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PURIFICATION AND CHARACTERIZATION OF PROTEIN SYNTHESIS INITIATION FACTOR eIF-3 FROM WHEAT GERM

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Summary

eIF-3 from wheat germ is a large multicomponent factor. It sediments at 15 S and is comprised of ten different polypeptides with an M_r value ranging from 26 000 to 135 000; five out of the ten seem to be present in a 1 : 1 stoichiometric ratio, whereas the others appear to occur approximately in a 0.5 to 1 ratio.

The factor is active in a partially purified cell-free system derived from wheat germ and in a mammalian model assay system for the synthesis of methionyl puromycin.

Introduction

By far the most complex protein factor involved in eukaryotic protein synthesis is initiation factor eIF-3. It is a multi-component assemblage, consisting of at least eight polypeptides, with a total mass of approximately 700 000 daltons [1—5]. The factor plays an important role in the initiation process, since it interacts with the small ribosomal subunit at a very early stage, thereby preventing premature formation of 80-S ribosomes [6—8]. The resulting eIF-3-40-S complex is the starting material in a sequence of events which eventually lead to the construction of an 80 S initiation complex. The presence of the factor on 40 S has been found to be required for optimal Met-tRNA and mRNA binding [6—9] (see for a review, Ref. 10).

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Abbreviation: eIF, eukaryotic initiation factor.

The complex nature of the factor may be a reflexion of its multiple functions [8], or be connected with specificity of messenger selection [11]. Most of the information on the factor has been derived from rabbit reticulocytes [1—3, 6—9] and, less detailed, from rat liver [4] and wheat germ [5].

In order to obtain a better-defined cell-free system for protein synthesis from wheat germ, we have undertaken the task of purifying and characterizing the most essential initiation factors. A previous paper [12] described the purification of the Met-tRNA$_f$ binding factor eIF-2. Here we present a thorough characterization of eIF-3, with emphasis on the number, molecular weight and stoichiometry of its subunits, information which has not been available so far (see Ref. 5).

Materials and Methods

Materials

[^{35}S]Methionine (600 Ci/mmol) and [^{3}H]methionine (8.8 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, U.K., Sephadex G-25 and Sepharose 6B from Pharmacia, DEAE-cellulose (DE-52) from Whatman, and commercial wheat germ from General Mills (Vallejo, U.S.A.).

Buffers

The compositions of these were: buffer A, 20 mM Tris-HCl, pH 7.6/2 mM magnesium acetate/7 mM 2-mercaptoethanol/120 mM KCl; buffer B, 20 mM Tris-HCl, pH 7.6/4 mM magnesium acetate/7 mM 2-mercaptoethanol/100 mM KCl/0.25 M sucrose; buffer C, 20 mM Tris-HCl, pH 7.6/7 mM 2-mercaptoethanol/10% glycerol; buffer D, 20 mM Tris-HCl, pH 7.6/7 mM 2-mercaptoethanol/100 mM KCl.

Biological components

The preparation of the following components has been described: Sepharose-heparin [13], hemoglobin 9 S mRNA [14], crude and purified initiation factors from rabbit reticulocytes [15—18], pH 5 enzymes from rat liver [19], ribosomal subunits from rat liver [20], [^{3}H]Met-tRNA 4000 cpm/pmol [12], 23 000 × g homogenate from wheat germ [21].

Wheat-germ S23 homogenate prepared from 62.5 g of dry wheat germ was passed through a 1.8 l Sephadex G-25 column equilibrated in buffer A according to Spremulli et al. [5]. Fractions eluting at the void volume of the column were combined (total volume of four preparations: 1770 ml), clarified, and centrifuged in 84-ml portions through 10 ml 1 M sucrose in buffer A in a Beckmann Ti 45 rotor for 16 h at 35 000 rev./min. The resulting ribosomal pellet was resuspended in buffer B and used as a source of wheat germ ribosomes devoid of initiation factors. The S100 supernatant was used as the starting material for the preparation of initiation factors (see Table I). pH 4.5 enzymes from wheat germ were prepared from the protein fraction that did not adsorb to Sepharose-heparin (see Ref. 19).

