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PURIFICATION OF FREE EUKARYOTIC INITIATION FACTORS eIF-4A AND eIF-4D ON CIBACRON BLUE F3G-A

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Summary

The eukaryotic initiation factors eIF-4A and eIF-4D are almost exclusively found in the supernatant of a reticulocyte lysate. The basic steps of general purification schemes failed because these factors do not bind to Sepharose-heparin or, in the case of eIF-4A, to phosphocellulose and a new procedure had to be devised. Cibacron Blue F3G-A proved to be a successful alternative in the purification of these factors from the excessive load of proteins found in the supernatant.

Introduction

The protein synthesis initiation factors eIF-4A and eIF-4D * are almost exclusively found in the supernatant of lysates from rabbit reticulocytes [1]. Both factors are not adsorbed to Sepharose-heparin whereas the purification of eIF-4A is further complicated by its lack of binding to phosphocellulose. Cibacron Blue F3G-A [2] has been used for the isolation of various enzymes possessing the dinucleotide fold or binding sites for adenine-containing nucleotides such as hydrogenases and kinases [3], restriction enzymes [4], RNA ligase [5] and DNA polymerase [6]. Therefore, interaction of the chlorotriazine dye with initiation factors could be in line with the observations mentioned in these papers [3—6]. A procedure is described which isolates both factors in sufficient quantity and with a purity of 98%. One molecule of eIF-4A is present per two ribosomes in the original lysate.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

* Nomenclature in accordance with Anderson et al. [7].

Materials and Methods

Materials

The following components were prepared as described: ribosomal subunits by Schreier and Staehelin [8]; pH 5 enzymes by Falvey and Staehelin [9]; hemoglobin 9 S mRNA by Krystosek et al. [10]; reticulocyte lysate by Schreier and Staehelin [8] with a few modifications [11]; tRNA from rat liver, rabbit liver or rabbit reticulocytes by Rogg et al. [12] and [^{3}H]methionyl-tRNA by Stanley [13]. Crude initiation factors were precipitated with ammonium sulphate from the high salt ribosomal wash according to Schreier and Staehelin [14]: fraction A (0—40% saturation of ammonium sulphate); fraction B (40—50%) and fraction C (50—70%). The following buffer was used: 20 mM Tris-HCl, pH 7.6, 10% glycerol and 5 mM 2-mercaptoethanol (buffer D). DEAE-cellulose DE 52 was purchased from Whatman.

Methods

Cibacron Blue F3G-A (Serva) was coupled to Sepharose according to the method of Heyns and de Moor [15] with a few modifications: Sepharose CL-6B (Pharmacia) was used instead of Sepharose 6B and the coupling reaction was performed at 65°C for 2 h. The blue gel was washed extensively with water, then with 1.5 M KCl and again with water. It was stored in 0.1 M KCl with the addition of 0.05% NaN_3. Approx. 1 mg of the dye was bound to 1 ml of the wet gel as determined by the method of Chambers [16].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed on 12.5% slab gels according to Laemmli [17] at 200 V for 4 h.

Protein concentrations were estimated from the absorbance ratio at 280 nm and 260 nm as described by Layne [18] or measured according to Bradford [19]. Affinity chromatography on Sepharose 6B-heparin was performed as reported previously [11]. Volumes of gel used in the purification steps to be described refer to the volume that the gel occupies in a column under normal running conditions.

