Removal of β subunit of the eukaryotic polypeptide chain initiation factor 2 by limited proteolysis

(eukaryotic initiation factor 2 subunits/initiation complex formation/lability of protease-treated initiation factor 2)

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ABSTRACT

It is generally considered that the eukaryotic polypeptide chain initiation factor 2 (eIF-2) from rabbit reticulocytes consists of three nonidentical subunits termed α, β, and γ, in order of increasing molecular weight. However, a recent report (Stringer, E. A., Chaudhuri, A., Valenzuela, D. & Maitra, U. (1980) Proc. Natl. Acad. Sci. USA 77, 3536–3539) suggested that this factor is made up of only two subunits. In this paper we show that limited proteolysis of rabbit reticulocyte eIF-2 leads to loss of the β subunit. This modified eIF-2 has the same activity as the native factor in promoting ternary (eIF-2-GTP-Met-tRNA<sub>1</sub>) and 40S (eIF-2-GTP-Met-tRNA<sub>1</sub>–40S ribosome) initiation complex formation. Like native eIF-2, the protease-treated factor can restore translation in heme-deficient lysates. On the other hand, the treated factor is less stable than the native protein.

The structure and properties of the eukaryotic polypeptide chain initiation factor 2 (eIF-2) have been studied in a number of laboratories. eIF-2 from rabbit reticulocytes has been isolated as a protein consisting of three nonidentical subunits, α, β, and γ, in a molar ratio of 1:1:1 (e.g., see refs. 1–4). In our laboratory these subunits have been found to have apparent molecular masses of approximately 38,000, 52,000, and 54,000 daltons (4) as analyzed by polyacrylamide/NaDodSO<sub>4</sub> gel electrophoresis in gels containing 10% acrylamide and 0.26% N,N'-methylenebisacrylamide (cf. refs. 1 and 3). On the other hand, eIF-2 from pig (5) and calf (6) liver has been isolated as a protein consisting of only two subunits, and this has also recently been reported (7) for eIF-2 from rabbit reticulocytes.

We find that the β subunit of our three-subunit rabbit reticulocyte eIF-2 can be digested away by short treatment with pancreatic protease I or trypsin to yield a two-subunit (αγ) factor that, like native eIF-2, is active in ternary and 40S initiation complex formation. The protease-digested factor is also effective in decreasing translational inhibition in heme-deficient lysates. One difference that has been found between native and αγ eIF-2 is a lesser stability of the latter.

MATERIALS AND METHODS

Preparations. Rabbit reticulocyte lysate for translation assays was prepared as described by Hunt et al. (8). Lysate used for preparation of eIF-2, eIF-2 stimulating protein (ESP), and eIF-2-β kinase (casein kinase) was prepared in a similar way by using cells purchased from Pel-Freez (Rogers, AR). eIF-2 (about 75% pure) was prepared according to Benne et al. (1) through the phosphocellulose step. Phosphorylation of the β subunit of eIF-2 was performed with casein kinase as described (4), and the phosphorylated factor was reisolated by heparin-Sepharose chromatography (9). The preparation of GDF-free GTP has been described (4). Ribosomal subunits were prepared from Artemia salina embryos by the procedure of Zasloff and Ochoa (10). ESP was prepared as outlined by Siekerka et al. (11). Capped, [3H]methyl-labeled reovirus mRNA was kindly provided by Aaron Shatkin (Roche Institute). [3H]Met-tRNA<sub>1</sub> was prepared as in previous work (4). Pancreatic protease (type I), trypsin, and carboxypeptidase Y were purchased from Sigma. Chymotrypsin and Staphylococcus aureus V8 protease were from Miles. Other preparations were as in previous work (4).