Assays

Formation of methionyl puromycin: this assay system has been described

elsewhere [12]. Protein synthesis in a mammalian pH 5 system: for this assay see Ref. 22. Protein synthesis in a wheat germ pH 4.5 system: a typical assay mixture of 25 μl contained: 80 mM KCl, 0.6 mM magnesium acetate, 20 mM Hepes/KOH, pH 7.6, 1 mM ATP · Mg^{2+}, 0.4 mM GTP · Mg^{2+}, 5 mM creatine phosphate, 1 mM dithiothreitol, 0.2 unit of creatine kinase, 100 μM spermine, 0.3 μM [^{35}S]methionine, 50 μM each of 19 unlabeled amino acids, 1 μg globin mRNA, 10 pmol of wheat germ ribosomes, 30 μg of crude initiation factors fraction BC (see Refs. 18, 22), 2 μg of purified eIF-4B from rabbit reticulocytes, 19 μg of pH 4.5 fraction from wheat germ and eIF-3 as indicated.

The samples were incubated for 30 min at 25°C. Incorporation of [^{35}S]-methionine was measured by hot trichloroacetic acid precipitation. Protein concentrations were determined according to Layne [23].

Results and Discussion

The purification of eIF-3 from wheat germ

The S100 fraction from 4 × 62.5 g of wheat germ was prepared as described under Materials and Methods. This material (16.7 g of protein in 2200 ml) was applied onto a 200 ml column of Sepharose-heparin equilibrated in buffer C containing 100 mM KCl. The proteins that did not adsorb were used as a source of pH 4.5 enzymes. Adsorbed proteins were eluted with buffer C containing 500 mM KCl. The fractions were pooled (445 ml, 3.9 mg/ml) and the proteins fractionated with ammonium sulphate between 0—40% (fraction A) and 40—70% (fraction BC) as described [16,17]. Fraction BC contained no eIF-3 and was further used in the assays as a source of all other initiation factors (except eIF-4B).

Fraction A (6.9 ml, 84.0 mg/ml) was dialyzed against buffer D and applied onto gradients of 15—31% sucrose in buffer D in portions of 30 mg protein per gradient. Centrifugation was carried out in a Beckman SW 27 rotor for 41 h at 26 000 rev./min. The gradients were collected in fractions of 2 ml, and 10 μl of each fraction was tested for eIF-3 activity (see Materials and Methods and Figs. 2 and 3). Active fractions were combined and concentrated by ammonium sulphate precipitation (50% saturation) to 7.9 ml, 7.0 mg/ml, in buffer C containing 50 mM KCl.

This fraction was applied onto a 30 ml column of DEAE-cellulose equilibrated in the same buffer. Adsorbed proteins were eluted with a 120 ml gradient of 50—300 mM KCl in buffer C.

100-μl aliquots of each 2 ml fraction were dialyzed against buffer C containing 100 mM KCl. 10-μl of these aliquots were assayed for eIF-3 activity (see Materials and Methods and Figs. 2 and 3). eIF-3 activity eluted between 180 and 250 mM KCl. Active fractions were pooled and concentrated with ammonium sulphate and dialyzed against buffer C containing 100 mM KCl.

Small (250-μl) portions of this fraction (1.3 ml, 12.8 mg/ml) were applied onto 15—31% gradients of sucrose in buffer D and centrifuged for 18 h at 32 000 rev./min in a Beckman SW 50 rotor. 10-μl aliquots of each fraction (250-μl) were assayed for eIF-3 activity (see Fig. 2).

The eIF-3 containing fractions were kept separate and used for the elucidation of the subunit composition of the factor (see next section, and Fig. 2). A

TABLE I

SUMMARY OF PURIFICATION OF eIF-3

1 unit of activity is the amount of eIF-3 which stimulates the formation of 1 pmol of [^{3}H]methionyl puromycin under standard assay conditions as described [12]. Limiting amounts of factor (5—10 μg, see Fig. 3) were tested for each purification step. eIF-3 activity in the S100 Sepharose-heparin fractions could not be determined because these fractions contained components inhibitory to the assay system.

Step	Protein (mg)	Activity (units)	Recovery (%)	Specific activity (units/mg)
S100	16 900	—	—	—
Sepharose-heparin	1735	—	—	—
Ammonium sulphate (0—40%)	580	12 470	100	21.5
Sucrose gradient I	55.1	2914	23.4	52.9
DEAE-cellulose	16.1	1318	10.6	81.9
Sucrose gradient II	6.0	926	7.4	154.3

summary of the purification procedure of eIF-3 is presented in Table I. 6 mg of purified eIF-3 was obtained from 250 g of wheat germ.