Assays

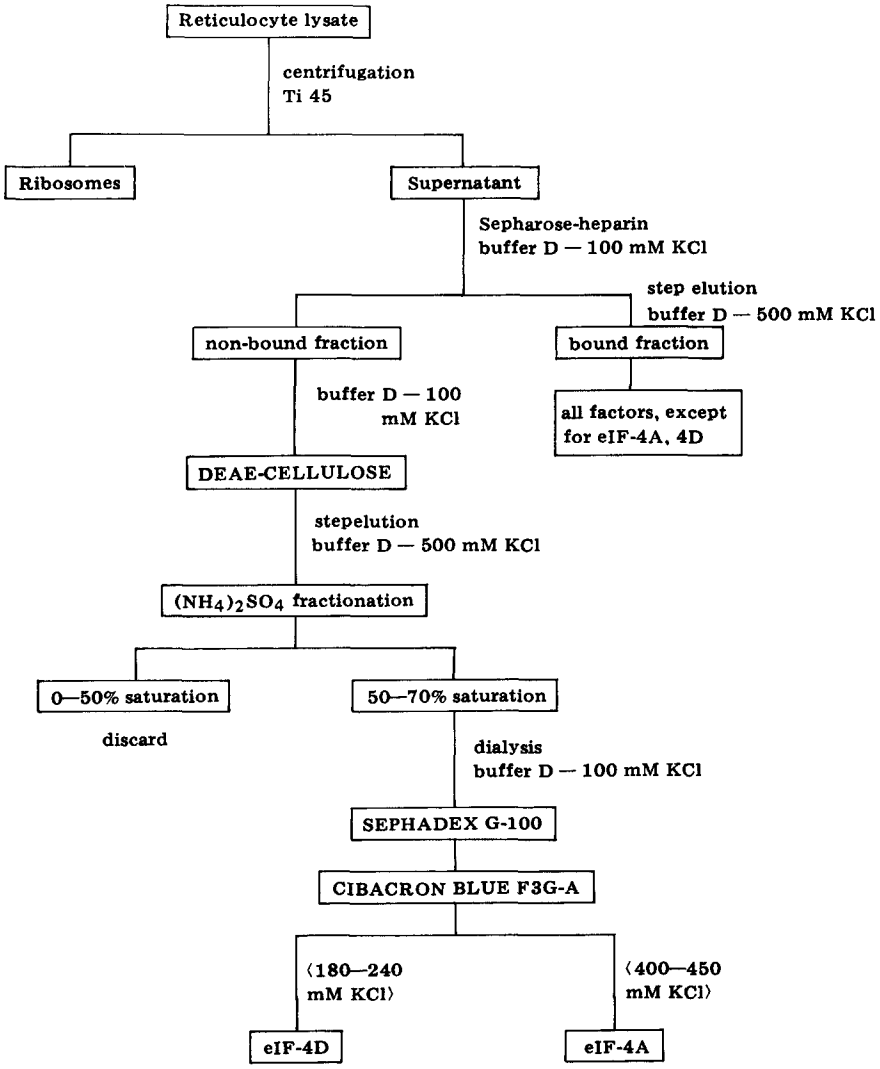
eIF-4A activity was assayed in a mRNA-dependent cell-free system. A 10 μl incubation mixture contained 20 mM Hepes/KOH, pH 7.6, 1 mM ATP-$Tris_3$, 0.4 mM GTP-$Tris_3$, 5 mM creatine phosphate-$Tris_2$, 1 mM dithiothreitol, 0.2 unit of creatine kinase (EC 2.7.3.2), 2 mM magnesium acetate, either 120 mM potassium acetate or 95 mM KCl, 100 μM spermine-HCl_4, 7 μM [^{14}C]leucine (spec. act. 330 Ci/mol), 50 μM each of 19 nonlabelled amino acids, 0.03 A_{260} unit of 40 S subunits and 0.06 A_{260} unit of 60 S subunits, 0.01 A_{260} unit of globin mRNA, 0.04 A_{260} unit of tRNA, 4 μg initiation factors of fraction A (see Materials), 5 μg of fraction B and 0.16 μg of eIF-4C. The samples were incubated for 60 min at 30°C and [^{14}C]leucine incorporation was measured as hot trichloroacetic acid insoluble counts [1]. Methionyl-puromycin formation was used in the assay for eIF-4D activity and was carried out as described by Thomas et al. [1].

Results

The purification of both factors proceeded according to Scheme I.

The non-bound fraction of the Sepharose-heparin column, 2000 ml with a protein content of 80 mg/ml, was treated in a batch operation with 330 ml of DEAE-cellulose for 24 h with continuous stirring. The gel was collected on a Buchner funnel by suction and washed with 4 l of buffer D containing 100 mM KCl. Re-extraction of the non-adsorbed material yielded only small quantities of eIF-4A and no eIF-4D and this step can be omitted.

The gel paste was poured into a column and, after settling, a step elution with buffer D with 500 mM KCl was carried out. After dialysis the proteins



Scheme I. Flow sheet for the purification of free eIF-4A and eIF-4D.

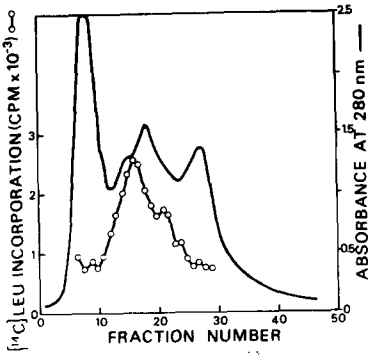


Fig. 1. The activity of eIF-4A in an elution pattern, on Sephadex G-100, of proteins precipitated with $(NH_4)_2SO_4$ between 50—70% saturation. The column, 94 X 2.6 cm, was developed at a flow rate of 20 ml/h, the fraction volume was 10 ml. Protein synthesis was carried out with 4 µl aliquots from the fractions as described in Materials and Methods. [^{14}C]LEU, [^{14}C]leucine.

