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Mode of action of the hemin-controlled inhibitor of protein synthesis: Studies with factors from rabbit reticulocytes*

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

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ABSTRACT **Previously [de Haro, C., Datta, A. & Ochoa, S. (1978) *Proc. Natl. Acad. Sci. USA* 75, 243–247] it was shown with initiation factors from *Artemia salina* embryos that the activity of the initiator methionyl-tRNA binding factor eIF-2 is stimulated by another factor (ESP, for eIF-2 stimulating protein) present, like eIF-2, in ribosomal salt washes. Incubation of eIF-2 with translational inhibitor from rabbit reticulocytes, in the presence of ATP, abolished the ESP effect. At physiological concentrations eIF-2 was virtually inactive without ESP. These observations indicated that the translational inhibitor acts by converting eIF-2 to a form that is not stimulated by ESP. The same observations have now been made with eIF-2 and ESP from rabbit reticulocytes but, in this case, the dependence of eIF-2 activity on ESP is much more pronounced than with the *A. salina* factors. eIF-2 from reticulocytes interacts with ESP from *A. salina* and conversely.**

The hemin-controlled inhibitor of protein synthesis in reticulocyte lysates is a cyclic AMP-independent protein kinase that catalyzes the phosphorylation of the small subunit of the initiation factor eIF-2 (1–4). Incubation of eIF-2 with ATP and eIF-2 kinase does not interfere with its ability to form ternary ($Met\text{-}tRNA_i$·eIF-2·GTP) or 40S ($Met\text{-}tRNA_i$·eIF-2·GTP·40S) initiation complexes under the usual assay conditions, which involve the use of rather large amounts of eIF-2 (1, 5), but it abolishes the interaction of eIF-2 with a protein (eIF-2 stimulating protein or ESP) that is essential for complex formation at the low concentrations of eIF-2 found in lysates (5).‡ Our previous work with factors from *Artemia salina* embryos (5) has now been confirmed with eIF-2 and ESP isolated from rabbit reticulocytes. ESP from reticulocytes interacts with eIF-2 from *A. salina* and conversely, but the dependence of eIF-2 activity on ESP is much greater when both factors are derived from reticulocytes, possibly because of better separation from each other. Reticulocyte and *A. salina* ESP have similar sensitivity to SH-binding reagents. The molecular weight of partially purified ESP from either source is about the same, in the vicinity of 200,000.

MATERIALS AND METHODS

Assays. The stimulation of ternary complex formation by ESP (5) was the basis of the standard assay for this factor. When no eIF-2 kinase was present (assay A) the incubation samples (50 μl) contained the following components added at 0° in the order listed: 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes) buffer (pH 7.6), 100 mM KCl, 3 mM $Mg(OAc)_2$, 1 mM dithiothreitol, and, when present, ESP and/or eIF-2 in the amounts specified in the legends. [^{35}S]$Met\text{-}tRNA_i$ (as specified in the legends) and 0.14 mM GTP, when present, were added last. After incubation for 5 min at 30°, the reaction was stopped by dilution with 2 ml of ice-cold wash buffer [20 mM Tris·HCl, pH 7.5/100 mM KCl/3 mM $Mg(OAc)_2$] and the samples were filtered through nitrocellulose membranes (Millipore Hawpo, 0.45-μm pore size). The filters were washed twice, each time with 4 ml of wash buffer, and dried, and the retained radioactivity was measured in Omnifluor in a Beckman LS-100 scintillation counter. Three samples (all containing GTP) were run simultaneously: (*a*) with eIF-2, (*b*) with ESP, and (*c*) with eIF-2 and ESP. Three corresponding blanks without GTP were also run and their values (0.002–0.004 pmol of [^{35}S]$Met\text{-}tRNA_i$ retained) were subtracted from the experimental ones to give the net GTP-dependent formation of ternary complex.

