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Protein synthesis in rabbit reticulocytes: Mechanism of protein synthesis inhibition by heme-regulated inhibitor*

(translational regulation/Met-tRNA$_f$ binding factor/eukaryotic initiation factors/protein phosphorylation)

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ABSTRACT **Partially purified Met-tRNA$_f$ binding factor, eIF-2, was phosphorylated by using heme-regulated inhibitor (HRI). Phosphorylated eIF-2 was freed from HRI by phosphocellulose column chromatography. Analysis by isoelectric focusing showed 100% phosphorylation of the 38,000-dalton subunit of eIF-2. Both eIF-2 and eIF-2(P) formed ternary complexes with Met-tRNA$_f$ and GTP with almost the same efficiency, and in both cases the ternary complex formation was drastically inhibited by prior addition of Mg^{2+}. However, whereas the ternary complexes formed with eIF-2 could be stimulated by Co-eIF-2C at 1 mM Mg^{2+} and dissociated by Co-eIF-2B at 5 mM Mg^{2+}, the ternary complexes formed with eIF-2(P) were unresponsive to both Co-eIF-2B and Co-eIF-2C. Also, under conditions of eIF-2 phosphorylation, HRI drastically inhibited AUG-dependent Met-tRNA$_f$ binding to 40S ribosomes. However, HRI (in the presence of ATP) had no effect on the joining of preformed Met-tRNA$_f$·40S·AUG complex to the 60S ribosomal subunit to form Met-tRNA$_f$·80S·AUG complex. These studies suggest that HRI inhibits protein synthesis initiation by phosphorylation of the 38,000-dalton subunit of eIF-2. HRI-phosphorylated eIF-2 does not interact with at least two other protein factors, Co-eIF-2B and Co-eIF-2C, and is thus inactive in protein synthesis initiation.**

During heme deficiency, protein synthesis in reticulocyte lysates is inhibited due to activation of a latent protein synthesis inhibitor, heme-regulated inhibitor (HRI) (7–9). HRI is a protein kinase that specifically phosphorylates the 38,000-dalton subunit of Met-tRNA$_f$ binding factor, eIF-2 (10–14). HRI also inhibits the following partial initiation reactions and in each case, the inhibition requires ATP: (*i*) Co-eIF-2B-promoted dissociation of Met-tRNA$_f$·eIF-2·GTP complex at high Mg^{2+} and low temperature (0°C) (TDF activity) (3, 15, 16); (*ii*) Co-eIF-2C stimulation of ternary complex formation at 1 mM Mg^{2+} (5, 16–18); and (*iii*) Met-tRNA$_f$ binding to 40S ribosomes (15, 16, 19). Based on these results, it has been proposed that HRI phosphorylation causes conformational modification of eIF-2 and that phosphorylated eIF-2 [eIF-2(P)] does not interact with two factors, Co-eIF-2B and Co-eIF-2C, and is inactive in peptide chain intiation. However, these studies do not conclusively establish the precise role of eIF-2(P) in the inhibition process and the possibility that the phosphorylation of other components such as HRI, Co-eIF-2B, and Co-eIF-2C also is involved in this inhibition has not been ruled out. Moreover, Trachsel and Staehelin (20) used prephosphorylated eIF-2 (HRI catalyzed) and observed that both eIF-2(P) and eIF-2 were equally active in all the partial initiation reactions studied including Met-tRNA$_f$ binding to 40S ribosomes. Also, the possibility that HRI can inhibit more than one step in peptide chain initiation has been indicated (21).

To clearly establish the mechanism of HRI inhibition of peptide chain initiation and the precise role of HRI-phosphorylated eIF-2 in this inhibition process, we prepared eIF-2(P) by using purified HRI and studied its interaction with Co-eIF-2B and Co-eIF-2C. Our results clearly establish that eIF-2(P) does not interact with Co-eIF-2B and Co-eIF-2C and that phosphorylation of other component(s) is not involved in this loss of interaction. Also, we report that HRI and ATP strongly inhibit Met-tRNA$_f$·40S·AUG complex formation but do not have any effect on subsequent joining of preformed Met-tRNA$_f$·40S·AUG complex with the 60S ribosomal subunit to form 80S initiation complex. These results thus suggest that inhibition of protein synthesis initiation by HRI is entirely due to phosphorylaton of eIF-2 and subsequent loss of interaction of eIF-2(P) with other initiation factors.