were precipitated with ammonium sulphate as follows: precipitation between 0—50% saturation gave 979 mg of protein which contained only minor amounts of both factors, whereas the proteins precipitated between 50—70% (313 mg) showed factor activity. This protein fraction was dissolved in buffer D with 100 mM KCl, dialysed against the same buffer and layered onto a Sephadex G-100 column, whereafter elution took place in buffer D supple-

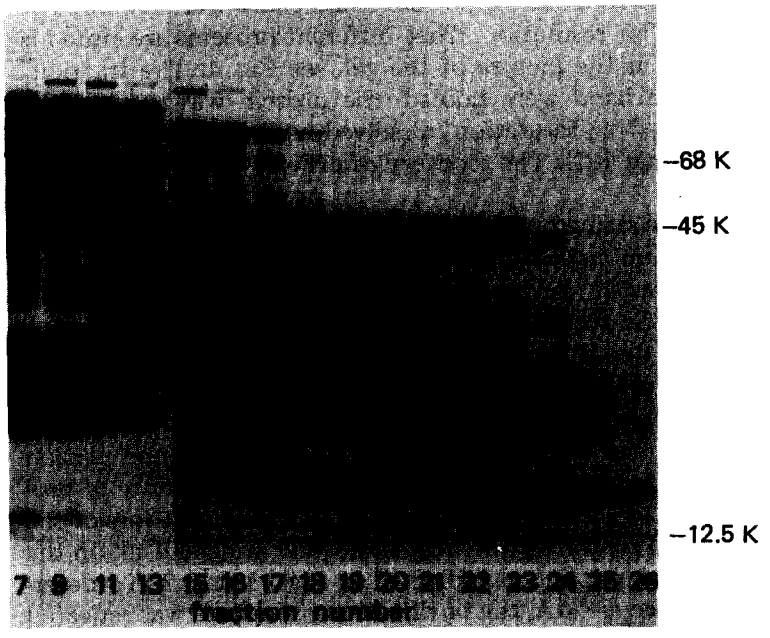


Fig. 2. Sodium dodecyl sulphate gel electrophoresis of the Sephadex G-100 fractions. The fractions 16—23 were selected for further purification. The fractions preceeding fraction 16 were omitted because of heavy contamination by other proteins. Aliquots of 25 µl were added to 75 µl of sample buffer and applied on a 12.5% slab gel. The electrophoresis usually lasted 4 h. The gels were stained overnight with 0.05% Fast Green in 10% acetic acid and 40% methanol in water and destained in the same solution without the dye.

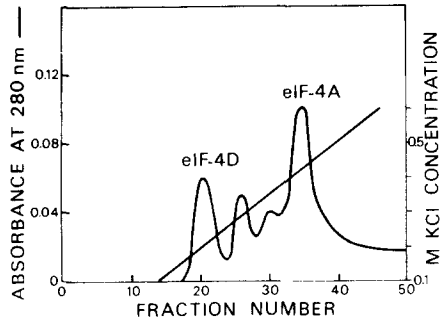


Fig. 3. The elution pattern of proteins desorbed from Cibacron Blue F3G-A with a salt gradient. From the Sephadex fractions, 68 ml was brought on the blue gel. Unadsorbed proteins were removed overnight with buffer D containing 100 mM KCl. The salt gradient of 100 to 600 mM KCl was delivered over a period of 24 h and was monitored with a conductivity meter. eIF-4D eluted at 220 mM KCl and eIF-4A at 430 mM KCl. Column size 30.5 × 2.5 cm, flow rate 20 ml/h, fraction size 10 ml.

mented with 100 mM KCl. The results are shown in Fig. 1.

Comparison of the proteins by analysis on slab gel electrophoresis revealed the presence of eIF-4D in the fractions which already contained eIF-4A (Fig. 2). The fractions from 16 to 23 were pooled, applied onto the Cibacron Blue column and eluted with buffer supplemented with 100 mM KCl until the nonadsorbed material had been effectively removed.

The bound proteins were eluted with a linear gradient from 100 to 600 mM KCl in buffer D. As can be seen from Fig. 3, four small peaks were recorded at 280 nm absorbance with a fair resolution. That different proteins are eluted is demonstrated more clearly in the picture of the gel, see Fig. 4. The fractions containing only one protein band were pooled, the protein was precipitated with ammonium sulphate at 75% saturation, dissolved, and dialysed overnight against buffer D with 100 mM KCl. The recovery of eIF-4A was 6.5 mg and of eIF-4D 1.6 mg.