Assays. Because αγ eIF-2 is less stable than the native factor (see below), it always was freshly prepared for activity assays. Ternary complex formation (assay A) was assayed as described (4) except that the samples were supplemented with 5 μg of bovine serum albumin as a stabilizer. The formation of 40S initiation complexes was determined by sucrose density gradient centrifugation. To form the ternary complex (eIF-2-GTP-Met-tRNA<sub>1</sub>), samples (0.1 ml) containing 20 mM Hepes buffer (pH 7.6), 100 mM KCl, 1 mM dithiothreitol, 44 μM GTP, 25 pmol of [3H]Met-tRNA<sub>1</sub> (52,000 cpm/pmol), and 10 pmol of either native or protease-digested eIF-2 were incubated for 6 min at 30°C. The samples were then supplemented with 5 mM MgCl<sub>2</sub>, 0.72 A<sub>250</sub> unit of A. salina 40S ribosomal subunits, and 0.2 A<sub>250</sub> unit of AUG (final volume, 0.15 ml). After incubation for a further 6 min at 30°C, the samples were chilled, layered on a 5-ml, 5–20% (wt/vol) linear sucrose gradient containing 20 mM Hepes (pH 7.6), 100 mM KCl, 5 mM MgCl<sub>2</sub>, and 2 mM dithiothreitol, and centrifuged for 100 min at 3°C and 50,000 rpm in the SW 56 rotor of the Spinco ultracentrifuge. The gradients were analyzed in an Isco gradient fractionator. Fractions (0.2 ml) were collected and their radioactivity was measured in Aquasol.

NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis was routinely performed as described by Laemmli (12) in disc or slab gels containing 10% acrylamide and 0.26% N,N'-methylenebisacrylamide. Under these conditions, the α, β, and γ subunits of eIF-2 migrate with apparent molecular masses of approximately 38,000, 52,000, and 54,000 daltons (4). In 15% acrylamide gels the rate of migration of β and γ subunits is reversed (3). The β subunit is characterized unequivocally by its specific phosphorylation by reticulocyte casein kinase (3, 4).

Translation (25 μl samples) was assayed as described (8) with 10 μl of 1:1 lysate and [14C]lysine as the labeled amino acid, with or without 20 μM hemin. Incubation was at 30°C. Protein was

Abbreviations: eIF-2, eukaryotic initiation factor 2; Met-tRNA<sub>1</sub>, eukaryotic initiator methionyl transfer RNA; ESP, eIF-2 stimulating protein; RF, translation restoring factor; AUG, the trinucleoside diphosphate ApUpG; HCl, heme-controlled translational inhibitor.

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determined by the Bradford procedure (13) with bovine serum albumin as the standard.

RESULTS

Protease Treatment. eIF-2 was dialyzed against 20 mM Hepes, pH 7.6/0.1 mM EDTA/2 mM 2-mercaptoethanol/5% (vol/vol) glycerol (buffer A) containing 100 mM KCl (buffer A-100). Part of the dialyzed eIF-2 (227 μg) was incubated with 0.325 μg of pancreatic protease (Sigma, type I) for 15 min at 26°C (final volume, 1.6 ml). Another portion of the dialyzed eIF-2 (227 μg), to serve as a control, was similarly incubated but without protease. The incubated solutions were loaded onto heparin-Sepharose columns (0.7 × 2 cm) equilibrated with A-100 buffer. After the column was washed with the same buffer until A280 was <0.01, eIF-2 was eluted with buffer A containing 400 mM KCl; 0.8-ml fractions were collected. The factor eluted in both cases as a sharp, symmetric peak of coincident A280 and ternary complex formation activities (Fig. 1A). Electrophoretic analysis in 10% acrylamide gels (Fig. 1B) showed that the control eIF-2 had the usual α, β, and γ peptide bands whereas the protease-treated factor showed the presence of the α and γ bands only. A densitometer scanning of the stained gels is shown in Fig. 1C. The molecular weight of native eIF-2 is taken as α (38,000) + β (52,000) + γ (54,000) = 144,000, and that of the αγ factor as α (38,000) + γ (54,000) = 92,000. Electrophoresis in 15% polyacrylamide/0.09% N,N'-bismethyleneacrylamide gels (not shown) yielded three polypeptides with apparent molecular masses of approximately 35,000, 48,000, and 53,000 daltons for native eIF-2 (cf. ref. 3) and two polypeptides of 35,000 and 48,000 daltons for the protease-treated factor. Because protease treatment removes the β subunit, it appears that the bands at 35,000, 48,000, and 53,000 daltons in 15% gels correspond to the α, γ, and β polypeptides, respectively, of native eIF-2.