The increase in specific activity of the eIF-3 fractions upon purification is not spectacular: a 7.5-fold enrichment was obtained (compare the ammonium sulphate fraction to the sucrose gradient II fraction).

Apparently, the bulk of contaminating proteins has been removed during the first steps of the purification procedure, suggesting that the ammonium sulphate A fraction is already relatively rich in eIF-3. This assumption is supported by gel analysis of the different eIF-3 fractions (see next section, Fig. 1). Furthermore, a similar observation has been made in rabbit reticulocytes (compare Ref. 1).

Physical characterization of eIF-3 from wheat germ

The analysis on sodium dodecyl sulphate slab gels of eIF-3 fractions from each purification step is shown in Fig. 1, lanes I—V. The relative intensity of a number of bands increases during purification. These bands could be tentatively considered as subunits of eIF-3 and are numbered 1—9 (Fig. 1, lane V). A strong indication that these proteins indeed compose eIF-3 came from a comparison of the activity profiles and gel analysis of the fractions from the sucrose gradient I step (not shown), the DEAE-cellulose step (not shown) and the sucrose gradient II step (Fig. 2). eIF-3 activity in the two assay-systems tested (Fig. 2A) seems to coincide with those fractions containing the numbered polypeptides of Fig. 1, lane V (Fig. 2B).

These polypeptides appear to sediment at approximately 15 S as a large complex comparable to the factor from reticulocytes [1—3], although most of its subunits seem to be different (Fig. 1, lanes V and VI). The interaction between these polypeptides is sufficiently strong to survive a sucrose gradient centrifugation step at 0.5 M KCl, since gel analysis of the fractions of such a gradient shows a pattern indistinguishable from the one of Fig. 2B.

Table II lists the tentative subunits of eIF-3 from wheat germ together with their molecular weights and apparent stoichiometry as derived from the absorbance tracings of the gels of Fig. 2B. The molecular weights range from 26 000
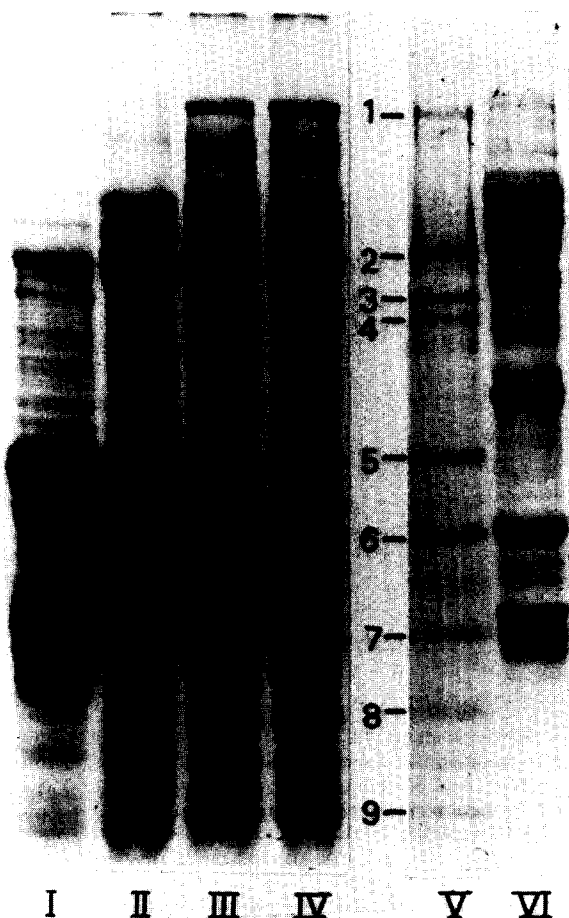
Fig. 1. Gel analysis of eIF-3 fractions. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed on 10% slab gels according to Laemmli [24]. The slots contained in 50 μl sample buffer (see Ref. 24): I, 180 μg eIF-3 following Sepharose-heparin; II, 170 μg eIF-3 following ammonium sulphate fractionation; III, 210 μg eIF-3 following sucrose gradient I; IV, 130 μg eIF-3 following DEAE-cellulose; V, 16 μg eIF-3 following sucrose gradient II; VI, 35 μg eIF-3 from rabbit reticulocytes. The numbers in the figure next to lane V correspond to the positions of the subunits of eIF-3 from wheat germ (see Table II).

to 135 000. It is striking that five out of the ten subunits (2, 5, 6, 8 and 9) appear to be present in equimolar amounts, whereas 1, 3, 4, 7a and 7b occur approximately in a 0.5 to 1.0 ratio with respect to the others.

