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Structure and function of peptide initiation factor 2: Differential loss of activities during proteolysis and generation of a terminal fragment containing the phosphorylation sites of the α subunit

(protein synthesis/protein kinase/translational control)

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ABSTRACT **A procedure is described by which the 38,000-dalton α subunit of native eukaryotic peptide initiation factor 2 (eIF-2) can be cleaved by trypsin to yield a 34,000-dalton fragment and a peptide of about 4,000 daltons after elimination of the β subunit. Under nondenaturing conditions the 4,000-dalton peptide remains bound to the modified eIF-2 and still can be phosphorylated by the heme-controlled eIF-2α kinase from reticulocytes. All of the phosphorylation sites for this protein kinase are located on the 4,000-dalton peptide. The ability of eIF-2 to form a ternary complex with GTP and Met-tRNA$_f$ and the ability to promote binding of Met-tRNA$_f$ to 40S ribosomal subunits are lost differentially during the proteolysis. Loss of the latter activity occurs rapidly and appears to be correlated with loss of the β subunit. Loss of activity for ternary complex formation is correlated with the appearance of the 4,000-dalton peptide.**

Eukaryotic polypeptide chain initiation factor 2 (eIF-2) from reticulocytes has been shown to consist of three nonidentical subunits (1–4), designated α, β, and γ according to their increasing isoelectric points. One mechanism of translational control involves phosphorylation and dephosphorylation of the α subunit. Two protein kinases from rabbit reticulocytes have been characterized that phosphorylate eIF-2α, apparently at the same serine residues (5). One is a ribosome-bound enzyme that is activated by double-stranded RNA (5, 6) and appears to be similar or identical to the enzyme that is induced by interferon in other cells (7–10). The other protein kinase is activated in the absence of heme (2, 5, 11, 12) and is associated with the heme-controlled repressor, HCR (13). Phosphorylation of eIF-2α by either protein kinase blocks peptide initiation at a step that is dependent upon eIF-2. Translational regulation involving phosphorylation and dephosphorylation of eIF-2 has been reviewed recently (14); however, the details of the mechanism by which phosphorylation of the α subunit of eIF-2 leads to inhibition of peptide initiation are poorly understood.

A form of eIF-2 containing only the α and γ subunits but apparently active in peptide initiation has been isolated from liver (15, 16) and reticulocytes (17). Recently, Mitsui *et al.* (18) presented data indicating that the β subunit of eIF-2 appears to be more sensitive to limited proteolysis than are the other two subunits, thus providing a possible mechanism by which the two-subunit form of eIF-2 may have been generated. Here we describe experiments using limited proteolysis of eIF-2 in which under certain conditions as described below the β subunit is rapidly lost from the molecule and eIF-2α is cleaved into a large and a small fragment at a slower rate. All phosphorylation sites for the heme-controlled eIF-2α protein kinase are on the small fragment. Activity of the eIF-2 for formation of the eIF-2·GTP·Met-tRNA$_f$ ternary complex and for binding of Met-tRNA$_f$ to 40S ribosomal subunits are lost at different rates during the proteolysis.

METHODS AND MATERIALS

Trypsin (EC 3.4.21.4, treated with tosylamidophenylethyl chloromethyl ketone), soybean trypsin inhibitor, and Sephadex G-100 were purchased from Sigma. [γ-^{32}P]ATP was obtained either from ICN Life Science Group or from Schwarz/Mann. X-ray No Screen film was from Eastman Kodak.

Preparation of eIF-2, eIF-2α Kinase, and Met-tRNA$_f$. eIF-2 was isolated from the ribosomal salt wash fraction from rabbit reticulocyte polysomes as published previously (19). A highly purified fraction containing the heme-regulated eIF-2α kinase was isolated from the postribosomal supernatant of rabbit reticulocytes as described (20). The purification procedure involved chromatography on DEAE-cellulose, hydroxyapatite, DEAE-cellulose in the presence of cAMP, and finally phosphocellulose. [^{35}S]Met-tRNA$_f$ was prepared as described (19).

