

# Regulation of protein synthesis in rabbit reticulocyte lysates

## Thiophosphorylation of initiation factor eIF-2 by heme-regulated protein kinase

Rajinder Singh Ranu

*Department of Microbiology, Colorado State University, Fort Collins, CO 80523, USA*

Received 6 August 1986; revised version received 25 August 1986

The heme-regulated protein kinase, which specifically phosphorylates the 38-kDa subunit of initiation factor eIF-2, can utilize adenosine 5'-O-(3-thiotriphosphate) (ATP[ $\gamma$ S]) as a substrate. The rate of thiophosphorylation is 5–6-times slower than that observed with ATP. It is of special interest that thiophosphorylated derivatives of eIF-2 are resistant to dephosphorylation catalyzed by eIF-2 phosphoprotein phosphatase. The thiophosphorylated eIF-2 is less effective in promoting protein synthesis in hemin-deficient lysates under physiological conditions. In addition, ATP[ $\gamma$ S] could also be utilized by the self-phosphorylation activity intrinsically associated with HRI.

*Heme-regulated protein kinase      Initiation factor eIF-2      Protein synthesis      Phosphorylation      Thiophosphorylation*

### 1. INTRODUCTION

Protein synthesis in rabbit reticulocytes and their lysates is regulated by heme (reviews in [1–3]). In heme deficiency a heme-regulated translational inhibitor (HRI) is activated that blocks protein chain initiation. The second inhibitor present in reticulocytes is activated by low concentrations of double-stranded RNA [4]. The HRI and double-stranded RNA activated inhibitors have been identified as adenosine 3':5'-cyclic monophosphate-independent protein kinases (eIF-2 protein kinases), which specifically phosphorylate the same site on the 38-kDa subunit ( $\alpha$ -subunit) of initiation factor eIF-2 [5–14]. As a consequence, catalytic reutilization of eIF-2 is inhibited [15–24]. The eIF-2 promotes formation of the ternary complex (eIF-2·GTP·Met-tRNA<sub>f</sub>) with GTP and initiator tRNA, Met-tRNA<sub>f</sub>. This is the first rate-limiting reaction in the protein synthesis initiation cycle.

The eIF-2 phosphorylation by HRI can be detected readily in vitro in a purified system

[5–7,25]. The dephosphorylation reaction in situ in lysates, however, restricts reaction conditions to short-term incubations [26]. There are major difficulties in analyzing reactions in complex systems because of the near impossibility of dissociating the two competing events, namely phosphorylation and dephosphorylation under physiological conditions. Cyclic AMP-dependent protein kinases that phosphorylate a wide variety of substrates have been shown to utilize adenosine 5'-[thio]triphosphate (ATP-[ $\gamma$ S] or ATP[S]) in phosphorylation reactions [27,28]. Thiophosphorylated proteins in these systems have been shown to be metabolically stable [27,28]. Unlike cyclic AMP-dependent protein kinases, which phosphorylate a wide spectrum of substrates, HRI catalyzed phosphorylation is highly specific and is restricted to eIF-2 [5–8]. Considerations of these facts led me to investigate whether ATP[ $\gamma$ -S] can be used as a substrate by HRI. Here I report that ATP[ $\gamma$ S] is an effective substrate for the HRI catalyzed thiophosphorylation of eIF-2 and that this thio derivative of eIF-2 is resistant to dephosphorylation catalyzed by eIF-2 phosphoprotein phosphatase.

## 2. EXPERIMENTAL

The following procedures have been described: preparation of rabbit reticulocyte lysates, protein synthesis reaction mixtures, assay of protein synthesis, preparation of purified HRI, SDS-polyacrylamide gel electrophoresis, autoradiography of polyacrylamide gel, and preparation of purified eIF-2 [25].

