Regulation of protein synthesis in rabbit reticulocyte lysates

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

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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[γS]) 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[γS] 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 (α-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$_f$) with GTP and initiator tRNA, Met-tRNA$_f$. 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–[γS] 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[γ-S] can be used as a substrate by HRI. Here I report that ATP[γS] 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.

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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 μl) containing 20 mM Tris-HCl (pH 7.6), 60 mM KCl, 10 mM magnesium acetate, 1 mM DTT, eIF-2 (0.5–1 μg), HRI (0.01–0.05 μg) and 0.2 mM [γ-^{32}S]ATP[S] (spec. act. 9000 cpm/pmol) or [γ-^{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% β-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 μl) 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 μl) 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[γS]) was obtained from Boehringer Mannheim (Indianapolis, IN); [γ-^{32}S]ATP[S] (spec. act. 61 Ci/mmol) and [γ-^{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 [γ-^{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

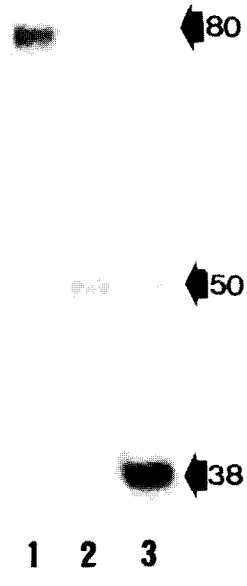

Fig.1. Thiophosphorylation of eIF-2. The reaction mixture (10 μl) containing 20 mM Tris-HCl (pH 7.6), 60 mM KCl, 10 mM magnesium acetate, 1 mM DTT, 0.2 mM [γ-^{35}S]ATP[S] (9000 cpm/pmol), 0.5 μg eIF-2 and 0.05 μg HRI, was incubated at 30°C for 40 min. The reaction was terminated by the addition of denaturing solution (50 mM Tris-HCl, pH 7.8; 1% SDS, 5% β-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[γS] 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 [γ-^{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 [γ-^{32}P]ATP leveled off rapidly after 40 min with a half-maximum of 16 min (fig.3). With ATP[γS], 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[γS] is 4-times higher (12.5 μM) 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

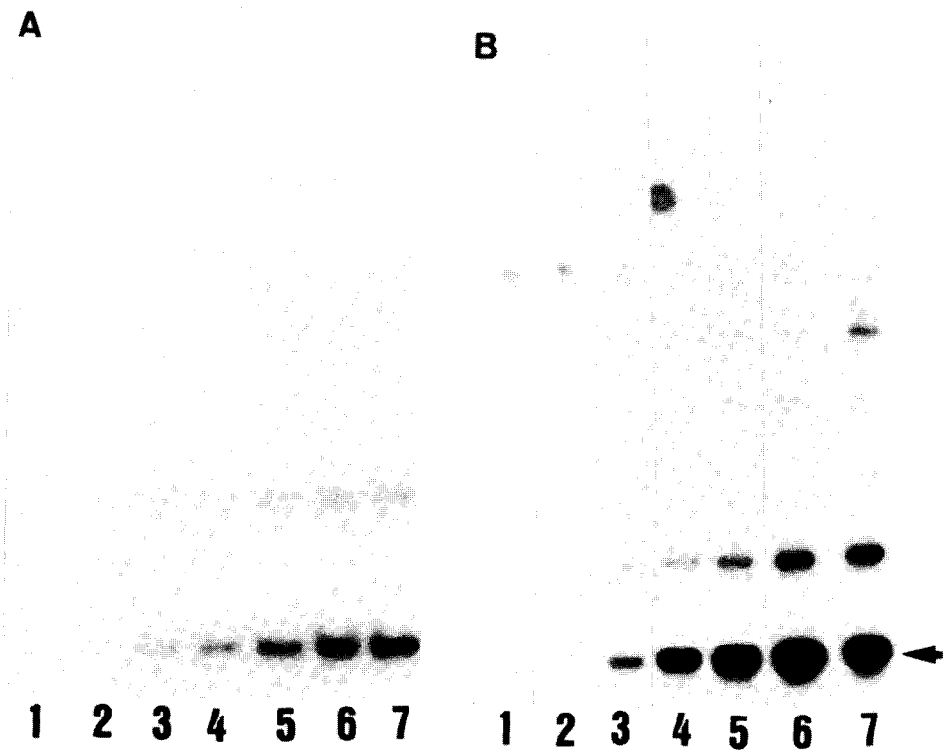

Fig.2. Kinetics of phosphorylation and thiophosphorylation. The reaction mixture (32 μl) was prepared similarly to that described in fig.1 with the addition of 0.2 mM [γ-^{35}S]ATP[S] (9000 cpm/pmol) or 0.2 mM [γ-^{32}P]ATP (5000 cpm/pmol), 8 μg eIF-2 and 0.25 μg HRI. Samples were incubated at 30°C, at intervals aliquots (4 μl) were removed, and phosphorylation and thiophosphorylation of eIF-2 were assayed as described in fig.1. (A) Lanes 1–7, samples incubated with [γ-^{32}P]ATP for 0.5, 1, 5, 10, 20, 40 and 80 min; (B) lanes 1–7, samples incubated with [γ-^{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.