Phosphorylation of eIF-2 and Isolation of eIF-2(α-*P*). The reaction mixture (300 μl) contained 20 mM Tris·HCl at pH 7.5, 2.5 mM dithioerythritol, 5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (4–8 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), 1 mg of eIF-2, and 720 μg of protein of the eIF-2α kinase fraction. After incubation at 37°C for 5 min, the mixture was loaded on phosphocellulose that had been packed in a Pasteur pipette and equilibrated with 20 mM Tris·HCl, pH 7.5/100 mM KCl/5 mM 2-mercaptoethanol/1 mM dithioerythritol/0.1 mM EDTA. The eIF-2α kinase and ATP or phosphate are not absorbed under these conditions. eIF-2 phosphorylated in its α subunit [eIF-2(α-*P*)] was eluted by 500 mM KCl in the same solution and used immediately.

Proteolysis and Gel Filtration Chromatography of eIF-2. The conditions for trypsin digestion of eIF-2 are given in *Results*. Incubation time was as indicated, and the reaction was stopped by the addition of a 40-fold molar excess of soybean trypsin inhibitor. A 125-μl aliquot of the reaction mixture was loaded on a Sephadex G-100 column (0.7 × 25 cm) that had been equilibrated either in 20 mM Tris·HCl, pH 7.5/100 mM KCl/5 mM 2-mercaptoethanol (nondenaturing conditions) or the same solution containing 8 M urea (denaturing conditions). In the latter case the column was run at room temperature, in the former case at 4°C. Fractions (350 μl) were collected. Where applicable, their radioactivity was determined from a 3-μl aliquot. The void volume of the column was determined with blue dextran.

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Abbreviations: eIF-2, eukaryotic peptide initiation factor 2; eIF-2α, the α subunit of eIF-2; eIF-2(α-*P*), eIF-2 phosphorylated in its α subunit.

Analysis of eIF-2 by Gel Electrophoresis. Peptides were separated on 15% polyacrylamide slab gels containing 180:1 (wt/wt) acrylamide/bisacrylamide in Tris/glycine buffer (pH 8.3) with 0.1% $NaDodSO_4$. This is a modification (21) of the procedure described by Laemmli (22). The details of the electrophoresis and autoradiography have been described (2, 23).

Activity of eIF-2. Formation of the ternary complex between eIF-2, GTP, and Met-tRNA$_f$ was assayed as described (19). About 39 pmol of [^{35}S]Met-tRNA$_f$ (1.9 Ci/mmol) was used per reaction mixture. Conditions for binding of Met-tRNA$_f$ to 40S ribosomal subunits are given in the same reference. Again, 39 pmol of [^{35}S]Met-tRNA$_f$ (1.9 Ci/mmol) was used with 8 μg of f2-fraction protein (cf. ref. 19), 12 pmol of reticulocyte 40S ribosomal subunits, and the indicated amounts of eIF-2.

Protein Determination. The amidoschwarz method described by Schaffner and Weissmann (24) was used.