When incubations were done in the presence of eIF-2 kinase and/or ATP, the assay (assay B) was conducted in two steps. *Step 1.* eIF-2 was preincubated without or with ESP and/or eIF-2 kinase for 6 min at 30°. *Step 2.* The samples were supplemented with [^{35}S]$Met\text{-}tRNA_i$, without or with GTP, and incubated for 5 more min at 30°. GTP-dependent ternary complex formation was determined as in assay A. The step 1 samples (40 μl) contained (added at 0° in the order listed): 25 mM Hepes buffer (pH 7.6), 50 mM KCl, 3.75 mM $Mg(OAc)_2$, 1.25 mM dithiothreitol, 0.5 mM ATP, and, when present, ESP, eIF-2, and eIF-2 kinase as specified in the legends. The step 2 samples (50 μl) contained 100 mM KCl, [^{35}S]$Met\text{-}tRNA_i$ as specified in the legends, and, when present, 0.14 mM GTP. Two sets of four samples each (one set without, one with GTP) were run simultaneously: (*a*) with eIF-2, (*b*) with eIF-2 and eIF-2 kinase, (*c*) with eIF-2 and ESP, and (*d*) with eIF-2, eIF-2 kinase, and ESP.

40S complex formation was analyzed by sucrose density gradient centrifugation as described (5). Samples (60 μl) containing 35 mM Hepes buffer (pH 7.6), 100 mM KCl, 3.5 mM $Mg(OAc)_2$, 3.5 mM dithiothreitol, 0.5 mM ATP, 15 μg of eIF-2, and, when present, 36 μg of ESP and/or 2 μg of eIF-2 kinase were preincubated for 2 min at 30°. They were then supplemented with 10 pmol of [^{35}S]$Met\text{-}tRNA_i$ (34,360 cpm/pmol), 0.12 mM GTP, and 0.5 A_{260} unit of *A. salina* 40S ribosomal subunits and brought to a final volume of 105 μl, maintaining the concentration of KCl at 100 mM. Incubation for 10 min at

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Abbreviations: $Met\text{-}tRNA_i$, initiator species of eukaryotic methionyl-tRNA; eIF-2, eukaryotic initiation factor 2; ESP, eIF-2 stimulating protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

* This is paper 2 in a series. Paper 1 is ref. 5.

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‡ Two errors were found in ref. 5. Fig. 3*B*, the ordinate should read "pmol [^{35}S]$Met\text{-}tRNA_i$ in ternary complex." On line 12 of *Discussion* the last two words should be "40S complex" not "ternary complex."