Densitometric scans of the stained proteins are given in Fig. 5. The biological activity of pure eIF-4A is demonstrated in the experiments depicted in Fig. 6. The specific activity measured in the methionyl-puromycin formation is 0.8 pmol methionyl puromycin formed per 0.15 μg eIF-4D added.

As loss of activity occurs due to incomplete pooling of the factor-containing fractions, no real estimate can be given of the actual amount of factors present. When the preceding fractions were included (see Fig. 1), the resolution of the peaks was severely impaired and resulted in no binding of eIF-4D and elution of eIF-4A at a lower KCl concentration, probably due to the presence of proteins with a higher affinity for Cibacron Blue in these fractions. The loss of the factors can be minimized by rerunning the high molecular weight peak on the Sephadex G-100 column and pooling as indicated in Figs. 1 and 2. To determine the total amount of free eIF-4A, the purification procedure was repeated with 530 ml of the non-bound fraction which ran through on the Sepharose-heparin column. Fractionation by ammonium sulphate was now carried out between 45—75% and this fraction contained all eIF-4A. After dialysis against buffer D with 100 mM KCl, the sample was directly applied onto Cibacron Blue F3G-A and eluted with a KCl gradient. All fractions containing eIF-4A

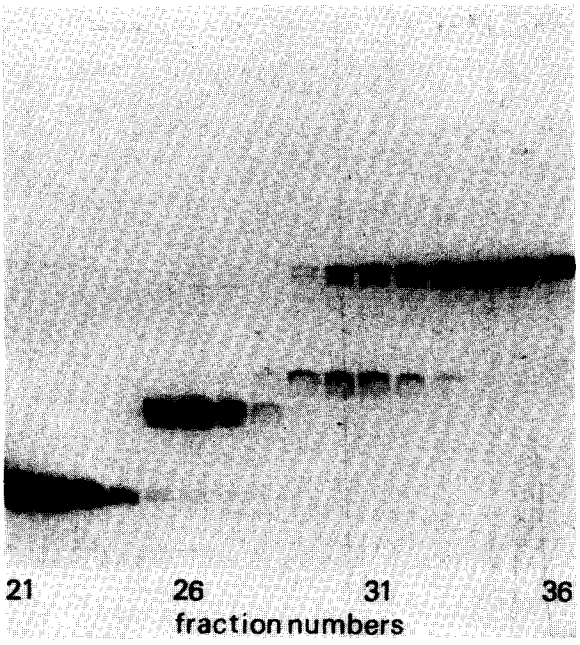


Fig. 4. Sodium dodecyl sulphate gel electrophoresis of some Cibacron Blue fractions used for the selection of the uncontaminated factors. The four proteins elute in order of increasing molecular weight with eIF-4D in the leading fractions and eIF-4A in the trailing ones. The fractions were directly applied to the gel. Due to the high salt load the stacking was incomplete and band broadening occurred. After precipitation of the pooled fractions followed by dialysis this phenomenon disappeared, indicating the absence of impurities within the band width of the depicted proteins. Aliquots of 50 µl were added to 50 µl of sample buffer. Further particulars are given in Fig. 2.