### 2.1. Thiophosphorylation assay

Phosphorylation was carried out in a reaction mixture (10  $\mu$ l) containing 20 mM Tris-HCl (pH 7.6), 60 mM KCl, 10 mM magnesium acetate, 1 mM DTT, eIF-2 (0.5–1  $\mu$ g), HRI (0.01–0.05  $\mu$ g) and 0.2 mM [ $\gamma$ - $^{32}$ S]ATP[S] (spec. act. 9000 cpm/pmol) or [ $\gamma$ - $^{32}$ P]ATP (spec. act. 4000 cpm/pmol). After incubation at 30°C, the reaction was terminated by the addition of denaturing solution (50 mM Tris-HCl, pH 7.8; 1% SDS, 5%  $\beta$ -mercaptoethanol) and heated at 100°C for several minutes. Samples were applied to an SDS-polyacrylamide gel (10%) and electrophoresed at 100 V for 4.5 h. The proteins in the gel were stained with Coomassie brilliant blue. The gel containing  $^{35}$ S-labelled polypeptides was soaked in a solution of Fluoro-Hance (Research Products, International Corp.) enhancer for 30 min. The gel was then dried and autoradiographed.

### 2.2. Dephosphorylation of eIF-2

In situ dephosphorylation was carried out in lysates under conditions of protein synthesis [25,26]. Aliquots (38  $\mu$ l) of lysate protein synthesis mixture were incubated at 30°C with either [ $^{32}$ P]eIF-2 or [ $^{35}$ S]eIF-2. At intervals, aliquots (6  $\mu$ l) were removed and subjected to SDS-polyacrylamide gel electrophoresis to separate the polypeptides. The remaining details of treatment of the gel for autoradiography are described in section 2.1.

### 2.3. Materials

Adenosine 5'-O-(3-thiotriphosphate) (ATP[ $\gamma$ S]) was obtained from Boehringer Mannheim (Indianapolis, IN); [ $\gamma$ - $^{32}$ S]ATP[S] (spec. act. 61 Ci/mmol) and [ $\gamma$ - $^{32}$ P]ATP (spec. act. 2000 Ci/mmol) were from New England Nuclear (Boston, MA). The sources of other materials have been described [25].

## 3. RESULTS AND DISCUSSION

### 3.1. Thiophosphorylation of eIF-2

Incubation of eIF-2 with [ $\gamma$ - $^{35}$ S]ATP[S] and HRI resulted in thiophosphorylation of the 38-kDa subunit of eIF-2 (fig.1, lane 3). In addition, self-thiophosphorylation of HRI was observed (fig.1, lane 1) and the minute amount of casein protein kinase [30], present in the eIF-2 preparation, thiophosphorylated the 50-kDa subunit of eIF-2 as

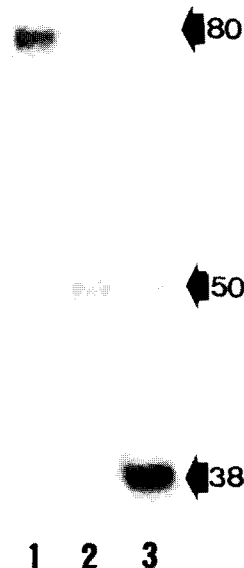


Fig.1. Thiophosphorylation of eIF-2. The reaction mixture (10  $\mu$ l) containing 20 mM Tris-HCl (pH 7.6), 60 mM KCl, 10 mM magnesium acetate, 1 mM DTT, 0.2 mM [ $\gamma$ - $^{35}$ S]ATP[S] (9000 cpm/pmol), 0.5  $\mu$ g eIF-2 and 0.05  $\mu$ g HRI, was incubated at 30°C for 40 min. The reaction was terminated by the addition of denaturing solution (50 mM Tris-HCl, pH 7.8; 1% SDS, 5%  $\beta$ -mercaptoethanol). Samples were heated at 100°C and then subjected to electrophoresis in a 10% polyacrylamide gel. Protein in the gel was stained and then autoradiographed, after being soaked for 30 min in enhancing solution. Lanes: 1, HRI; 2, eIF-2; 3, eIF-2 and HRI. Positions of 38-kDa, 50-kDa and HRI (80-kDa) bands are indicated.

well. These findings suggest that not only ATP[ $\gamma$ S] acts as a substrate for thiophosphorylation of the 38-kDa subunit of eIF-2, but that it could be utilized as a substrate by the self-phosphorylation activity associated with HRI [29] and by casein protein kinase [30].