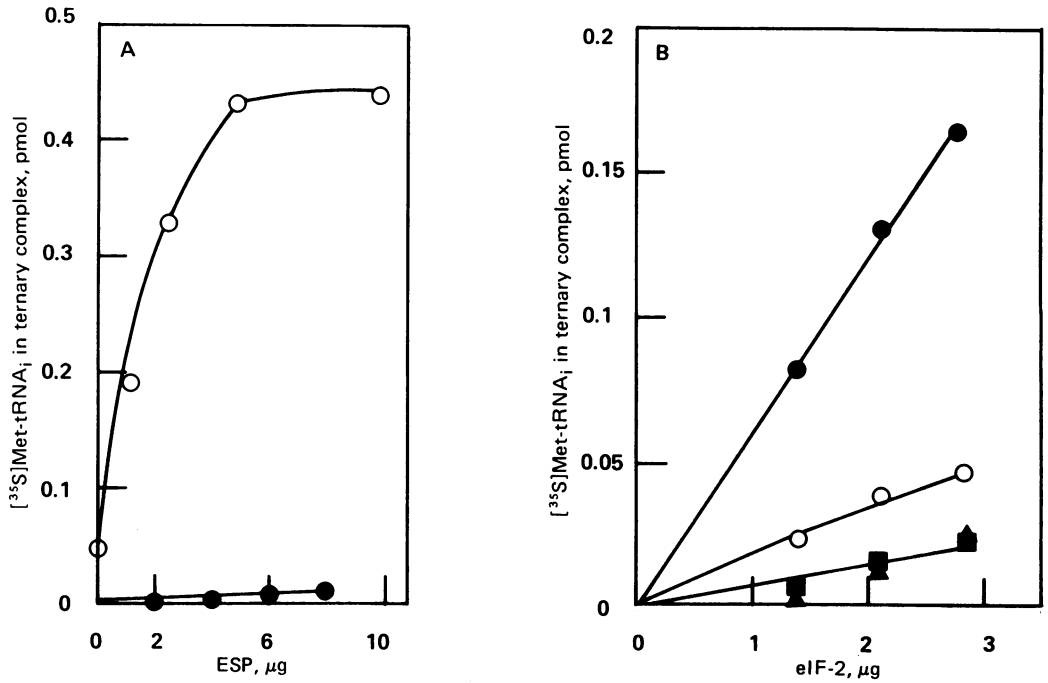

FIG. 1. (*A*) Ternary complex formation as a function of the concentration of ESP. Assay A with 2.5 pmol of [^{35}S]Met-tRNA$_i$ (20,075 cpm/pmol), 0.14 mM GTP, and increasing amounts of ESP, without (●) or with (○) 1.9 μg of eIF-2. (*B*) Effect of ESP and eIF-2 kinase on ternary complex formation as a function of the concentration of eIF-2. Assay B with 2.5 pmol of [^{35}S]Met-tRNA$_i$ (93,550 cpm/pmol), 0.14 mM GTP, 0.4 mM ATP, increasing amounts of eIF-2, and further additions as follows: ■, none; ▲, eIF-2 kinase (0.7 μg); ●, ESP (3.6 μg); ○, eIF-2 kinase (0.7 μg) and ESP (3.6 μg).

37° was followed by the addition of 3 μl of 110 mM $Mg(OAc)_2$ to each sample and further incubation for 5 min at 0°. The samples were processed as described by Nombela *et al.* (6). Protein was determined by the procedure of Lowry *et al.* (7), with bovine serum albumin as the standard.

Preparations. eIF-2 and ESP were isolated from the ribosomal salt wash of rabbit reticulocyte lysates essentially as described (5) for the *A. salina* factors. All operations were conducted at 0°–2°. One hundred forty milliliters of lysate (Gibco Laboratories) was centrifuged at 27,000 × *g* for 15 min in the SS 34 rotor of the Sorvall centrifuge and the supernatant was further centrifuged at 125,000 × *g* for 3.5 hr in the 60 Ti rotor of the Spinco preparative ultracentrifuge. The ribosomal pellet was suspended in 5 ml of buffer A [20 mM Hepes, pH 7.6/0.5 M KCl/5 mM $Mg(OAc)_2$/0.1 mM EDTA/2 mM dithiothreitol/5% (vol/vol) glycerol] for 2 hr and centrifuged for 2.5 hr at 235,000 × *g* in the Spinco 75 Ti rotor. The solution was dialyzed overnight against buffer B [20 mM Hepes, pH 7.6/0.1 mM EDTA/1 mM dithiothreitol/5% (vol/vol) glycerol] containing 80 mM KCl. The dialyzed ribosomal wash (2.8 ml, 24 mg of protein per ml) was applied to a DEAE-cellulose (Whatman DE 52) column (0.9 × 4 cm) equilibrated with buffer B containing 80 mM KCl. After the column was washed with the same buffer, eIF-2 and ESP were eluted with buffer B containing 180 mM KCl. The DE-180 fraction (3 ml) contained 3 mg of protein per ml. It was applied to a column (0.6 × 1 cm) of carboxymethyl-Sephadex C-50 (Pharmacia) equilibrated with buffer B containing 180 mM KCl. As with the *A. salina* factors (5), this step separates eIF-2, which is retained by the column, from ESP, which is not retained. The ESP CM-180 effluent fraction (3 ml) contained 2 mg of protein per ml. ESP-free eIF-2 was eluted with buffer B containing 350 mM KCl. This elution yielded 0.5 ml of solution containing 1 mg of protein per ml (eIF-2 CM-350). The specific activity of this preparation (assay A with excess ESP) was about 200 pmol/mg of protein. It was stored in small aliquots in liquid nitrogen. The protein in the CM-180 effluent fraction was concentrated by precipitation with ammonium sulfate at 80% saturation. The precipitate was dissolved in 1 ml of buffer B, 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, that had been equilibrated with buffer B. ESP (CM-0) is not retained. The ESP CM-0 fraction was chromatographed on Sephadex G-200 (0.9 × 57 cm column) with buffer B as eluant; 0.5-ml fractions were collected. The bulk of the ESP activity was eluted in fractions 32–40. These fractions were pooled; the ESP was concentrated by adsorption on a DEAE-cellulose column (0.6 × 1.5 cm) that had been equilibrated with buffer B, and eluted with a small volume of the same buffer containing 200 mM KCl. One milliliter of solution (ESP SE-200), containing 1 mg of protein per ml, was obtained. It was stored in small aliquots in liquid nitrogen. If 1 unit of ESP is taken as the amount that, in the presence of 2 μg of eIF-2, stimulates ternary complex formation in assay A by 1 pmol, the specific activity of ESP SE-200 (calculated from the initial protion of the ESP curve of Fig. 1*A*) was about 165 units/mg of protein. As determined by Sephadex G-200 filtration (see ref. 5), the molecular weight of reticulocyte ESP, like that of *A. salina* ESP, was about 200,000. eIF-2 CM-350 and ESP SE-200 were used throughout this work. Other preparations, including *A. salina* eIF-2, ESP, Met-tRNA$_i$, and 40S ribosomal subunits, as well as rabbit reticulocyte translational inhibitor (eIF-2 kinase), were as in previous work (5).