Similar heterogeneity was observed for the reticulocyte factor [1—3]. It has been postulated that the different forms of eIF-3 possess different biological properties (Refs. 8, 11; see Introduction). On the other hand, the possibility arises that subequimolar proteins originate from limited proteolysis of larger precursors.

It is not clear whether the other proteins present in at least a part of the active fractions of Fig. 2B play any role in eIF-3 functioning. Only those that closely follow the activity profile of Fig. 2A and other purification steps (not shown) are listed in Table II.
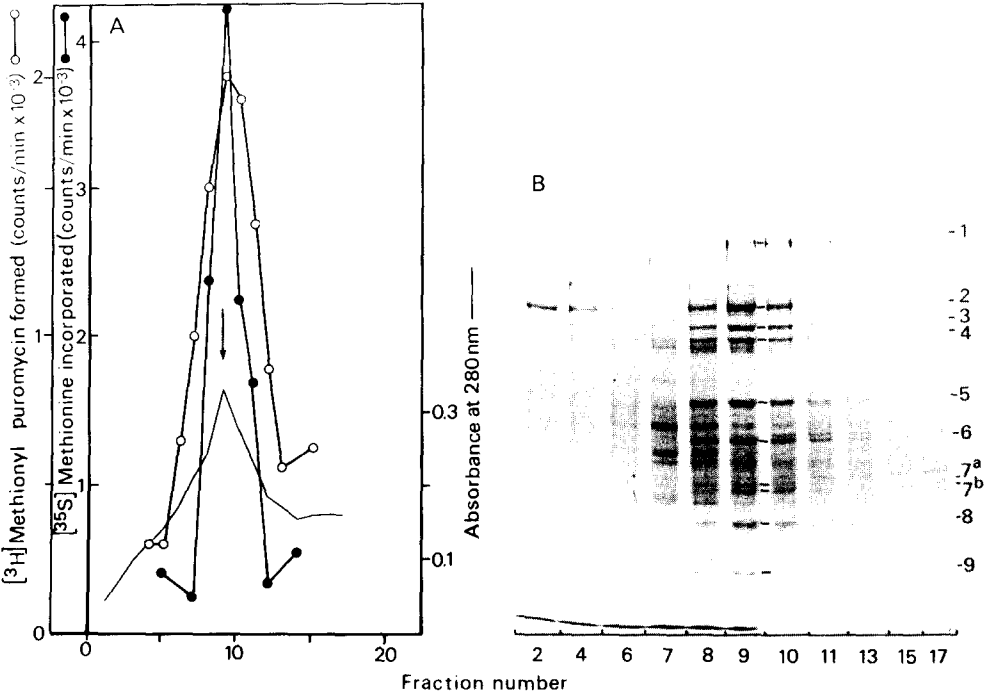
Fig. 2. Analysis of eIF-3 from wheat germ on sucrose gradient. 4.2 mg of eIF-3 following DEAE cellulose was subjected to sucrose gradient centrifugation as described in section Results and Discussion, 0.7 mg of eIF-3 was applied per gradient. A, Activity profile. 10 μl of each fraction were assayed for eIF-3 activity in the assay systems for methionyl puromycin formation (I) and for protein synthesis in a pH 4.5 system derived from wheat germ (II) as described under Materials and Methods. A background synthesis obtained in the absence of eIF-3 (3000 cpm (assay I, ○) or 4000 cpm (assay II, ●)) has been subtracted. The arrow in the figure corresponds to the position at which β-galactosidase sedimented in a control gradient. B, Gel analysis. 80 μl of each fraction were electrophoresed on a 10% slab gel according to Laemmli [24]. The lines in the figure indicate the position to which to supposed subunits of eIF-3 migrated.

TABLE II

SUBUNIT COMPOSITION OF eIF-3

Molecular weights were calculated from the gels of Figs. 1 and 2 with the following protein standards: phosphorylase b (90 000), bovine serum albumin (67 500), ovalbumin (45 000) and chymotrypsinogen (25 000). Stoichiometric determinations were made from densitometric tracings as described [1]. The values obtained were normalized to protein No. 2.

Protein No.	Stoichi- ometry	Molecular weight ($\times 10^{-3}$)
1	0.4	135
2	1.0	95
3	0.6	87
4	0.5	83
5	1.0	60
6	1.0	49
7a	0.7	39.5
7b	0.5	38.5
8	1.0	32
9	0.9	26

The molecular weight of the non-dissociated factor may vary, depending on the precise subunit composition, between 400 000 and 500 000, which is in good agreement with a sedimentation coefficient of 15 S.

