PARTIAL REACTION OF PEPTIDE INITIATION INHIBITED BY THE RETICULOCYTE HEMIN-CONTROLLED REPRESSOR

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The hemin-controlled repressor from rabbit reticulocytes inhibits binding of Met-tRNA$_f$ to reticulocyte 40S ribosomal subunits in a partial reaction containing these components, two initiation factor fractions and GTP. The inhibitor does not interfere with the formation of the Met-tRNA$_f$·initiation factor IF-E$_2$·GTP complex.

A translational inhibitor, the so-called hemin-controlled repressor (HCR)*, is formed in reticulocytes or their cell-free lysates under conditions of hemin deficiency (1-3). This inhibitor blocks peptide chain initiation. A decrease of [^{35}S]Met-tRNA$_f$ bound to 40S subunits was observed in lysates incubated in the absence of hemin (4,5). The inhibition of protein synthesis caused by HCR in reticulocyte lysates can be overcome by the addition of the initiation factor that forms a ternary complex with Met-tRNA$_f$ and GTP (6), IF-E$_2$ by Staehelin's nomenclature (7). It has been suggested (8) and demonstrated (9) that highly purified HCR contains a protein kinase that phosphorylates the smallest subunit of IF-E$_2$. This kinase activity is not affected by cAMP.

These observations implicate IF-E$_2$ dependent reactions in the mechanism of HCR inhibition. IF-E$_2$ functions in peptide initiation by formation of a Met-tRNA$_f$·IF-E$_2$·GTP ternary complex (10) which can be measured by its binding to Millipore filters. With additional initiation factors this ternary complex mediates binding of Met-tRNA$_f$ to 40S ribosomal subunits (10). Messenger RNA is bound to this 40S ribosomal initiation complex in subsequent reactions in which other initiation factors are involved (11).

Here we demonstrate that HCR activity can be monitored using purified components in a peptide initiation partial reaction system. Direct evidence is

*Abbreviation: HCR, hemin-controlled repressor.

provided that HCR does not prevent formation of the Met-tRNA$_f$·IF-E$_2$·GTP complex but that it does inhibit IF-E$_2$-dependent binding of Met-tRNA$_f$ to reticulocyte 40S ribosomal subunits.

METHODS

Preparation of HCR.

HCR was prepared from the postribosomal supernatant of rabbit reticulocytes by the procedure described by Gross and Rabinovitz (3) as modified by Kramer et al. (9). The preparation used in this study was purified by successive chromatography on DEAE cellulose, hydroxylapatite and phosphocellulose. The resulting HCR preparation gave 50% inhibition of protein synthesis in a reticulocyte lysate at a concentration of about 11 µg of protein/ml of reaction mixture.

Preparation of Components for the Formation of the 40S Initiation Complex.

Fractionation of the reticulocyte 0.5 M KCl ribosomal wash to prepare FI, FII and DE$_{100}$ fractions (12) and the purification of IF-E$_2$ (9) have been described. Reticulocyte ribosomal subunits were prepared by the procedure described by Falvey and Staehelin (13) as modified by Obrig et al. (14), except that the subunits were collected by centrifugation and suspended in a solution containing 20 mM Tris-HCl, pH 7.5; 8 mM $MgCl_2$; 0.15 M KCl. Deacylated yeast tRNA was charged with [^{35}S] methionine (Schwarz/Mann, Orangeburg, N.Y.) as described by Culp et al. (15). Met-tRNA$_f$ was separated from Met-tRNA$_m$ by chromatography on benzoylated DEAE cellulose (16).

Formation of the Met-tRNA$_f$·IF-E$_2$·GTP Complex.

Formation of the ternary complex between Met-tRNA$_f$, GTP and IF-E$_2$ was assayed by rentention on nitrocellulose filters (0.45-µm pore size, type HAWG, Millipore Corporation, Bedford, MA.). Reaction mixtures contained the following components in a final volume of 100 µl: 40 mM Tris-HCl, pH 7.5; 0.1 M KCl; 0.8 mM GTP; 1 mM DTE, 5 µg IF-E$_2$ and 14 pmol of [^{35}S]Met-tRNA$_f$ (2-4 Ci/mmol). They were incubated at 37° for 5 min, then diluted with 2 ml of ice-cold buffer containing 40 mM Tris-HCl, pH 7.5, plus 0.1 M KCl. These samples were passed through Millipore filters, which were then washed three times with 5 ml each of the same buffer. The filters were dried and counted in 10 ml of a counting fluid containing 5 g of 2, 5-diphenyloxazole per liter of toluene.