MATERIALS AND METHODS

Materials. The materials were obtained from the following sources: [^{35}S]methionine [900 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerels)] from Amersham/Searle; AUG from Miles Laboratories; phosphocellulose (P-11) from Whatman. [^{35}S]Methionine was diluted with unlabeled methionine to 10,000–20,000 cpm/pmol.

Initiation Factors, HRI, and Ribosomal Subunits. The peptide chain initiation factors eIF-2 and Co-eIF-2C were prepared from reticulocyte ribosomal salt (0.5 M KCl) wash. The crude ribosomal salt wash was further purified by using DEAE-cellulose chromatography and ammonium sulfate precipitation (fraction II) as described (22). The dialyzed fraction II preparation was then passed through a phosphocellulose column equilibrated with buffer A [20 mM Tris·HCl, pH 7.8/1 mM dithiothreitol/50 μM EDTA/10% (vol/vol) glycerol] containing 0.1 M KCl. The eIF-2 and Co-eIF-2C activities were adsorbed onto the column and were eluted by washing the column with buffer A containing 0.65 M KCl. The proteins were concentrated by ammonium sulfate (0–80% saturation) precipitation. The precipitate was dissolved in a minimal volume of buffer A containing 0.18 M KCl and the solution was dialyzed for 6–8 hr against the same buffer with one change. The dialyzed solution was then adsorbed onto a CM-Sephadex C-50 column previously equilibrated with buffer A containing 0.18 M KCl. Under these conditions, Co-eIF-2C

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Abbreviations: The present nomenclature for the peptide chain initiation factors and their past nomenclature (shown in parenthesis) (1) as once used by our laboratory are: eIF-2 (EIF-1), Met-tRNA$_f$ binding factor (2, 3); eIF-2(P), phosphorylated eIF-2 (HRI catalyzed); Co-eIF-2A (Co-EIF-1), a factor stimulating Met-tRNA$_f$ binding to eIF-2 (4); Co-eIF-2B (EIF-2, TDF), a factor promoting dissociation of ternary complex at high Mg^{2+} (3); Co-eIF-2C (EIF-2B), a factor reversing Mg^{2+} inhibition of ternary complex formation (5). HRI, heme-regulated translational inhibitor.

* This paper is no. 24 of a series. The previous paper in this series is ref. 6.

activity is not adsorbed onto the column and is eluted with the 0.18 M KCl wash of the column (17). The column wash was concentrated by using ammonium sulfate precipitation. The concentrated fraction was applied to a DEAE-cellulose column previously equilibrated with buffer A. The column was washed thoroughly with buffer A containing 0.1 M KCl. The adsorbed protein was eluted by using a linear KCl gradient (0.1–0.3 M KCl) in buffer A. The active fractions were pooled and concentrated by using another DEAE-cellulose column and one-step elution with buffer A containing 0.3 M KCl.

The eIF-2 activity was eluted from the CM-Sephadex column by using buffer A containing 0.4 M KCl. The active fractions were pooled, and the pooled fraction was diluted 1:4 with buffer A and was concentrated by passage through a small DEAE-cellulose column equilibrated with buffer A containing 0.1 M KCl and then one-step elution by using buffer A containing 0.3 M KCl.

Purification of Co-eIF-2A (4) and Co-eIF-2B (3) was as described. In some experiments, a partially purified initiation factor preparation, fraction II (23), was used.

Preparation of HRI and ribosomal subunits was as before (15, 23).

Preparation of eIF-2(P). A partially purified preparation of eIF-2 [CM-Sephadex purified preparation; specific activity, 1200–1500 (22)] was phosphorylated by using HRI. The reaction mixture (total volume, 0.8 ml) contained the following components: 20 mM Tris·HCl at pH 7.8, 100 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 2 mM dithiothreitol, 100 μg of eIF-2, and 30 μg of HRI. The reaction mixture was incubated at 37°C for 10 min and then was cooled in ice and applied to a small phosphocellulose column (bed volume, 1 ml) previously equilibrated with buffer A containing 0.3 M KCl. The column was thoroughly washed with the same buffer. eIF-2 activity was eluted from the column by using buffer A containing 0.65 M KCl. The protein concentrations of the active fractions were determined by the procedure of Lowry *et al.* (24) and the fractions were then mixed with bovine serum albumin (1 mg/ml) and stored in ice. The amounts of eIF-2 added in the experiment were calculated based on the original protein concentration of the solution before mixing with bovine serum albumin.