RESULTS AND DISCUSSION

Limited proteolysis of eIF-2

Peptide initiation factor eIF-2 was isolated from rabbit reticulocytes, then phosphorylated in its α subunit by the heme-regulated eIF-2α kinase from reticulocytes and chromatographed on phosphocellulose as described in *Methods and Materials*. This chromatographic procedure provides good separation of the components of the eIF-2α kinase and free ATP or phosphate from phosphorylated eIF-2 (compare tracks 1 and 2 of Fig. 1). The three subunits of eIF-2 are marked as α, β, and γ, with β migrating more slowly than γ in the gel system used here. Peptides of the eIF-2α kinase preparation are indicated as 90 and 100 in Fig. 1 *A* and *B*, respectively. eIF-2(α-*P*) was subjected to limited proteolytic digestion with trypsin. The incubation mixture consisted of 200 μg of eIF-2(α-*P*), 0.3 μg of trypsin, 20 mM Tris·HCl (pH 7.5), 100 mM KCl, 5 mM 2-mercaptoethanol, 1 mM dithioerythritol, and 0.1 mM EDTA in a final volume of 120 μl. Incubation was for 15 min at 25°C, then 10 μg of soybean trypsin inhibitor was added to stop the digestion. The resulting digest was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis with the results shown in Fig. 1. The peptide bands of the sample before and after digestion are shown in tracks 2 and 3, respectively. The γ subunit is in the same position in both tracks but the β subunit is not seen in the digested sample, track 3. Also, the α subunit, judged to be about 38,000 daltons is almost missing in the digested sample; however, a new peptide band is seen at about 34,000 daltons. The strongly stained peptide of about 21,000 daltons seen in track 3, Fig. 1*A* is the trypsin inhibitor. Autoradiography of the dried gel (Fig. 1*B*) shows an intense band corresponding to phosphorylated eIF-2α at the 38,000-dalton position, tracks 1 and 2. The intensity of this band is greatly reduced in the digested sample (Fig. 1*B*, track 3). This band apparently arises from a small amount of undigested eIF-2α that is barely visible on the stained gel. A new phosphorylated peptide of about 4,000 daltons is generated by the digestion. No [^{32}P]phosphate is detected at the position of the 34,000-dalton peptide. Comparison on the autoradiogram of the intensity of the 4,000-dalton peptide with the bands from the α and β peptides in the undigested sample indicates that the 4,000-dalton fragment was cleaved from the α subunit. The β subunit is hydrolyzed by trypsin into a number of small peptides (18) that are not detected in the system used here. Furthermore, the total molecular mass of the 4,000-dalton peptide plus the 34,000-dalton fragment seen on the stained gel corresponds closely to the 38,000-dalton molecular mass of intact eIF-2α.

The 4,000-dalton phosphorylated peptide that is generated by tryptic digestion can be separated from undigested eIF-2 and higher molecular weight components by chromatography on Sephadex G-100 in 8 M urea performed as described in *Methods and Materials*. A typical elution profile for radioactive phosphate is shown in Fig. 2. A small amount of radioactivity is eluted near the void volume, well separated from a larger peak that contains about 85% of the total radioactive phosphate applied to the column. Material from the V_o fraction and the major

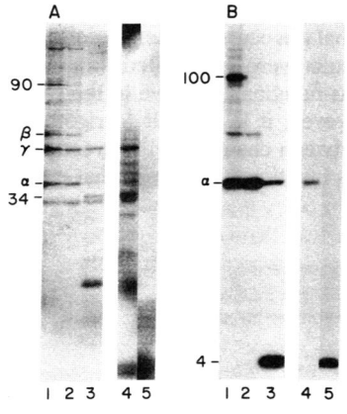

FIG. 1. Analysis of phosphorylated, trypsin-treated eIF-2. Phosphorylation of eIF-2 with the hemin-regulated eIF-2α kinase and [γ-^{32}P]ATP followed by isolation of eIF-2(α-*P*) was carried out as described in *Methods and Materials*. Details of the subsequent trypsin treatment are given in the text; chromatography of trypsin-treated eIF-2(α-*P*) under denaturing conditions is shown in Fig. 2. Samples from the different steps of eIF-2 incubation and chromatography are shown here after electrophoresis on $NaDodSO_4$/polyacrylamide gels and autoradiography. (*A*) Stained gel; (*B*) autoradiogram. Track 1, eIF-2 after phosphorylation; track 2, isolated eIF-2(α-*P*); track 3, eIF-2(α-*P*) after trypsin incubation (tracks 1–3 contain the equivalent of about 3 μg of eIF-2); track 4, trypsin-treated eIF-2 after chromatography on Sephadex G-100 (30 μl from fraction 6 of Fig. 2); track 5, 30 μl from fraction 14 of Fig. 2. The following positions are indicated: α, β, and γ are the three subunits of eIF-2; 34 and 4, eIF-2α fragments of 34,000 and 4,000 daltons, respectively; 90 and 100, 90,000- and 100,000-dalton peptides of the eIF-2α kinase preparation.