Binding of [^{35}S]Met-tRNA$_f$ to Reticulocyte 40S Ribosomal Subunits.

The reaction mixtures contained in a final volume of 100 µl: 20 mM Tris-HCl, pH 7.5; 4 mM $MgCl_2$; 85 mM KCl; 2.5 mM DTE; 0.2 mM GTP; 75 µM spermine; 2.1 µg of IF-E$_2$; 38 µg of FII; 0.2 A_{260} units of reticulocyte 40S subunits and 8 pmol of [^{35}S]Met-tRNA$_f$ (2-4 Ci/mmol). The reaction mixtures were incubated at 34° for 5 min, then at 0° for 5 min. Met-tRNAf bound to 40S subunits was analyzed by two methods:

a) Millipore filtration. The incubation mixtures were diluted with 2 ml of ice-cold buffer containing 20 mM Tris-HCl, pH 7.5; 4 mM $MgCl_2$; 85 mM KCl, then filtered through Millipore filters as described above. The dried filters were counted by liquid scintillation.

b) Sucrose density gradient centrifugation. The reactions were carried out as described above except that the incubation mixtures were enlarged to 150 µl. Each sample (140 µl) was layered on a linear 15-30% (w/v) sucrose gradient containing 20 mM Tris-HCl, pH 7.5; 4 mM $MgCl_2$; 85 mM KCl. The gradients were centrifuged at 56,000 rpm for 1 hour in a Spinco SW 60 Ti rotor. Fractions of about 250-µl were collected and filtered through Millipore filters as described above.

TABLE I

Effect of HCR on the Formation of the Met-tRNA$_f$·IF-E$_2$·GTP Complex

Additions	[^{35}S]Met-tRNA$_f$ bound to Filters (pmol)
Complete	4.47
+HCR	4.48
-GTP	0.38
-IF-E$_2$	0.03

Formation of the ternary complex between Met-tRNA$_f$, IF-E$_2$ and GTP was assayed as described under Methods. Where indicated 2.8 µg protein of the HCR fraction was included in the reaction mixture.

RESULTS

Rabinovitz and co-workers (17) reported that HCR did not block the retention of Met-tRNA$_f$ to Millipore filters in the presence of GTP and 0.5 M KCl ribosomal salt wash fraction. The data presented in Table I demonstrate that HCR does not inhibit the formation of the Met-tRNA$_f$·IF-E$_2$·GTP ternary complex formed with highly purified IF-E$_2$. Reaction mixtures containing the indicated components were incubated, then filtered through Millipore filters as described under Methods. Met-tRNA$_f$ is retained on the filter in the presence of IF-E$_2$ and GTP. The amount of IF-E$_2$ used in these experiments is in the linear range for IF-E$_2$ dependent retention of Met-tRNA$_f$ on the filters.

Gupta and co-workers (18) described a method for measuring IF-E$_2$-mediated binding of Met-tRNA$_f$ to 40S ribosomal subunits by filtration through Millipore filters. Their assay system is based on the observation that the Met-tRNA$_f$·IF-E$_2$·GTP complex is unstable at 0° in the presence of 4 mM $MgCl_2$, whereas Met-tRNA$_f$ remains bound to 40S ribosomal subunits under the conditions used. This assay was altered by Spremulli *et al.* (19) and finally modified for use

TABLE II

Requirements for Binding of Met-tRNA$_f$ to 40S Ribosomal Subunits

Initiation Factor Fractions	Reticulocyte 40S Subunits	[^{35}S]Met-tRNA$_f$ bound to Filters (pmol)	[^{35}S]Met-tRNA$_f$ bound to 40S (pmol)
IF-E$_2$	-	0.25	-
IF-E$_2$	+	0.28	0.03
IF-E$_2$ + FII	-	0.85	-
IF-E$_2$ + FII	+	1.56	0.71

The reaction mixtures contained the components specified under Methods. Bound Met-tRNA$_f$ was measured by Millipore filtration. FII and/or 40S subunits were omitted where indicated. Met-tRNA$_f$ bound to 40S ribosomal subunits was calculated as the difference between samples containing and lacking 40S subunits.

TABLE III

HCR Inhibition of Met-tRNA$_f$ Binding to Reticulocyte 40S Subunits as Assayed by Millipore Filtration

Additions	[^{35}S]Met-tRNA$_f$ bound to 40S (pmol)
None	1.30
HCR	0.38

Binding of Met-tRNA$_f$ to 40S subunits was assayed by the Millipore filtration procedure as outlined under Methods. Where indicated reaction mixtures contained 2.8 µg protein of the HCR fraction. A blank without 40S subunits gave 0.44 pmol. This value was subtracted.