In the control experiment, the same amount of eIF-2 was treated identically except that HRI was omitted from the incubation mixture. eIF-2 activity was similarly purified by passage through an identical phosphocellulose column. This eIF-2 preparation was used as control nonphosphorylated eIF-2 in all the experiments.

Millipore Filtration Assays for Peptide Chain Initiation Factors. eIF-2 was assayed by using a Millipore filtration assay method for ternary complex [^{35}S]Met-tRNA$_f$·eIF-2·GTP formation as described (22, 25). The cofactors Co-eIF-2A, Co-eIF-2B, and Co-eIF-2C were added as indicated. The reaction mixtures (total volume, 0.075 ml) contained: 20 mM Tris·HCl at pH 7.8; 100 mM KCl; 2 mM dithiothreitol; 0.26 mM GTP; 10 μg of bovine serum albumin; 8–10 pmol of [^{35}S]Met-tRNA$_f$; eIF-2; and where indicated, Co-eIF-2A, Co-eIF-2B, Co-eIF-2C, and magnesium acetate. The reaction mixtures were incubated at 37°C for 5 min. At the end of incubation, 3 ml of cold wash buffer (20 mM Tris·HCl, pH 7.8/100 mM KCl) was added to stop the reaction. The reaction mixtures were then filtered through Millipore filters. The filters were washed twice with the same buffer, dried, and assayed for radioactivity.

In some experiments, the reactions were carried out in two or more stages. Details of the experimental procedures are described in the legends.

In several Millipore filtration experiments, we used energy-regenerating systems such as creatine phosphate plus creatine kinase and observed some stimulation (10–15%) of ternary complex formation. However, the characteristics of cofactor stimulation of eIF-2 activity were unchanged by such additions. The cofactor stimulation of eIF-2 activity reported in this paper is not due to reversal of GDP inhibition of ternary complex formation (26).

Isoelectric Focusing. Isoelectric focusing in polyacrylamide gels was performed according to the method of O'Farrell (27) as modified by Farrell *et al.* (28). The gels were stained by using the procedure described (29) for sodium dodecyl sulfate gels.

RESULTS

Partially purified eIF-2 was phosphorylated by using HRI and ATP, and the eIF-2(P) thus formed was further purified by phosphocellulose chromatography. Under the experimental conditions, HRI did not adsorb onto the column and was eluted with the wash buffer. eIF-2(P) was eluted by washing the column with high-salt solution.

The extent of phosphorylation of eIF-2 was analyzed by isoelectric focusing. Almost complete phosphorylation of the 38,000-dalton subunit of eIF-2 was observed (Fig. 1). The control experiment, without HRI, showed no phosphorylation of the 38,000-dalton subunit of eIF-2.

We compared the activities of eIF-2 and eIF-2(P) in different partial initiation reactions. Both eIF-2 and eIF-2(P) formed ternary complexes with almost equal efficiency, and in both cases the complex formation was dependent on the presence of GTP (Fig. 2). Also, both eIF-2 and eIF-2(P) responded similarly to addition of Co-eIF-2A and Mg^{2+} (Table 1); Co-eIF-2A stimulated ternary complex formation by both eIF-2 and eIF-2(P) (Exp. 1), and prior addition of Mg^{2+} inhibited ternary complex formation with both factors (Exp. 3). No significant