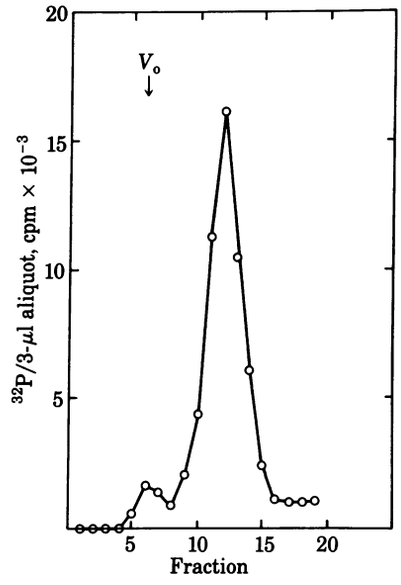

FIG. 2. Separation of the peptides derived from eIF-2α by gel filtration in urea. Radioactive eIF-2(α-*P*) was subjected to limited proteolysis and then chromatography on Sephadex G-100 in the presence of urea. The distribution of [^{32}P]phosphate radioactivity is shown as determined from a 3-μl aliquot of the individual fractions. V_o indicates the void volume of the column.

peak were analyzed by $NaDodSO_4$ gel electrophoresis, with the results shown in tracks 4 and 5, respectively, of Fig. 1. Phosphorylated material at the 38,000-dalton position is seen in the autoradiogram of the V_0 fraction, but only the band at the 4,000-dalton position is detected in the major peak (cf. tracks 4 and 5 of Fig. 1*B*). Bands seen in the stained gel of the V_0 fraction (Fig. 1*A*, track 4) include a well-defined peptide at the 34,000-dalton position. No [^{32}P]phosphate is detected at this position in the corresponding autoradiogram. The results appear to indicate that, in native eIF-2, a site or region of restricted size near one end of the α subunit is exposed to trypsin and that the phosphorylation site(s) for the eIF-2α kinase are located between the trypsin-sensitive site and the proximal end of the α subunit.

Under nondenaturing conditions the 4,000-dalton phosphorylated peptide that is generated by trypsin cleavage of eIF-2α remains tightly bound to the 34,000-dalton fragment and the γ subunit. Results leading to this conclusion are presented in Figs. 3 and 4. Limited digestion of eIF-2 was carried out as described above. Then the products were chromatographed on Sephadex G-100 as for the experiment represented by Fig. 2, except that urea was omitted from the solvent. [^{32}P]Phosphate was found in a single peak near the void volume of the column (Fig. 3). Aliquots of the column fractions were analyzed by $NaDodSO_4$ gel electrophoresis. The results shown in Fig. 4 (tracks 2–4) demonstrate that the phosphorylated 4,000-dalton peptide was eluted from the column with the γ subunit and 34,000-dalton fragment of eIF-2α.

The α subunit of eIF-2 can be cleaved by trypsin and then phosphorylated as shown by the results presented in Fig. 5. For these experiments native eIF-2 that had not been subjected to *in vitro* phosphorylation was incubated with trypsin for the times indicated under conditions similar to those used for the experiments of Fig. 1. Trypsin inhibitor was added to aliquots of the reaction mixture at the indicated times. Then aliquots of these samples were incubated with [γ-^{32}P]ATP and the eIF-2α kinase. Analysis by $NaDodSO_4$ gel electrophoresis was carried out without prior chromatography on phosphocellulose or Sephadex G-100. The results show that the β subunit disappears

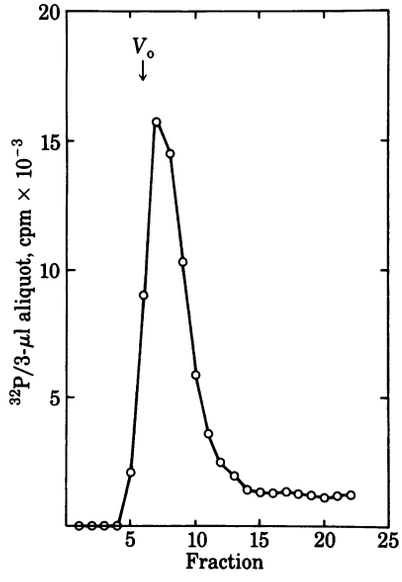

FIG. 3. Chromatography of trypsin-treated eIF-2(α-*P*) under nondenaturing conditions. The experiment was carried out as outlined in the legend of Fig. 2 except that urea was omitted during the chromatography.