The kinetics of phosphorylation and thiophosphorylation reactions are shown in fig.2A,B. In the presence of [ $\gamma$ - $^{35}$ S]ATP[S], incorporation of  $^{35}$ S increased progressively over a period of 160 min with a half-maximum of 90 min. In contrast, incorporation of  $^{32}$ P from [ $\gamma$ - $^{32}$ P]ATP leveled off rapidly after 40 min with a half-maximum of 16 min (fig.3). With ATP[ $\gamma$ S], a lag period of about 8–10 min is observed before significant incorporation of  $^{35}$ S takes place. The

data suggest that the initial rate of the thiophosphorylation reaction is 5–6-times slower than that observed with ATP, but the extent of the reaction is nearly the same. The  $K_m$  of HRI for ATP[ $\gamma$ S] is 4-times higher (12.5  $\mu$ M) than for ATP (not shown).

### 3.2. Susceptibility of thiophosphorylated and phosphorylated eIF-2 to eIF-2 phosphoprotein phosphatase

The results in fig.4 show that when phosphorylated eIF-2 is added to lysates under conditions of protein synthesis, eIF-2 is dephosphorylated rapidly (fig.4A). In contrast, thiophosphorylated eIF-2 is completely resistant to

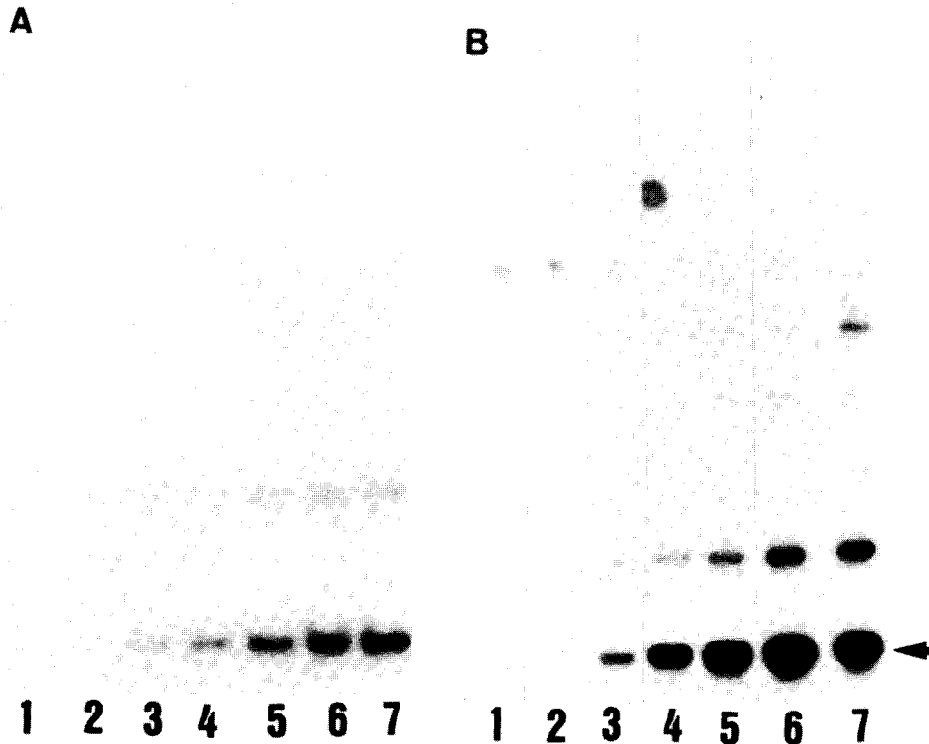


Fig.2. Kinetics of phosphorylation and thiophosphorylation. The reaction mixture (32  $\mu$ l) was prepared similarly to that described in fig.1 with the addition of 0.2 mM [ $\gamma$ - $^{35}$ S]ATP[S] (9000 cpm/pmol) or 0.2 mM [ $\gamma$ - $^{32}$ P]ATP (5000 cpm/pmol), 8  $\mu$ g eIF-2 and 0.25  $\mu$ g HRI. Samples were incubated at 30°C, at intervals aliquots (4  $\mu$ l) were removed, and phosphorylation and thiophosphorylation of eIF-2 were assayed as described in fig.1. (A) Lanes 1–7, samples incubated with [ $\gamma$ - $^{32}$ P]ATP for 0.5, 1, 5, 10, 20, 40 and 80 min; (B) lanes 1–7, samples incubated with [ $\gamma$ - $^{35}$ S]ATP[S] for 5, 10, 20, 40, 80, 120 and 160 min. The autoradiogram of  $^{32}$ P-labelled samples is underexposed deliberately; otherwise the bands for samples 3–7 appear as dark black bands. Significant incorporation of  $^{32}$ P takes place in 0.5 and 1 min of incubation. Data are presented in fig.3.

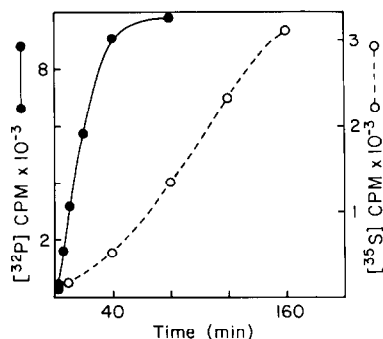


Fig. 3. Rate of phosphorylation and thiophosphorylation of eIF-2. Data are derived from fig.2. The 38-kDa polypeptides were cut out from the gel and the associated radioactivity was measured.

dephosphorylation under otherwise identical conditions (fig.4B).

The metabolic stability of thiophosphorylated derivative of eIF-2 provides a useful tool to study the effect of eIF-2 phosphorylation under physiological conditions. Therefore, the activity of thiophosphorylated eIF-2 on protein synthesis in hemin-deficient lysates under physiological conditions was examined. Consistent with earlier data [2], the control and phosphorylated eIF-2 showed little difference in promoting protein synthesis (table 1). The thiophosphorylated eIF-2 was, however, much less effective in maintaining protein synthesis (table 1).

We have observed that the heme-regulated protein kinase, which specifically phosphorylates the