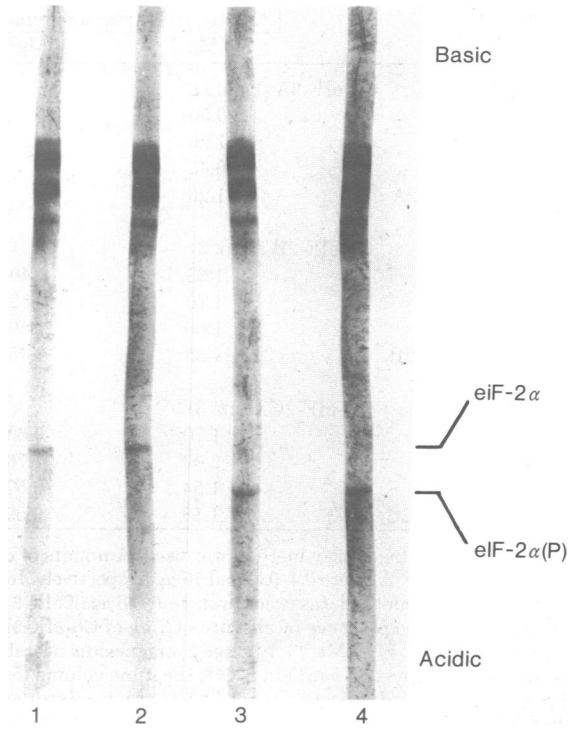

FIG. 1. Isoelectric focusing of eIF-2 and eIF-2(P). Lanes: 1 and 2, eIF-2, 1 and 1.4 μg; 3 and 4, eIF-2(P), 1.4 and 2 μg, respectively. α denotes the 38,000-dalton subunit of eIF-2. The large smeared band at the top of the gel is due to bovine serum albumin in the preparations.

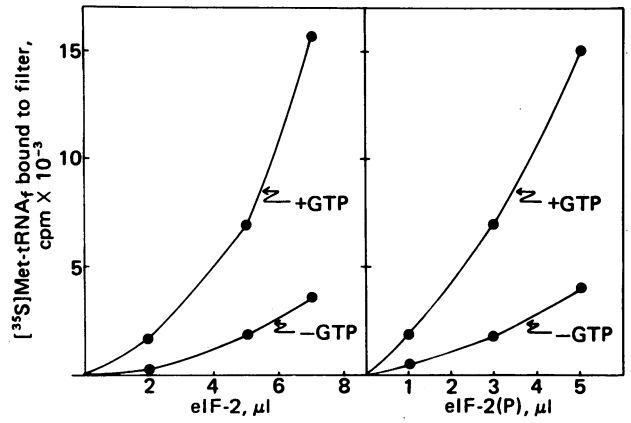

FIG. 2. GTP-dependent ternary complex formation by eIF-2 and eIF-2(P), assayed by the Millipore filtration assay method. eIF-2 and eIF-2(P) concentrations were 0.08 and 0.10 mg of protein per ml, respectively.

difference in Co-eIF-2A stimulation of ternary complex formation by eIF-2 or eIF-2(P) was observed when varying concentrations of Co-eIF-2A were used in these experiments. However, whereas the ternary complexes formed with eIF-2 could be dissociated by Co-eIF-2B at 5 mM Mg^{2+} and low temperature (TDF activity) (Exp. 2) and also could be stimulated by Co-eIF-2C at 1 mM Mg^{2+} (Exp. 3), the ternary complex formed with eIF-2(P) was not significantly affected by addition of Co-eIF-2B or Co-eIF-2C under similar experimental conditions. The effect of addition of different concentrations of Co-eIF-2C on ternary complex formation by eIF-2 and eIF-2(P) in the presence and absence of HRI is shown in Fig. 3. As expected, Co-eIF-2C stimulated ternary complex formation by eIF-2 and this stimulation was abolished by HRI in the presence of ATP. Under similar experimental conditions, formation of ternary complex with eIF-2(P) was neither stimulated by Co-eIF-2C nor inhibited by HRI.

Table 1. Effects of addition of Co-eIF-2A, Co-eIF-2B, and Co-eIF-2C on Met-tRNA$_f$ binding to eIF-2 and eIF-2(P)

Factors added	[35S]Met-tRNA$_f$ bound, pmol	
	No Mg^{2+}	+ Mg^{2+}
	Co-eIF-2A (Exp. 1)	
eIF-2	0.50	
eIF-2 + Co-eIF-2A	1.25	
eIF-2(P)	0.45	
eIF-2(P) + Co-eIF-2A	1.20	
	Co-eIF-2B (Exp. 2)	
eIF-2	1.35	1.40
eIF-2 + Co-eIF-2B	1.70	0.52
eIF-2(P)	1.66	1.50
eIF-2(P) + Co-eIF-2B	1.55	1.45
	Co-eIF-2C (Exp. 3)	
eIF-2	1.70	0.60
eIF-2 + Co-eIF-2C	2.03	1.70
eIF-2(P)	1.54	0.57
eIF-2(P) + Co-eIF-2C	1.58	0.59