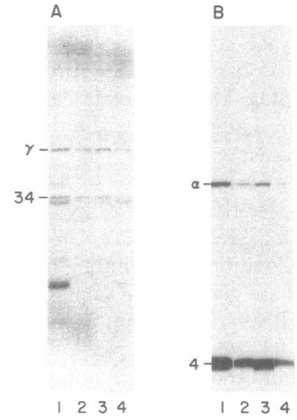

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis of fractions from the chromatography shown in Fig. 3. (*A*) Stained gel; (*B*) autoradiogram. Tracks 2, 3, and 4 contained 30-μl aliquots of fractions 6, 8, and 10, respectively, from the chromatogram shown in Fig. 3. Track 1 contains the trypsin-treated eIF-2(α-*P*) before chromatography. Position markers are the same as in Fig. 1.

rapidly during the first 3 min of hydrolysis and is not detectable after 6 min. Disappearance of the 38,000-dalton α subunit with a concomitant appearance of the 34,000-dalton fragment (Fig. 5*A*) and the phosphorylated 4,000-dalton peptide (Fig. 5*B*) appears not to be linear with time in that it proceeds slowly during the first 6 min. The results appear to indicate that prior modification of eIF-2, possibly elimination of the β subunit, must occur before clipping of the α subunit can occur efficiently.

Activity of trypsin-treated eIF-2

Although reticulocyte eIF-2 consisting of only the α and γ subunits was found to be less stable to thermal denaturation than the native factor (18), eIF-2 lacking the β subunit was able to support peptide initiation (17, 18) and reverse inhibition of peptide synthesis that was caused by hemin deficiency in cell lysates (18). These results were interpreted to indicate that the two-subunit eIF-2 is functionally active in the reactions of peptide initiation. However, it is difficult to rigorously establish the relationship between changes in the gross physical properties of eIF-2 and its functional activity in that most preparations of

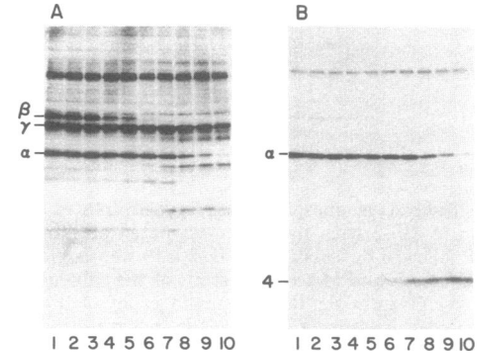

FIG. 5. Time course of trypsin digestion of unphosphorylated eIF-2 and subsequent phosphorylation by the eIF-2α kinase. Conditions for trypsin digestion were the same as for Fig. 1. Aliquots were withdrawn at the times indicated below and trypsin inhibitor was added to stop the reaction. After this treatment, an aliquot corresponding to about 4 μg of eIF-2 was incubated with the eIF-2α kinase and [γ-^{32}P]ATP. (*A*) Stained gel; (*B*) autoradiogram. Track 1, control without trypsin; tracks 2 to 10: trypsin incubation for 0.5, 1, 2, 3, 6, 8, 12, 25, and 35 min, respectively. Position markers are as in Fig. 1.

purified eIF-2 have less than the theoretical maximum activity. Typically, the molar ratio of Met-tRNA$_f$ bound in the eIF-2·Met-tRNA$_f$·GTP ternary complex may be about 25% of the total eIF-2 present (25). The efficiency of the unmodified eIF-2 used here is about 0.3 and 0.2, respectively, for ternary complex formation and binding of Met-tRNA$_f$ to 40S ribosomal subunits.

