Regulation of protein synthesis in rabbit reticulocyte lysates

Requirement of initiation factor eIF-2 holoprotein for substrate specificity of heme-regulated protein kinase

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The specificity of the heme-regulated protein kinase (HRI) was investigated further by utilizing the isolated 38000 Da subunit (α subunit) polypeptide of eIF-2 as the substrate. For this purpose, the three subunit polypeptides of eIF-2 (38000 Da, α; 50000 Da, β; and 52000 Da, γ) were resolved by reversed-phase high performance liquid chromatography (HPLC). Results show that HRI is incapable of phosphorylating the 38000 Da subunit separated from the other two eIF-2 polypeptides. Data suggest that the substrate specificity of HRI is determined by the quaternary structure assumed by the α subunit in association with the other two subunits in the eIF-2 holoprotein.

Protein kinase *Translational inhibitor* *Initiation factor eIF-2* *Phosphorylation* *Ternary complex* *Heme regulation*

1. INTRODUCTION

Protein synthesis in rabbit reticulocytes and their lysates is regulated by heme (reviews [1–3]). In heme deficiency, a heme-regulated translational inhibitor (HRI) is activated that blocks protein chain initiation. A second translational inhibitor in lysates 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) that 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 initiation factor eIF-2 is inhibited [15–24]. eIF-2 promotes formation of the ternary complex (eIF-2·GTP·Met-tRNA$_f$), the first rate limiting reaction in the protein synthesis initiation cycle.

Unlike cAMP-dependent protein kinases, which phosphorylate a wide spectrum of substrates [25], HRI-catalyzed phosphorylation is highly specific and is restricted to eIF-2 [5–8]. It is not known whether this specificity of phosphorylation resides in the eIF-2 holoenzyme or if HRI is capable of phosphorylating the 38 kDa subunit free of the other two eIF-2 subunit polypeptides (the 50 kDa (β subunit) and the 52 kDa (γ subunit) polypeptides). In order to examine this question, the three eIF-2 subunits were separated by reversed-phase high performance liquid chromatography (HPLC). The phosphorylation of the isolated eIF-2 38 kDa subunit by purified HRI was assayed. The results presented in this communication show that HRI is not able to phosphorylate the isolated 38 kDa subunit of eIF-2.

2. EXPERIMENTAL

The following procedures have been described: preparation of purified initiation factor eIF-2;

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preparation of purified HRI (spec. act. 5000 units per mg); SDS-polyacrylamide gel electrophoresis; eIF-2 phosphorylation assay; and autoradiography of polyacrylamide gels [26].

2.1. *Separation of eIF-2 subunits by high performance liquid chromatography*

Pure eIF-2 (40 μg) was suspended in 20% acetonitrile (ACN) buffered with 0.05% trifluoroacetic acid (TFA) and applied to a Baker Bond reversed-phase wide pore (330 Å) C_8 column (4.8 × 250 mm) pre-equilibrated with 40% ACN buffered with 0.1% TFA [27]. The polypeptides were then eluted with a 40–60% ACN gradient buffered with 0.1% TFA. The gradient was delivered over a period of 40 min at a flow rate of 0.5 ml/min. The eluent was monitored at 220 nm, with a signal input to the recorder of 100 mV and a sensitivity of 0.05 absorption units full scale. The eluent from the column was collected in 0.25 ml fractions and the polypeptides eluted from the column were identified by electrophoresis in SDS-polyacrylamide gel (10%) along with 1–2 μg of standard eIF-2 holoenzyme. The proteins in the gel were stained with Coomassie brilliant blue dye.

The fractions containing the 38 kDa subunit were lyophilized and resuspended in protein phosphorylation mixture (20 mM Tris-HCl, pH 7.6; 60 mM KCl; 1 mM dithiothreitol; 5 mM magnesium acetate and 0.2 mM [γ-^{32}P]ATP, spec. act. 6000 cpm/mmol). The protein phosphorylation was assayed after the addition of HRI as described [26].

