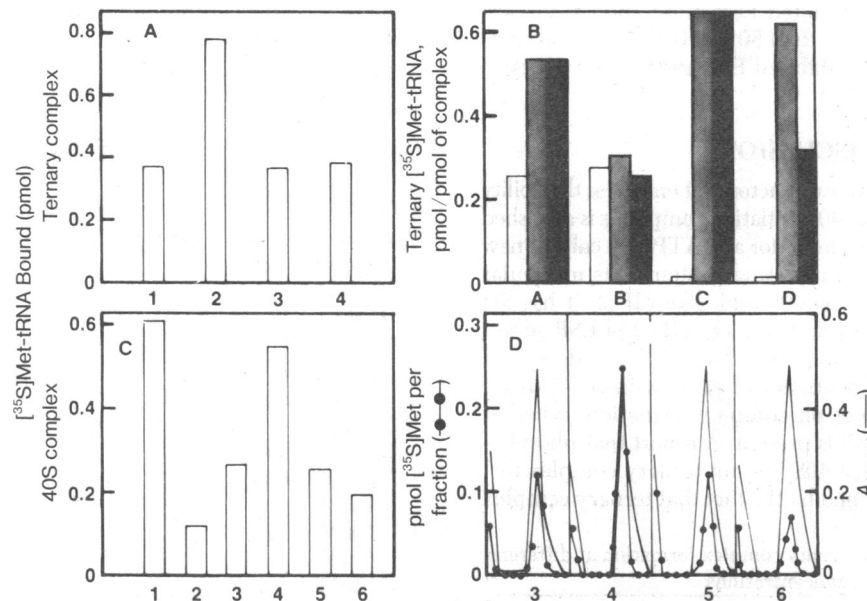


FIG. 3. Effect of ESP and inhibitor on initiation complex formation. (A) Effect on ternary complex formation. ATP (0.4 mM) and eIF-2 (6 μ g) were present throughout. Other reactants, when present, were: ESP, 6 μ g; inhibitor, 0.7 μ g. Bars: 1, eIF-2; 2, eIF-2 and ESP; 3, eIF-2 and inhibitor; 4, eIF-2, inhibitor, and ESP. (B) Inhibition of ESP effect on ternary complex formation requires interaction of inhibitor and ATP with eIF-2 only. Samples (175 μ l) containing either 35 μ g of eIF-2 (bars A and B) or 24 μ g of ESP (bars C and D) were preincubated with 0.057 mM ATP (bars A and C) or with 0.057 mM ATP and 3.5 μ g of inhibitor (bars B and D) for 6 min at 30°. Other components present in all samples during preincubation were Hepes buffer, pH 7.6, 28.5 mM; Mg(OAc)₂, 4.3 mM; KCl, 68.5 mM; and dithiothreitol, 2.8 mM. The ATP was removed with charcoal. Aliquots (35 μ l) of the supernatant were supplemented with GTP, 0.14 mM, and [³⁵S]Met-tRNA_i (25,000 cpm/pmol), 1.8 pmol, plus the following additions: In A, \square none, \blacksquare ESP (3.2 μ g), \blacksquare ESP (3.2 μ g) and inhibitor (0.7 μ g); in B, \square none, \blacksquare ESP (3.2 μ g), \blacksquare ESP (3.2 μ g), inhibitor (0.7 μ g), and ATP (0.04 mM); in C, \square none, \blacksquare eIF-2 (5 μ g), \blacksquare eIF-2 (5 μ g) and inhibitor (0.7 μ g); in D, \square none, \blacksquare eIF-2 (5 μ g), \blacksquare eIF-2 (5 μ g), inhibitor (0.7 μ g), and ATP (0.04 mM). Final volume, 50 μ l. After incubation for 5 min at 30° the samples were assayed for ternary complex formation. The concentration of the other components present in all samples during the final incubation were: Hepes buffer, pH 7.5, 20 mM; Mg(OAc)₂, 3 mM; KCl, 100 mM; and dithiothreitol, 2 mM. (C) Effect on 40S complex formation. Samples 1 and 2 had ESP-containing eIF-2 DE-180, 51 μ g; samples 3–6 had eIF-2 CM-400, 18 μ g. Other reactants, when present, were: ESP, 12 μ g, and/or inhibitor, 2 μ g in sample 2 or 1 μ g in samples 5 and 6. Bars: 1, eIF-2; 2, eIF-2 and inhibitor; 3, eIF-2; 4, eIF-2 and ESP; 5, eIF-2 and inhibitor; 6, eIF-2, inhibitor, and ESP. (D) Sucrose density gradient centrifugation profiles of samples 3–6 of C.