The ability of trypsin-modified eIF-2 to form the eIF-2·GTP·Met-tRNA$_f$ ternary complex and to promote binding of Met-tRNA$_f$ to 40S ribosomal subunits was measured in aliquots of the same reaction mixture that was analyzed by gel electrophoresis with the results shown in Fig. 5. The results presented in Fig. 6 indicate that activity for binding of Met-tRNA$_f$ to 40S ribosomal subunits is more sensitive to trypsin hydrolysis than activity for ternary complex formation. Loss of the former activity appears to be correlated with loss of the β subunit, which proceeds rapidly during the initial period of the incubation. After 6 min of incubation, about 70% of the original activity for Met-tRNA$_f$ binding to 40S subunits has been lost (Fig. 6*B*) and the β subunit is no longer detectable (Fig. 5*A*). Nearly all of the original activity for ternary complex formation is retained after 6 min (Fig. 6*A*), and it is appreciable after 25 min. The peptide bands in Fig. 5*A* show that most of the α subunit has been cleaved after 25 min. Also, a small loss of the γ subunit occurs during the longer incubations.

The rapid and differential loss of activity for binding of Met-tRNA$_f$ to 40S ribosomal subunits is surprising in the light of reports that eIF-2 consisting of only the α and γ subunit is active for peptide initiation (15–18). It is possible that the activity observed in these studies was due to a small amount of three-subunit eIF-2 that was present but undetected in preparations of the two-subunit factor. Alternatively, the purified components used in the study reported here may be deficient in a component that can activate or stabilize the two-subunit eIF-2. Free β subunit might have such an effect. We observed loss of activity for binding of Met-tRNA$_f$ to 40S ribosomal subunits when eIF-2 was phosphorylated in its α subunit but no effect on formation of the ternary complex (26). The system and components used were similar to those employed in the experiments reported here. An inhibitory effect of α subunit phosphorylation on the function of eIF-2β in binding of Met-tRNA$_f$ to 40S ribosomes appears to be an attractive hypothesis for the mechanism by which eIF-2α kinases block peptide initiation.

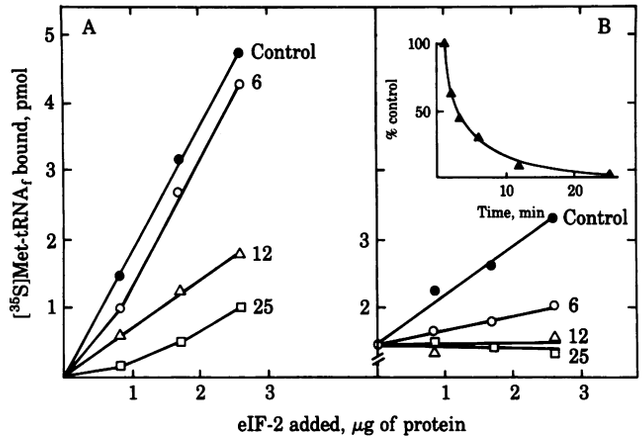

FIG. 6. Biological activity of trypsin-treated eIF-2. (*A*) Ternary complex formation with GTP and [^{35}S]Met-tRNA$_f$. (*B*) Binding of [^{35}S]Met-tRNA$_f$ to 40S ribosomal subunits. eIF-2 was the same sample analyzed in Fig. 5; aliquots from the control (●) or from the samples after 6 min (○), 12 min (△), and 25 min (□) of trypsin treatment were used in the amounts indicated on the abscissa. These amounts refer to undigested eIF-2. (*Inset*) Time-dependent decrease in Met-tRNA$_f$ binding to 40S ribosomal subunits with the equivalent of 2.7 μg of eIF-2 withdrawn after trypsin digestion for the given period of time. Control is set at 100% activity with the background (sample without 40S subunits added) subtracted.

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