In Exp. 1, a one-stage assay method was used. Amounts of eIF-2, eIF-2(P), and Co-eIF-2A were 0.4, 0.5, and 16 μg, respectively. In Exp. 2, a two-stage assay method was used. In stage 1, 0.8 μg of eIF-2 or 1.0 μg of eIF-2(P) in the presence or absence of 7 μg of Co-eIF-2B was incubated as in Exp. 1 (no Mg^{2+}). In stage 2, magnesium acetate was added to make the system 5 mM in Mg^{2+}; the same volume (5 μl) of water was added to duplicate tubes. The reaction mixtures were chilled in ice for 15 min. Reactions were terminated by addition of 3 ml of cold wash buffer (or wash buffer containing 5 mM Mg^{2+} for the tubes containing Mg^{2+}). In Exp. 3, a one-stage assay method was used. Amounts of eIF-2, eIF-2(P), and Co-eIF-2C were 0.8, 1.0, and 4 μg, respectively. Where indicated, 1 mM Mg^{2+} was present in the reaction mixture.

The above experiments were done with eIF-2 and prephosphorylated eIF-2 and no further additions of HRI and ATP were made during incubation. The results thus clearly establish that eIF-2(P) did not interact with Co-eIF-2B or Co-eIF-2C and no further phosphorylation of any other component or continued presence of HRI was necessary for such loss of interaction.

We have previously reported that at least two factor preparations, eIF-2 and Co-eIF-2B, are necessary for AUG-dependent Met-tRNA$_f$ binding to 40S ribosomes. The lack of interaction between eIF-2(P) and Co-eIF-2B could possibly explain HRI inhibition of Met-tRNA$_f$ binding to 40S ribosomes, in agreement with our previous hypothesis regarding the mechanism of HRI inhibition.

We also investigated the possibility that HRI may inhibit more than one step in peptide chain initiation reactions (21). We studied the effects of addition of HRI and ATP on the initial formation of Met-tRNA$_f$·40S·AUG complex and also on the subsequent joining step using preformed Met-tRNA$_f$·40S·AUG with the 60S ribosomal subunit. As expected, HRI strongly inhibited Met-tRNA$_f$·40S·AUG complex formation in the presence of ATP (Table 2). However, HRI and ATP had no significant effect on the subsequent joining of preformed Met-tRNA$_f$·40S·AUG with 60S ribosomes to form the 80S initiation complex.

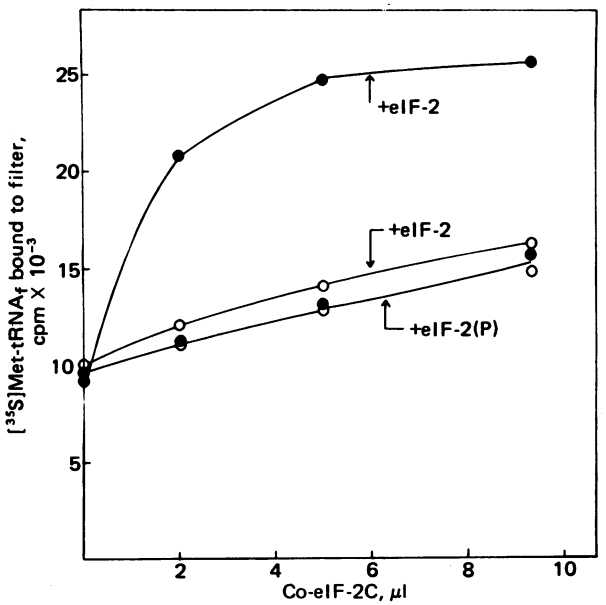

FIG. 3. Effect of HRI on Co-eIF-2C-dependent stimulation of ternary complex formation by eIF-2 and eIF-2(P). ○, with HRI; ●, without HRI. The standard two-stage Millipore filtration assay was used. In stage 1, the reaction mixtures (total volume, 0.05 ml) contained: 30 mM Tris·HCl at pH 7.8, 130 mM KCl, 10 μg of bovine serum albumin, 3 mM dithiothreitol, 1.5 mM magnesium acetate, 0.2 mM ATP, and 0.8 μg of eIF-2 or 1.0 μg of eIF-2(P) and Co-eIF-2C (protein concentration, 0.8 mg/ml). The reaction mixtures were incubated at 37°C for 5 min. In stage 2, the reaction mixtures were mixed with 0.01 ml of GTP (final concentration, 0.26 mM) and 0.015 ml of [35S]Met-tRNA$_f$ (8–10 pmol) and were incubated for another 5 min at 37°C. The reactions were terminated by addition of 3 ml of cold wash buffer.