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in the experiments described here as detailed under Methods. As determined with this system the requirements for binding of [^{35}S]Met-tRNA$_f$ to reticulocyte 40S ribosomal subunits are shown in Table II. Another initiation factor fraction, FII (12), in addition to IF-E$_2$ is required for stable binding of Met-tRNA$_f$ to reticulocyte 40S ribosomal subunits. In the absence of FII, IF-E$_2$ over a concentration range from 1 to 10 µg does not promote 40S subunit-dependent re-

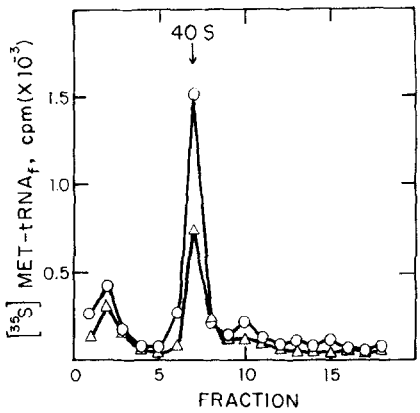

Figure 1. HCR Inhibition of Met-tRNA$_f$ Binding to Reticulocyte 40S Subunits as Analyzed by Sucrose Gradients. The incubation and analysis of Met-tRNA$_f$ bound to 40S subunits was carried out as described under Methods. O-O = control; Δ——Δ = with HCR (4.2 μg protein/150-μl reaction mixture).

tention of Met-tRNA$_f$ on the filters. This binding of [^{35}S]Met-tRNA$_f$ to reticulocyte 40S subunits is strongly reduced when HCR is included in the reaction mixture as shown in Table III. The amount of HCR used in these experiments is two to three times that required for 50 per cent inhibition of protein synthesis in a reticulocyte lysate containing similar amounts of 40S ribosomal subunits. This difference in sensitivity to HCR may be related to differences in the amount of IF-E$_2$ in the two systems.

Equivalent results can be demonstrated using sucrose gradient centrifugation, as shown in Figure 1. For these experiments reaction mixtures identical to those used in the experiment of Table III were layered directly on sucrose gradients containing 4 mM $MgCl_2$. Fractions were filtered through Millipore filters. In the absence of HCR, Met-tRNA$_f$ sediments with 40S ribosomal subunits. The amount of Met-tRNA$_f$ bound to 40S subunits is reduced when HCR is added to the reaction mixture.

Table IV presents evidence that the decrease in [^{35}S] radioactivity observed in the presence of HCR is not caused by Met-tRNA hydrolase which might contaminate the HCR preparation. Met-tRNA hydrolase promotes the rapid hydrolysis of

TABLE IV

Effect of HCR on Preformed Met-tRNA$_f$·40S Complex

Additions	[^{35}S]Met-tRNA$_f$ bound to 40S (pmol)
None	0.78
HCR	0.84

Met-tRNA$_f$ was bound to reticulocyte 40S subunits as described under Methods. Then 2.8 µg HCR was added where indicated and the reaction mixtures incubated for another 5 minutes at 37°. [^{35}S] radioactivity retained on Millipore filters was determined. A background value of 0.52 pmol for incubation mixtures without 40S subunits was subtracted.

Met-tRNA$_f$ bound to 40S ribosomal subunits (20, 21). Addition of HCR to reaction mixtures after the Met-tRNA$_f$·40S complex is formed, does not reduce the amount of [^{35}S]methionine bound to 40S subunits. We conclude from these data that HCR inhibits binding of the Met-tRNA$_f$·IF-E$_2$·GTP complex to reticulocyte 40S ribosomal subunits.

DISCUSSION

In a recent paper (9) we presented evidence demonstrating that preparations of highly purified HCR contain cAMP-independent kinase activity for phosphorylation of the smallest subunit of IF-E$_2$ and several proteins associated with 0.5 M KCl-washed reticulocyte 40S ribosomal subunits. Anti-HCR IgG caused a parallel decrease in these kinase activities and HCR inhibition of protein synthesis in a reticulocyte lysate. Our unpublished results indicate that both IF-E$_2$ and 40S ribosomal subunits are phosphorylated under conditions of the assay system used in the experiments presented here. It is not clear whether this involves GTP or contaminating ATP. In either event, the experiments described here provide a direct demonstration that the Met-tRNAf·IF-E$_2$·GTP complex formed in the presence of HCR cannot be bound efficiently to 40S ribosomal subunits.

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