RESULTS

ESP has no effect in the absence of eIF-2 but markedly stimulates ternary complex formation by eIF-2 (Fig. 1*A*). In one experiment (Table 1), 3.6 μg of ESP increased ternary complex

Table 1. Effect of ESP on ternary complex formation at different eIF-2 concentrations

ESP, μg/50 μl	eIF-2, μg/50 μl	[35S]Met-tRNAi in ternary complex, pmol		Stimulation by ESP, -fold
		Without ESP	With ESP	
3.6	1.4	0.01	0.36	36
3.6	2.1	0.03	0.49	16
3.6	2.8	0.07	0.53	7
10	2.0	0.04	0.72	18
10	7.0	0.30	0.89	3
10	12.0	0.54	0.96	2

Assay A with 3 pmol of [35S]Met-tRNAi (94,320 cpm/pmol) and the indicated amounts of ESP and eIF-2.

formation nearly 40-fold with 1.4 μg of eIF-2 but only 7-fold with 2.8 μg of the factor. Incubation with eIF-2 kinase and ATP is without effect on ternary complex formation with eIF-2 alone (Fig. 1*B*, lower curve) but markedly inhibits the stimulation normally elicited by ESP (Fig. 1*B*; compare upper and middle curves). The time course of ternary complex formation, in the absence or presence of ESP and/or eIF-2 kinase and ATP, is shown in Fig. 2. As observed with the *A. salina* factors (5), ESP increases both the extent and the rate of ternary complex formation. Again, incubation with eIF-2 kinase and ATP has no effect on complex formation by eIF-2 alone but severely depresses the ability of ESP to enhance this reaction. Fig. 3 shows that what is true of ternary complex is also true of 40S initiation complex formation. Thus, the small amount of complex formed in the absence of ESP is not affected by preincubation with eIF-2 kinase and ATP (bars and samples 1 and 2) whereas such preincubation virtually abolishes the considerable stimulation produced by ESP (bars and samples 3 and 4).

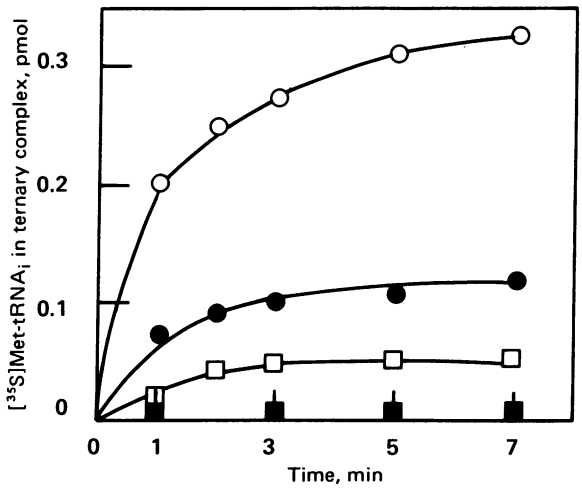

FIG. 2. Kinetics of ternary complex formation in the absence or presence of ESP and/or eIF-2 kinase. Samples containing 2.5 pmol of [35S]Met-tRNAi (92,890 cpm/pmol), 0.14 mM GTP, 0.4 mM ATP, and other additions as indicated below were incubated for various times under the conditions of assay B. ■, ESP (3.6 μg); □, eIF-2 (2.1 μg) without or with eIF-2 kinase (0.7 μg); ●, eIF-2 (2.1 μg), eIF-2 kinase (0.7 μg), and ESP (3.6 μg); ○, eIF-2 (2.1 μg) and ESP (3.6 μg).