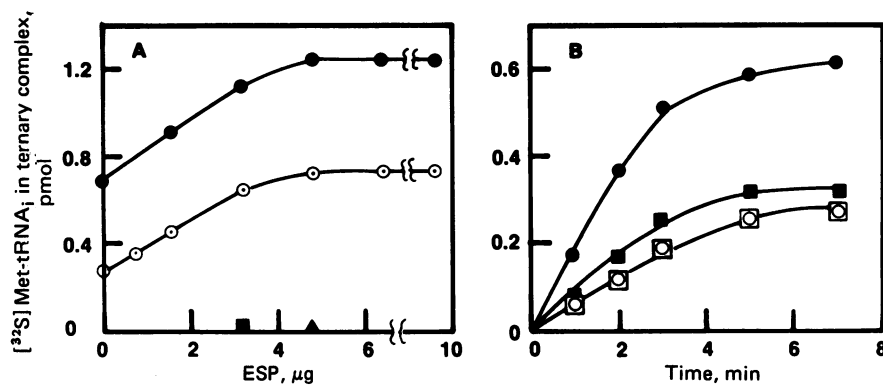


FIG. 4. (A). Ternary complex formation as a function of the concentration of ESP. Samples containing GTP and $[^{35}\text{S}]\text{Met-tRNA}_i$ were incubated with the amounts of eIF-2 indicated below and increasing amounts of ESP for 5 min at 30° and assayed for ternary complex formation. O, eIF-2, 3 μg ; ●, eIF-2, 6 μg ; ■, eIF-2, 6 μg ; ESP, 3.2 μg ; no GTP. ▲, No eIF-2; ESP, 4.8 μg . (B). Kinetics of ternary complex formation in the absence or present of ESP and/or inhibitor. Samples containing GTP, $[^{35}\text{S}]\text{Met-tRNA}_i$, eIF-2 (3 μg) and other additions as indicated below were incubated for various times at 30° and assayed for ternary complex formation. O, ATP, 0.4 mM; ●, ATP, 0.4 mM, and ESP, 3.2 μg ; □, ATP, 0.4 mM, and inhibitor, 0.7 μg ; ■, ATP, 0.4 mM, inhibitor, 0.7 μg , and ESP, 3.2 μg .

ternary complex formation as much as 20-fold at low eIF-2 concentrations but was virtually without effect at high concentrations (Table 2). We have also isolated from rabbit reticulocyte lysate by the same procedure used for the *A. salina* factors, eIF-2 and ESP free from each other and have obtained similar results. Moreover, reticulocyte eIF-2 interacted with *A. salina* ESP and conversely (unpublished data).

Sensitivity of ESP to SH-Binding Reagents. ESP was as sensitive as eIF-2 (11, 17, 18) to SH-binding reagents (Table 3). *N*-Ethylmaleimide at 20 mM or *p*-chloro-mercuribenzenesulfonic acid at 1.0 mM inhibited ESP stimulation of ternary complex formation by more than 80%; 2.0 mM *p*-chloro-mercuribenzenesulfonic acid inhibited ESP and eIF-2 activity to the same extent, 95%.

DISCUSSION

The activity of a ribosomal wash factor that enhances the ability of eIF-2 to form ternary or 40S initiation complexes is abolished by incubation of eIF-2 with inhibitor and ATP. We call the new factor "ESP" for "eIF-2 stimulating protein." Its molecular weight is approximately 180,000 and, like eIF-2, it has SH groups that are essential for activity. The effect of ESP on ternary complex formation is similar to that of a factor described by Dasgupta *et al.* (19). Incubation of eIF-2 with eIF-2 kinase and ATP is without effect on complex formation (whether ternary or 40S) unless ESP is present. A report that phosphorylation of eIF-2 inhibited 40S but not ternary complex formation (20) can be explained by the fact that ternary complex

Table 2. Effect of ESP on ternary complex formation at different eIF-2 concentrations

eIF-2 $\mu\text{g}/50 \mu\text{l}$	$[^{35}\text{S}]\text{Met-tRNA}_i$ in ternary complex, pmol		Stimulation by ESP, -fold
	Without ESP	With ESP	
0.24	0.0002	0.004	20
0.60	0.001	0.02	20
1.2	0.015	0.07	5
2.4	0.05	0.16	3
4.8	0.18	0.34	2
7.2	0.29	0.47	1.6
10.8	0.48	0.57	1.2

Standard assay with 3 pmol of high specific radioactivity $[^{35}\text{S}]\text{Met-tRNA}_i$ (104,600 cpm/pmol), the indicated amounts of eIF-2, and when present, 3.2 μg of ESP.