The time course of proteolysis was followed with eIF-2 la-
FIG. 2. Effect of different proteases on eIF-2. Samples (6.2 μg) of eIF-2 32P-labeled β subunit were incubated (volume, 10 μl) for 10 min at 26°C with 10 ng of pancreatic protease (track 3), trypsin (track 4), chymotrypsin (track 5), or carboxypeptidase (track 7) or for 60 min at the same temperature with 10 ng of S. aureus protease (track 6), and electrophoresed as in Fig. 1D. The gel was stained with Coomassie blue (A) and then dried and autoradiographed (B). Track 1, molecular weight markers phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000) (each 2 μg); track 2, eIF-2 incubated without protease. The stronger degradation of the 32P-labeled β subunit is readily apparent in B (track 4) by the greater release of small labeled peptide(s) moving to the gel front.

beled with 32P in the β subunit. The gradual disappearance of the β subunit was accompanied by the appearance of smaller peptides in the stained gels (Fig. 1D). The autoradiogram confirmed that the subunit that was degraded was indeed the β subunit and showed, in addition, that the bulk of the radioactive label was released with a small peptide(s) that formed quite early and migrated to the gel front (Fig. 1D, tracks 2 to 5).

Pancreatic protease (type I) is a crude preparation that contains trypsin and chymotrypsin among other proteases. We therefore incubated eIF-2 32P-labeled β subunit with different proteases including trypsin, chymotrypsin, carboxypeptidase, and a bacterial protease. Dialyzed aliquots of labeled β subunit eIF-2 were incubated with each of the enzymes and processed as described in the legend to Fig. 2. The β subunit was more sensitive to trypsin than to chymotrypsin and it was not susceptible to S. aureus protease or carboxypeptidase (Fig. 2).

Properties of Protease-Treated eIF-2. The β subunit does not seem to be required for eIF-2 activity but the eIF-2 without β subunit is less stable than the native factor. Fig. 1A shows that protease-treated eIF-2 was active in ternary complex formation. This point is examined in more detail in Fig. 3. In the absence of ESP (4), ternary complex formation was promoted as efficiently by αγ as by native eIF-2. Moreover, Mg2+ inhibited ternary complex formation (11, 14, 15) to about the same extent in both cases, and ESP largely prevented the Mg2+ inhibition (11, 14, 15) with native and with eIF-2 lacking β subunit (Fig. 3B). Formation of 40S complex was also promoted to a similar extent by native and β subunit-free eIF-2 (Fig. 4A). It has been reported (e.g., refs. 16 and 17) that eIF-2 can bind mRNA or Met-tRNAf in the absence of GTP, and the β subunit has been implicated in this binding (18). We examined the binding of reovirus mRNA by native or protease-treated eIF-2 (Fig. 4B) and found that, at 2 pmol of eIF-2 per sample, the latter factor bound approximately half as much mRNA as did the native protein.

eIF-2 is known to prevent or to overcome translational inhibition in reticulocyte lysates caused by heme deficiency or by the addition of heme-controlled translational inhibitor (HCl) (for a review, see ref. 19), and it was of interest to ascertain whether eIF-2 lacking β subunit had a similar effect. The protease-treated eIF-2 exhibited somewhat lower activity in this assay than did the native factor (Fig. 5). However, the protease-treated factor is less stable (see below) and may decay more rapidly under the conditions of these experiments.