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

Treatment	Ternary complex formation: ESP-treated, Due to ESP, pmol	ESP-treated, Inhibition, %	eIF-2-treated, Total, pmol	eIF-2-treated, Inhibition, %
Control			0.10	
N-Ethylmaleimide				
1 mM			0.88	20
5 mM			0.001	99
20 mM			0	100
20 mM + 40 mM dithiothreitol			0.09	10
Control	0.33		0.07	
p-Chloromercuribenzenesulfonic acid				
1.0 M	0.02	94	0.04	43
2.0 mM	—	—	0.007	90
2.5 mM	0.009	97	—	—
2.5 mM + 20 mM dithiothreitol	0.31	6	—	—

eIF-2 was preincubated without or with *N*-ethylmaleimide at the stated concentrations for 15 min at 25°; after the unreacted reagent was neutralized with excess dithiothreitol (20 mM), the activity of the factor in ternary complex formation was assayed (assay A). In the case of *p*-chloromercuribenzenesulfonic acid, ESP or eIF-2 was preincubated with the stated concentrations of reagent for 15 min at 25° prior to assaying for ternary complex formation (assay A). The final concentration of *p*-chloromercuribenzenesulfonate in the assay was less than 0.25 mM, a noninhibitory concentration. Controls were run in which excess dithiothreitol was present during incubation of the factors with the SH-binding reagent. In ESP sensitivity tests, the samples contained 2.4 μg of eIF-2, 4.8 μg of ESP, and 2.5 pmol of [35S]Met-tRNAi (21,495 cpm/pmol); in the eIF-2 sensitivity tests with *p*-chloromercuribenzenesulfonate, they contained 3.4 μg of eIF-2 and 2.5 pmol of [35S]Met-tRNAi (18,655 cpm/pmol); in the eIF-2 sensitivity tests with *N*-ethylmaleimide, they contained 4.9 μg of eIF-2 and 2.5 pmol of [35S]Met-tRNAi (87,800 cpm/pmol). Values are given in pmol of [35S]Met-tRNAi 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.

The sensitivity of reticulocyte eIF-2 and ESP to SH-binding reagents (Table 2) is similar to that of the *A. salina* factors (5). Moreover, as seen in Table 3, reticulocyte ESP stimulates ternary complex formation by *A. salina* eIF-2 and conversely. However, the extent of complex formation and its enhancement by ESP were greater when both factors were derived from reticulocytes.

Table 3. Cross-interaction between eIF-2 and ESP from *A. salina* and rabbit reticulocytes

A. salina eIF-2	*A. salina* ESP	Reticulocyte lysate eIF-2	Reticulocyte lysate ESP	[35S]Met-tRNAi in ternary complex, pmol	Stimulation by ESP, -fold
2.4	—	—	—	0.08	—
2.4	1.6	—	—	0.16	2.0
2.4	3.2	—	—	0.18	2.2
2.4	—	—	1.2	0.14	1.7
2.4	—	—	2.4	0.16	1.9
—	—	2.8	—	0.06	—
—	1.6	2.8	—	0.16	2.7
—	3.2	2.8	—	0.18	3.0
—	4.8	2.8	—	0.20	3.3
—	—	2.8	1.2	0.18	3.0
—	—	2.8	2.4	0.33	5.5
—	—	2.8	3.6	0.45	7.5

Conditions of assay A with 2 pmol of [35S]Met-tRNAi (94,400 cpm/pmol) and the indicated amounts (μg/50 μl) of *A. salina* or reticulocyte eIF-2 and ESP. At the time these experiments were done the reticulocyte factors were about 1 month old, the *A. salina* factors about 3 months old.