Table 2. Effects of addition of HRI and ATP on [^{35}S]Met-tRNA$_f$·40S·AUG complex formation and joining of preformed [^{35}S]Met-tRNA$_f$·40S·AUG with 60S ribosomal particle to form [^{35}S]Met-tRNA$_f$·80S·AUG complex

Additions	Radioactivity, cpm Exp. 1 40S region	Exp. 2 80S region
None	2,740	890
AUG	12,850	7097
HRI + AUG	12,010	6069
ATP + AUG	10,920	6706
HRI + ATP + AUG	3,520	6034

40S and 80S initiation complexes were assayed by using the sucrose density gradient centrifugation method (23). *40S initiation complex formation*: The reaction mixtures (0.075 ml) in stage 1 contained: 20 mM Tris·HCl at pH 7.8, 100 mM KCl, 2 mM dithiothreitol, 10 μg of bovine serum albumin, 1 mM Mg^{2+}, 20 μg of fraction II initiation factors, and, where indicated, 0.5 μg of HRI and 0.2 mM ATP. The reaction mixtures were incubated at 37°C for 5 min. The reaction mixtures were placed in an ice bath and 0.26 mM GTP, 0.04 A_{280} unit of AUG, 0.16 A_{260} unit of 40S ribosomes, and 10–12 pmol of [^{35}S]-Met-tRNA$_f$ were added. At this stage, the volume of the reaction mixture was 0.14 ml and the composition was adjusted to 20 mM Tris·HCl at pH 7.8, 100 mM KCl, 2 mM dithiothreitol, and 2 mM Mg^{2+}. The reaction mixtures were incubated for an additional 5 min at 37°C. Then additional Mg^{2+} (0.01 ml) was added to make the final Mg^{2+} concentration 5 mM. After the mixture was incubated for an additional 10 min at 0°C, 0.1 ml was layered on top of a 14–27% linear sucrose density gradient (containing 20 mM Tris·HCl at pH 7.8, 100 mM KCl, 5 mM Mg^{2+}, and 2 mM dithiothreitol) and centrifuged at 45,000 rpm for 90 min in an SW 50.1 rotor. The gradients were fractionated by using an ISCO density gradient fractionator, and 7-drop fractions were collected into 3 ml of cold wash buffer containing 5 mM Mg^{2+}. The samples were filtered through Millipore filters; the filters were washed twice with 10 ml of the same buffer, dried, and assayed for radioactivity. *80S initiation complex formation*: Formation was carried out in four stages. In stage I, the 40S ribosome complex was formed; The reaction mixture (total volume, 0.125 ml) contained 24 mM Tris·HCl at pH 7.8, 100 mM KCl, 10 μg of albumin, 2.4 mM dithiothreitol, 2 mM Mg^{2+}, 0.26 mM GTP, 0.16 A_{260} unit of 40S ribosomes, 20 μg of fraction II initiation factor, 10–12 pmol of [^{35}S]Met-tRNA$_f$, and 0.04 A_{260} unit of AUG where indicated. The reaction mixture was incubated at 37°C for 5 min. In stage II, 0.5 μg of HRI and 0.2 mM ATP were added where indicated and the reaction mixtures were incubated at 37°C for 5 min. In stage III, 0.32 A_{260} unit of 60S ribosome was added and incubated for another 5 min at 37°C. In stage IV, additional Mg^{2+} was added to adjust the final Mg^{2+} concentration to 5 mM and the reaction mixtures were incubated in ice for 10 min. A 0.1-ml aliquot of this reaction mixture was layered on top of a sucrose density gradient and analyzed as described for the 40S complex formation assay.

DISCUSSION

The results presented in this paper define the precise locus of HRI inhibition. HRI phosphorylates the 38,000-dalton subunit of eIF-2. In this work, we provide evidence that eIF-2(P) does not interact with two other factors, Co-eIF-2B and Co-eIF-2C, and the further presence of HRI or additional phosphorylation of HRI or any other component(s) is not necessary for this loss of interaction.