Although eIF-2 activity did not seem to be markedly affected by loss of its β subunit, it was conceivable that this loss might impair the stability of the factor. This appears to be the case. Storing eIF-2 at 0°C for 5 days resulted in about 10% loss of ternary complex formation activity but, under the same conditions, protease-treated eIF-2 lost 74%. In line with this observation, heating eIF-2 for 6 min at 32°C, a treatment that hardly affected the ternary complex formation capacity of the native factor, decreased the activity of protease-treated eIF-2 by about 70% (Fig. 6).
DISCUSSION

The β subunit of the three-subunit (αβγ) eIF-2 from rabbit reticulocyte lysates is susceptible to protease digestion, after which a two-subunit (αγ) factor can be recovered by heparin-Sepharose chromatography. The β subunit is characterized as the subunit of intermediate mobility when eIF-2 is analyzed by NaDodSO₄/polyacrylamide gel electrophoresis in gels containing 10% acrylamide and 0.26% N,N'-bis(methylene)acrylamide (3). This subunit is specifically phosphorylated by a cyclic AMP-dependent protein kinase (casein kinase) from reticulocyte lysates (3, 4). The β subunit of reticulocyte eIF-2 is more sensitive to trypsin than to chymotrypsin. This might be related to the high content of basic amino acids reported by Lloyd et al. (20) for this subunit. The properties of native eIF-2 are largely retained by its β subunit-free counterpart. However, the β subunit-free factor is less stable. The fact that all three eIF-2 subunits are present in the 40S ribosomal complex (1, 21) and that they are also present in the recently described translation restoring factor-eIF-2 complex (11) would be consistent with the idea that the three-subunit eIF-2 is the native form of the factor in which the β subunit may play a stabilizing role.

Our observation (Fig. 4B) that αγ eIF-2 binds significantly less mRNA than does the native factor may be in line with the report (18) that the isolated β subunit binds mRNA and Met-tRNA. However, because the capacity to form a ternary complex is retained by αγ eIF-2, the binding of Met-tRNA by the isolated β subunit may be nonspecific. The same may also be true of the mRNA binding because the primary function of eIF-2—i.e., formation of the ternary complex—does not involve mRNA. One wonders whether RNA binding by the β subunit might not be a consequence of its high basic amino acid content.

In the light of our finding that the protease-treated αγ eIF-2 has α and γ bands at 38,000 and 54,000 daltons in 10% polyacrylamide gels and at 35,000 and 48,500 daltons in 15% gels, it would appear that the two-subunit eIF-2 isolated (and analyzed by NaDodSO₄/polyacrylamide gel electrophoresis in 15% gels) in other laboratories is also αγ eIF-2. The molecular weights of the two subunits, given as 35,000 and 48,500 for the pig liver factor (5) and as 38,000 and 48,000 for the factors from calf liver (6) and rabbit reticulocytes (7), are reasonably close to our values for protease-treated eIF-2. It has in fact been reported (22) that the two-subunit eIF-2 from rabbit reticulocytes (7) lacks the polypeptide that is specifically phosphorylated by reticulocyte casein kinase—i.e., the β subunit. Thus, the two-subunit eIF-2 isolated by others (5-7) is αγ eIF-2 probably formed by limited proteolysis of the kind described in this paper. This is not unlikely in the case of the pig (5) and calf (6) liver eIF-2 because no protease inhibitors were used early during isolation. However, phenylmethylsulfonyl fluoride reportedly was used in the preparation of the ribosomal salt wash and at later steps in the isolation of the rabbit reticulocyte factor (7). Perhaps the β subunit is intrinsically labile and was damaged during isolation.

Note Added in Proof. Meyer et al. (23) recently reported that two eIF-2 preparations purified from rabbit reticulocyte lysate prepared in the laboratory had about equimolar amounts of α, β, and γ subunits but one made from commercial lysate (cf. ref. 7) was deficient in the β subunit.
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