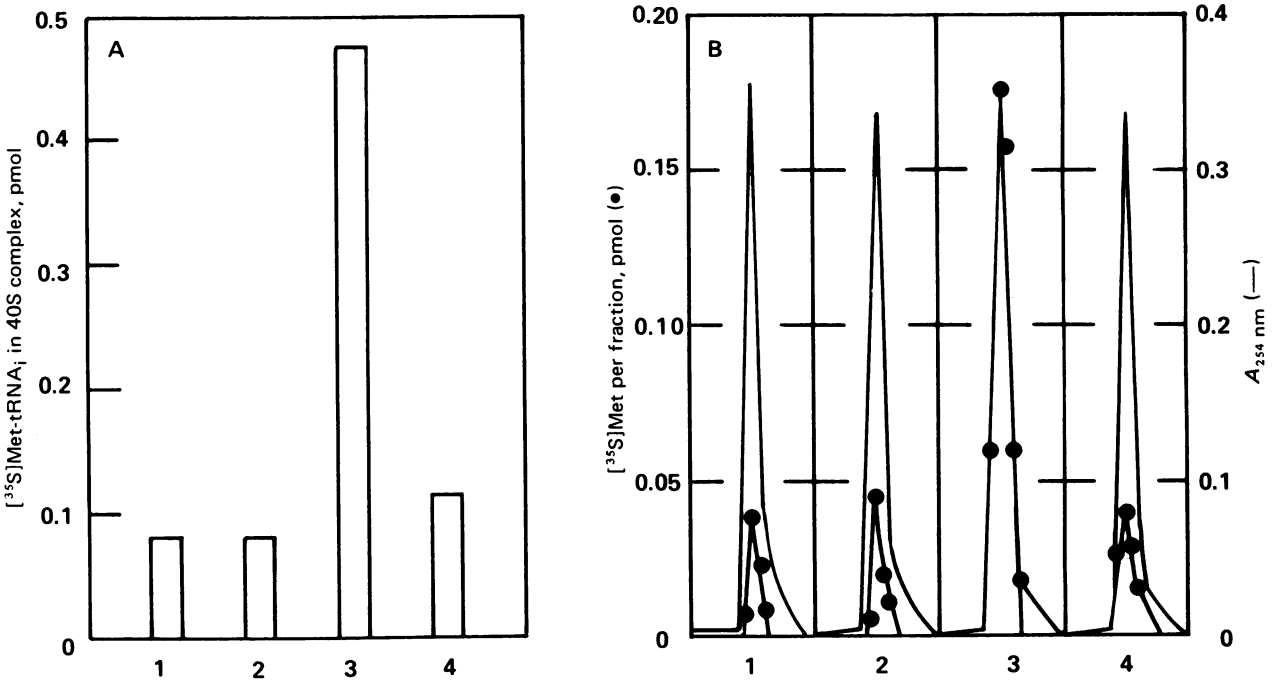

FIG. 3. Effect of ESP and eIF-2 kinase on 40S complex formation. (*A*) [^{35}S]Met-tRNA$_i$ bound in the 40S region of the gradient. (*B*) Sucrose density gradient centrifugation profiles. The experimental procedure is described in *Materials and Methods*. All samples had ATP and eIF-2. Other additions were as follows: 1, none; 2, eIF-2 kinase; 3, ESP; 4, eIF-2 kinase and ESP.

DISCUSSION

The results presented in this paper lend added weight to our earlier results (5) on the mechanism of inhibition of polypeptide chain initiation by the hemin-controlled translational inhibitor, eIF-2 kinase. Phosphorylation of the 38,000-dalton subunit of eIF-2, catalyzed by eIF-2 kinase (1–4), abolishes the action of ESP, a protein essential for eIF-2 activity at physiological concentrations of the factor. However, eIF-2 assays are usually carried out at high, unphysiological concentrations and, under these conditions, phosphorylated eIF-2 forms substantial amounts of ternary or 40S initiation complexes (5). This prompted the suggestion (8) that phosphorylation of eIF-2 might interfere with eIF-2 recycling, thus impairing its catalytic efficiency. This is unlikely in the light of recent evidence that phosphorylated eIF-2 enters the 40S complex and is released upon formation of the 80S initiation complex (i.e., it recycles) as well as the nonphosphorylated factor (9). However, much larger amounts of eIF-2 are required for translation when the factor is phosphorylated. Thus, through elimination of the stimulatory effect of ESP, phosphorylation sharply curtails the catalytic efficiency of eIF-2.

As pointed out earlier (5), the effect of ESP on ternary complex formation is similar to that of a ribosomal wash factor described by Dasgupta *et al.* (10). Whether the two factors are identical or not remains to be established.

A plausible model is that ESP complexes with intact, but not phosphorylated, eIF-2 and displaces the equilibrium of ternary complex formation in favor of this reaction (5).

We thank Christa Melcharick for excellent technical assistance.

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