Biological characterization of eIF-3 from wheat germ

The activity of eIF-3 from wheat germ was compared to that of the analogous factor from rabbit reticulocytes in a number of assay systems. The results are shown in Fig. 3. The wheat germ factor was virtually inactive in the pH 5 assay system derived from mammalian sources (reticulocytes and liver) which is shown in Fig. 3B. However, the factors from wheat germ and reticulocytes were comparably active in the pH 4.5 system derived from wheat germ (Fig. 3A).

The activity of the wheat germ factor in the assay for methionyl puromycin formation is approximately 15% of that observed for the reticulocyte factor (Fig. 3C).

Similar observations have been made for eIF-2 from wheat germ [12], which turned out to be considerably less active than the reticulocyte factor when tested in assay systems derived from mammalian sources.

A possible explanation for this difference in activity could lie in the presence of contaminating components in the wheat germ factor preparations which are inhibitory to these (heterologous) assay systems. An alternative explanation is provided if one assumes that species-specific differences exist between the factors from the two sources which cause them to interact poorly with heterologous initiation factors and ribosomes. Their basic mode of action, however, is
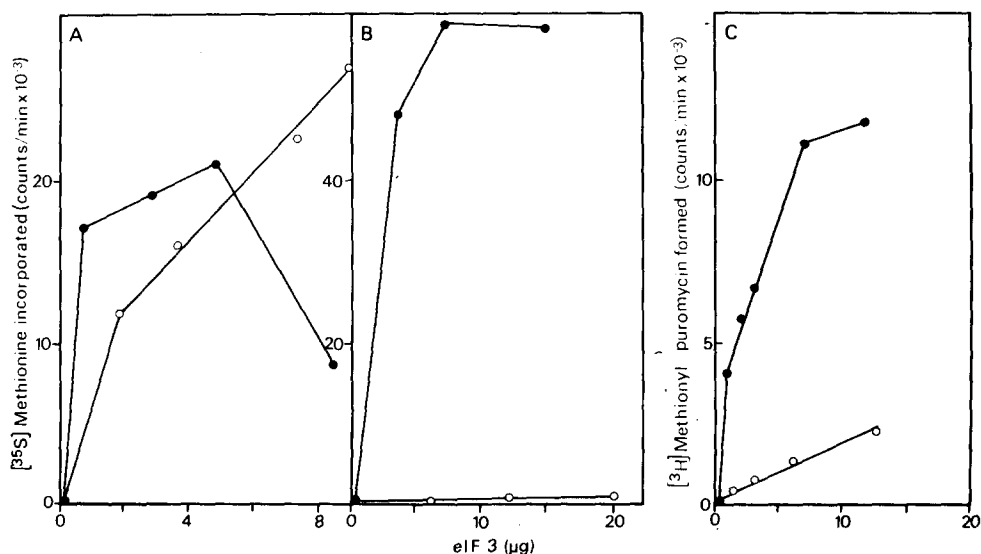

Fig. 3. Activity of eIF-3 from wheat germ and from rabbit reticulocytes. Amounts as indicated of eIF-3 from wheat germ (○) or from rabbit reticulocytes (●) were tested in various assay systems: A, pH 4.5 assay system derived from wheat germ; B, pH 5 assay system derived from mammalian sources; and C methionyl puromycin synthesis. The experimental details have been described under Materials and Methods and in the legend to Fig. 2B.

similar since eIF-3 from wheat germ shows activity (albeit little) in a model assay system for protein-synthesis initiation (such as the methionyl puromycin formation) derived exclusively from mammalian sources.

eIF-3 from rabbit reticulocytes [8,25] or rat liver [4] is capable of dissociating 80-S ribosomes or preventing the reassociation of the 40-S and 60-S ribosomal subunits.

A recent report [26] indicates that in wheat germ such an activity is displayed by a protein distinct from eIF-3. Therefore, we checked the dissociation and anti-association activity of our eIF-3 preparation from wheat germ, utilizing wheat germ ribosomes.

No activity of wheat germ eIF-3 could be detected in these assays (results not shown), indicating that in wheat germ the dissociation of ribosomes into subunits is not mediated by eIF-3. However, the possibility still exists that eIF-3 stimulates the dissociation activity of other factors.

Acknowledgements

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