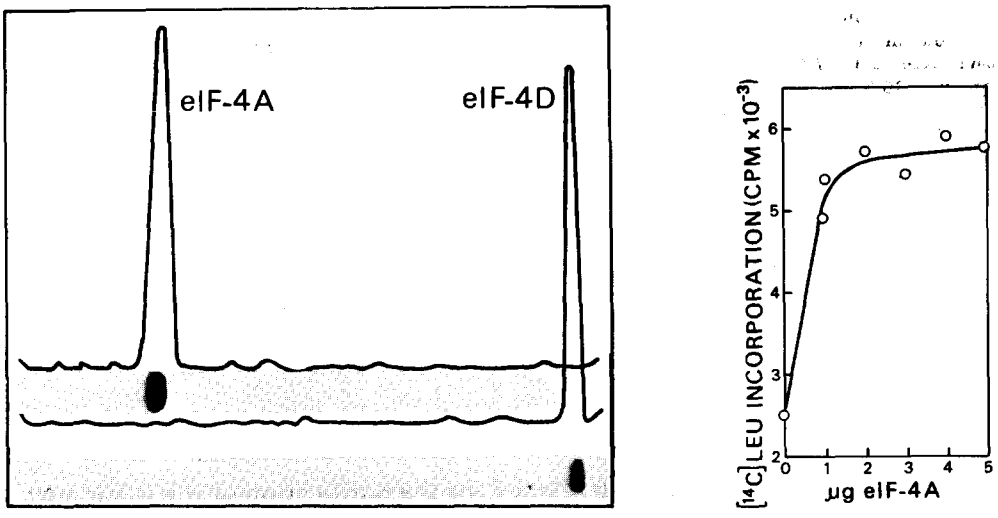


Fig. 5. Densitometric scans of the two initiation factors after pooling of the pure Cibacron Blue fractions and precipitation with 75% ammonium sulphate followed by dialysis against buffer D supplemented with 100 mM KCl. The proteins, 20 µg of eIF-4A and 7 µg of eIF-4D, were stained as described in Fig. 2. The destained gels were scanned at 610 nm in a Vitatron spectrophotometer. Integration of the scans showed both factors to have a purity of 98%.

Fig. 6. The dependence of the protein synthesizing system on pure eIF-4A. The high blank value is probably due to the presence of eIF-4A in fraction B (see Materials and Methods). [^{14}C]LEU, [^{14}C]leucine.

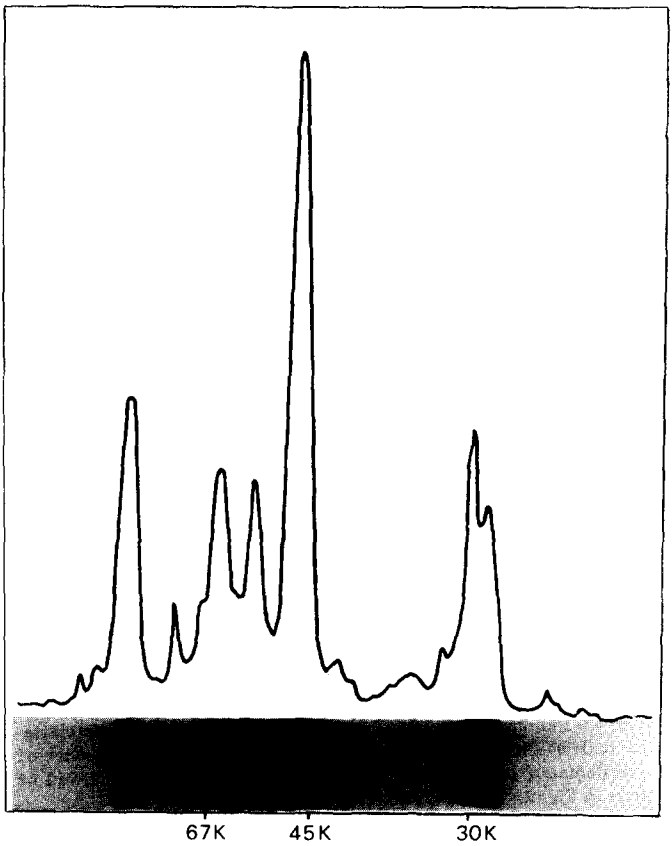


Fig. 7. Densitometric scan of the pooled fractions containing all eIF-4A eluted from a 75 ml Cibacron Blue column in an experiment to determine the total amount of free eIF-4A in 530 ml supernatant. Scans were made of four tracks containing different amounts of protein. The purity of eIF-4A in these scans was 30.5, 32, 32 and 36%. All proteins with a molecular weight lower than approx. 25 000, i.e. eIF-4D, are absent.

were pooled, whereafter this sample was analysed by gel electrophoresis. The purity of eIF-4A in this preparation is 32% (Fig. 7) with a total amount of protein of 14.1 mg. From these data, the actual amount of eIF-4A can be calculated to be 4.5 mg. Using a M_r of 50 000 for eIF-4A and the presence of 300 pmol of ribosomes per ml of lysate this indicates the existence of one molecule of free eIF-4A for every two ribosomes.

Discussion

A rapid procedure has been developed for the purification of the eukaryotic initiation factors eIF-4A and eIF-4D which both occur almost exclusively in the supernatant of reticulocyte lysates. When care is taken to avoid the high molecular weight proteins of the Sephadex step which show increased affinity for Cibacron Blue (see Fig. 7), gradient elution on this affinity column results in almost pure eIF-4A and eIF-4D. In investigations to elucidate the function of eIF-4A, the presence of excessive amounts of this factor in the supernatant

have to be taken into consideration. Until now no clue can be given as to whether such an excess is necessary to fulfil its role in mRNA binding to the 40 S subunit as proposed by several investigators [14,20]. Care must be taken in the interpretation of these data as to the possession of specific affinity regions for adenine-containing nucleotides on the two factors as the dye molecule has been shown to exhibit nonspecific interactions [21,3].

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