3. RESULTS AND DISCUSSION

The results in fig.1 show the resolution of the three subunit polypeptides of eIF-2 by chromatography on a C_8 wide pore HPLC column. A comparison of these polypeptides and standard eIF-2 polypeptides upon electrophoresis in SDS-polyacrylamide gels identified the 6 min retention time fraction polypeptide as the 50 kDa subunit, the 38 min retention time peak as the 38 kDa polypeptide and the 42.5 min retention time peak as the 52 kDa polypeptide.

The separated 38 kDa subunit polypeptide was dried under vacuum. To determine if the separated subunit could be phosphorylated by HRI, aliquots of eIF-2 phosphorylation reaction mixture and

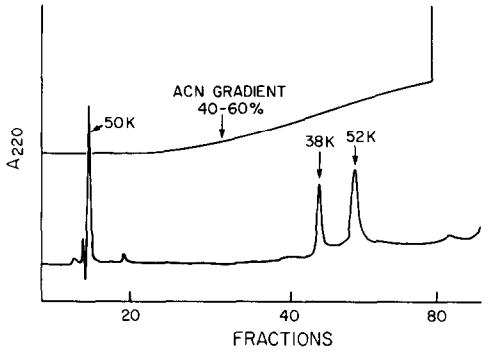

Fig.1. Separation of eIF-2 polypeptides by reversed phase HPLC. Details are provided in section 2.

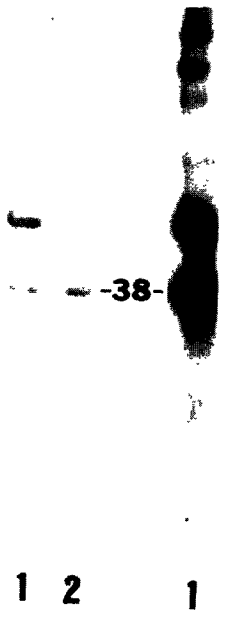

Fig.2. Phosphorylation of eIF-2 and isolated 38 kDa eIF-2 polypeptide by HRI. eIF-2 holoenzyme or the 38 kDa polypeptide was incubated with HRI under the phosphorylation reaction conditions described in section 2 [26]. After 1 h incubation at 30°C, the reaction was terminated by the addition of denaturing solution and the mixture heated at 100°C for several minutes. Samples were subjected to electrophoresis in SDS-polyacrylamide gel (10%). The proteins in the gel were stained with Coomassie brilliant blue dye (left) and then autoradiographed (right). Lanes: 1, eIF-2 holoprotein (1 μg) + HRI (0.05 μg); 2, eIF-2 38 kDa polypeptide (0.25 μg) + HRI (0.05 μg). The position of the 38 kDa subunit is indicated.

HRI were mixed with the 38 kDa polypeptide. Samples were incubated at 30°C for 1 h and analyzed by SDS-polyacrylamide gel elec-

trophoresis. Results are presented in fig.2. Under these conditions, no detectable phosphorylation of the 38 kDa subunit was observed (fig.2). These results would suggest that HRI is incapable of phosphorylating the 38 kDa subunit when separated from the other subunits. Consequently, the specificity of 38 kDa subunit phosphorylation by HRI is determined by the quaternary structure of the holoenzyme (assumed by the α subunit in association with the other subunits). When this structure is disrupted, HRI is unable to phosphorylate the eIF-2 α subunit.

In an earlier study, this laboratory [28,29] showed that a specific site on the 38 kDa subunit is phosphorylated by eIF-2 protein kinases irrespective of the source of these enzymes. This would suggest that the eIF-2 site phosphorylated by such protein kinases is not only unique but is also highly conserved. Recently, Wettenhall et al. [30] have sequenced the phosphopeptide of the 38 kDa subunit phosphorylated by HRI. Consistent with the high specificity of HRI-catalyzed eIF-2 phosphorylation, this phosphopeptide exhibits a unique sequence

(Arg-Ile-Leu-Leu-Ser^{48}-Glu-Leu-Ser^{51}-Arg) [30].
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