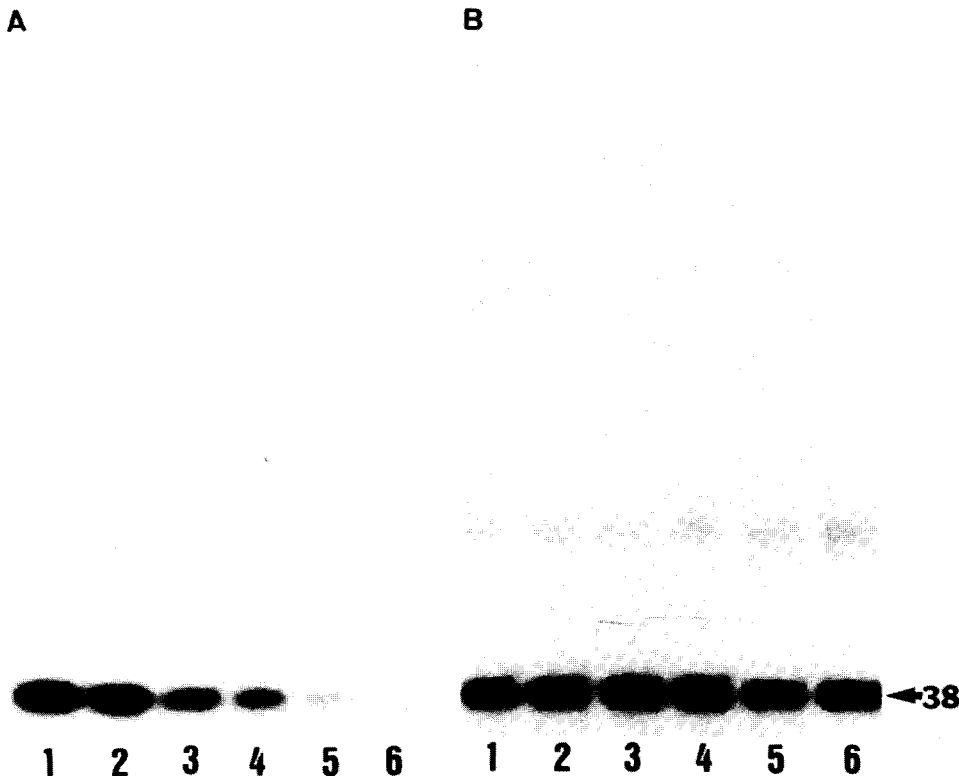


Fig.4. Dephosphorylation of phosphorylated and thiophosphorylated eIF-2. Protein synthesis lysate reaction mixtures (38  $\mu$ l) were prepared and incubated with  $^{32}$ P- or  $^{35}$ S-labelled eIF-2 at 30°C. At intervals, aliquots (6  $\mu$ l) were removed, denatured and subjected to electrophoresis in SDS-polyacrylamide gel (10%). The gels were then autoradiographed. (A) Lanes 1-6, sample of [ $^{32}$ P]eIF-2 incubated for 0, 1, 5, 10, 20 and 30 min; (B) lanes 1-6, samples of [ $^{35}$ S]eIF-2 incubated for 0, 1, 5, 10, 20 and 30 min.

Table 1

Effect of thiophosphorylation on the activity of eIF-2 in hemin-deficient lysates

Sample	Protein synthesis (cpm)
Minus hemin lysate	5780
Minus hemin lysate + eIF-2	14711
Minus hemin lysate + [P]eIF-2	14302
Minus hemin lysate + [S]eIF-2	8509

eIF-2 (10  $\mu$ g) was phosphorylated or thiophosphorylated as described in the legend to fig.2 for 4 h. Lysate protein synthesis reaction mixtures without hemin (40  $\mu$ l) were incubated without or with 1  $\mu$ g eIF-2 or phosphorylated eIF-2 or thiophosphorylated eIF-2 at 30°C for 40 min. Aliquots (5  $\mu$ l) were removed and assayed for protein synthesis [25]

38-kDa subunit of eIF-2, is also capable of transferring the  $\gamma$ -thiophosphoryl group from ATP[ $\gamma$ S] to its substrate. However, the rate of the reaction is substantially slower. This finding suggests that affinity of the enzyme for ATP[ $\gamma$ S] is much lower than for ATP; this is consistent with  $K_m$  measurements. The thiophosphate derivatives of eIF-2 are completely resistant to dephosphorylation catalyzed by eIF-2 phosphoprotein phosphatase in situ under conditions which dephosphorylate phosphorylated eIF-2 completely. This stability of thiophosphorylated eIF-2 derivatives is also supported by the limited promotion of protein synthesis in heme-deficient lysates by thiophosphorylated eIF-2.

In addition, unexpectedly we observed that heme-regulated protein kinase can also utilize ATP[ $\gamma$ S] for self-phosphorylation and that the casein protein kinase [30], often found as a contaminant in eIF-2 preparations, can transfer the thiophosphoryl group from ATP[ $\gamma$ S] to the 50-kDa subunit of eIF-2. The physiological significance, if any, of this latter phosphorylation is not known.

#### ACKNOWLEDGEMENT

This work was supported by grant NSF DMB 8302915.

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