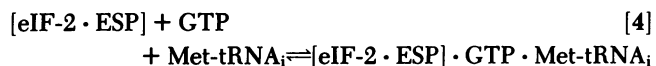
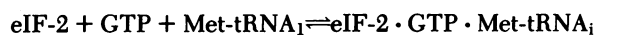
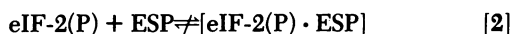
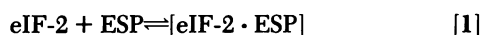
formation was assayed in the presence of an additional ribosomal wash fraction that probably contained ESP. It could be argued that ESP might be contaminated with a factor that inactivates phosphorylated eIF-2, the kind of factor that we originally set out to isolate. We think that is unlikely because the ratio of stimulation of complex formation by ESP to inhibition of complex formation by eIF-2 kinase plus ATP in the presence of ESP remained constant (i) on purification, (ii) on heating at various temperatures, (iii) on exposure to different concentrations of -SH binding reagents, and (iv) over a wide range of ESP concentrations (data not shown).

Table 3. Sensitivity of ESP and eIF-2 to SH-binding reagents

Treatment	Ternary complex formation			
	ESP treated		eIF-2 treated	
	Due to ESP, pmol	Inhibi- tion, %	Total, pmol	Inhibi- tion, %
Control	0.23			
<i>N</i> -Ethylmaleimide:				
1 mM	0.15	35		
5 mM	0.07	69		
20 mM	0.04	83		
20 mM plus dithiothreitol, 40 mM	0.23	0		
Control	0.27		0.16	
<i>p</i> -Chloromercuriben- zenesulfonic acid:				
0.1 mM	0.25	7	—	—
0.5 mM	0.20	26	0.13	19
1.0 mM	0.03	89	—	—
2.0 mM	0.01	96	0.01	94
2.0 mM plus dithiothreitol, 10 mM	—	—	0.16	0

ESP or eIF-2 was preincubated without or with *N*-ethylmaleimide or *p*-chloromercuribenzenesulfonate at the stated concentrations for 15 min at 25° : after the unreacted reagent was neutralized with excess dithiothreitol (20 or 10 mM, respectively), the activity of the factors in ternary complex formation was assayed. Controls were run in which excess dithiothreitol was present during preincubation of the factors with the SH-binding reagent. In the ESP sensitivity tests, the samples contained 3 μg of eIF-2 and 3.2 μg of ESP; in the eIF-2 sensitivity tests they contained 3 μg of eIF-2 but no ESP. Values are given in pmol of $[^{35}\text{S}]\text{Met-tRNA}_i$ in ternary complex. In the ESP experiments, the difference between samples with ESP and a control without ESP—i.e., the stimulation of ternary complex formation by ESP—is given.

At the concentrations routinely used in this work, eIF-2 was able to form an initiation complex in the absence of ESP; complex formation was increased 2- to 3-fold by ESP. However, at low eIF-2 concentrations (0.24–0.6 μg of eIF-2 CM-400 per 50 μl), ESP stimulated complex formation as much as 20-fold. We have determined the eIF-2 content of rabbit reticulocyte lysates and have estimated that protein synthesis assay samples (50 μl , 25 μl of lysate) contain the equivalent of about 0.4 μg of eIF-2 CM-400. At this concentration (see Table 2), the ability of eIF-2 to form a ternary complex is almost negligible in the absence of ESP. Because the effect of ESP is prevented by incubation of eIF-2 with inhibitor and ATP, the observations reported in this paper fully elucidate the mechanism by which phosphorylation of the eIF-2 present in reticulocyte lysates inhibits chain initiation. They also explain why addition of large amounts of eIF-2 relieves this inhibition (9). The following model for the mode of action of ESP is consistent with our observations. ESP complexes with eIF-2 (reaction 1), but not with phosphorylated eIF-2 (reaction 2), whereby the equilibrium of reaction 3 is displaced toward ternary complex formation (reaction 4).



A better understanding of the properties of ESP must await further purification of this protein.

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