Also, our results using partial initiation reactions and artificial mRNA (AUG codon) strongly suggest that HRI inhibition is limited to phosphorylation and subsequent inactivation of eIF-2 and that HRI has no further inhibitory effect on any other step in peptide chain initiation. Addition of HRI and ATP did not have any effect on the subsequent joining of preformed Met-tRNA$_f$·40S·AUG with 60S ribosomes to form the Met-tRNA$_f$·80S·AUG complex.

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1. Chatterjee, B., Dasgupta, A., Majumdar, A., Palmieri, S. & Gupta, N. K. (1979) *Methods Enzymol.* **60**, 256–265.
2. Anderson, W. F., Bosch, L., Cohn, W. E., Lodish, H., Merrick, W. C., Weissbach, H., Wittman, H. G. & Wool, I. G. (1977) *FEBS Lett.* **76**, 1–10.
3. Majumdar, A., Roy, R., Das, A., Dasgupta, A. & Gupta, N. K. (1977) *Biochem. Biophys. Res. Commun.* **78**, 161–169.
4. Dasgupta, A., Das, A., Roy, R., Ralston, R., Majumdar, A. & Gupta, N. K. (1978) *J. Biol. Chem.* **253**, 6054–6059.
5. Gupta, N. K., Das, A., Das, H., Ghosh-Dastidar, P., Palmieri, S., Ralston, R., Roy, R., Samiullah, Md. & Yaghmai, B. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 464.
6. Ralston, R. O., Das, A., Dasgupta, A., Roy, R., Palmieri, S. & Gupta, N. K. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4858–4862.
7. Rabinovitz, M., Freedman, M. L., Fisher, J. M. & Maxwell, C. R. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 567–578.
8. Howard, G. A., Adamson, S. D. & Herbert, E. (1970) *Biochim. Biophys. Acta* **213**, 237–240.
9. Hunt, T., Vanderhoff, G. & London, I. M. (1972) *J. Mol. Biol.* **66**, 471–481.
10. Ranu, R. S. & London, I. M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4349-4353.
11. Levin, D. H., Ranu, R. S., Ernst, V. & London, I. M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3112–3116.
12. Kramer, G., Cimadevilla, J. M. & Hardesty, B. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3078–3082.
13. Farrell, P. J., Balkow, J., Hunt, T., Jackson, R. J. & Trachsel, H. (1977) *Cell* **11**, 187–200.
14. Gross, M. & Mendelenski, J. (1977) *Biochem. Biophys. Res. Commun.* **74**, 559–569.
15. Das, A. & Gupta, N. K. (1977) *Biochem. Biophys. Res. Commun.* **78**, 1433–1441.
16. Ranu, R. S., London, I. M., Das, A., Dasgupta, A., Majumdar, A., Ralston, R., Roy, R. & Gupta, N. K. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 745–749.
17. deHaro, C., Datta, A. & Ochoa, S. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 243–247.
18. Ranu, R. S. & London, I. M. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1079–1083.
19. Kramer, G., Henderson, A. B., Pinphanichakarn, P., Walis, M. H. & Hardesty, B. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1445-1449.
20. Trachsel, H. & Staehelin, T. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 204–208.
21. Gross, M. (1979) *J. Biol. Chem.* **254**, 2370–2377.
22. Majumdar, A., Reynolds, S. & Gupta, N. K. (1975) *Biochem. Biophys. Res. Commun.* **67**, 689–695.
23. Chatterjee, B., Dasgupta, A., Palmieri, S. & Gupta, N. K. (1976) *J. Biol Chem.* **251**, 6379–6387.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
25. Majumdar, A., Dasgupta, A., Chatterjee, B., Das, H. K. & Gupta, N. K. (1979) *Methods Enzymol.* **60**, 35–52.
26. Benne, R., Amesz, H., Hershey, W. B. & Voorma, H. O. (1979) *J. Biol. Chem.* **254**, 3201–3205.
27. O'Farrell, P. (1975) *J. Biol. Chem.* **250**, 4007–4021.
28. Farrell, P., Hunt, T. & Jackson, R. (1978) *Eur. J. Biochem.* **89**, 517–521.
29. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.