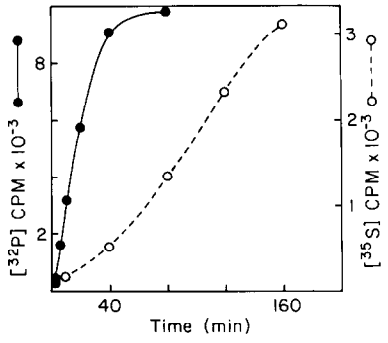

Fig.3. Rate of phosphorylation and thiophosphorylation of eIF-2. Data are derived from fig.2. The 38-kDa polypeptides were cut out from the gel and the associated radioactivity was measured.

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

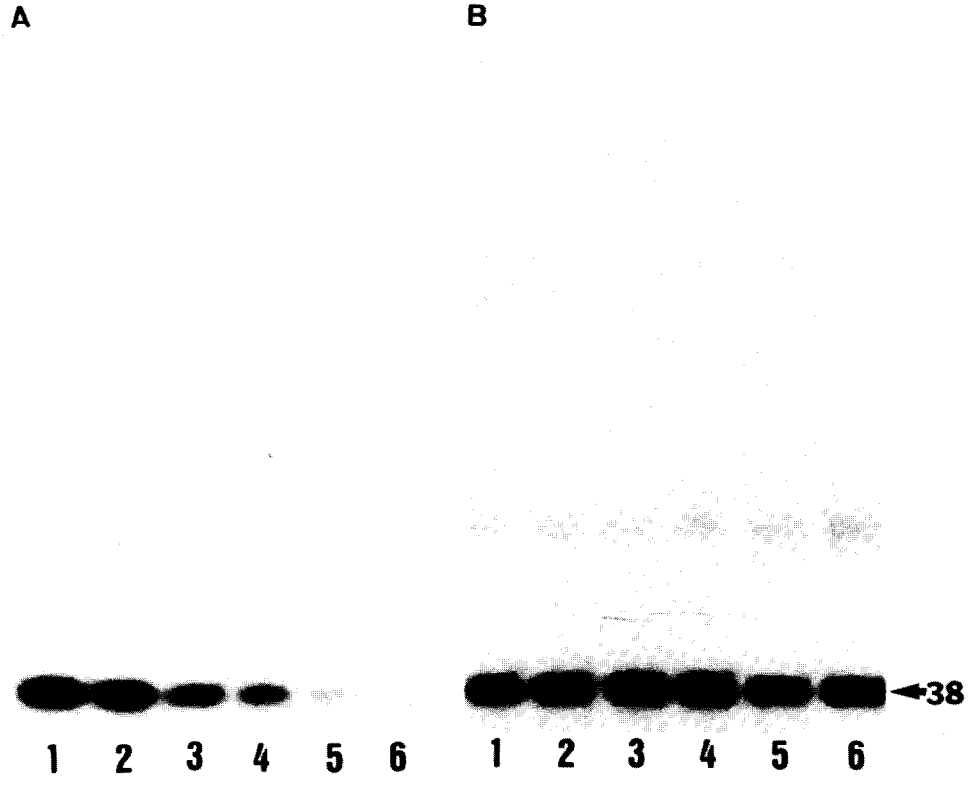

Fig.4. Dephosphorylation of phosphorylated and thiophosphorylated eIF-2. Protein synthesis lysate reaction mixtures (38 μl) were prepared and incubated with ^{32}P- or ^{35}S-labelled eIF-2 at 30°C. At intervals, aliquots (6 μl) were removed, denatured and subjected to electrophoresis in SDS-polyacrylamide 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 μg) was phosphorylated or thiophosphorylated as described in the legend to fig.2 for 4 h. Lysate protein synthesis reaction mixtures without hemin (40 μl) were incubated without or with 1 μg eIF-2 or phosphorylated eIF-2 or thiophosphorylated eIF-2 at 30°C for 40 min. Aliquots (5 μl) were removed and assayed for protein synthesis [25]

38-kDa subunit of eIF-2, is also capable of transferring the γ-thiophosphoryl group from ATP[γS] to its substrate. However, the rate of the reaction is substantially slower. This finding suggests that affinity of the enzyme for ATP[γS] 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[γS] 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[γS] to the 50-kDa subunit of eIF-2. The physiological significance, if any, of this latter phosphorylation is not known.

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