Purification and Some Properties of a Protein Factor Binding and Deacylating Initiator Transfer Ribonucleic Acid

By PETR POHLREICH,* OLDŘICH KRÍŽ, ZDENA TUHÁČKOVÁ,
ZDENĚK DUŠEK and JAN HRADEC

Department of Biochemistry, Oncological Institute, 18000 Prague 8, Czechoslovakia

(Received 8 August 1978)

1. A protein factor promoting the binding of initiator tRNA to the 40S ribosomal subunit was purified to homogeneity (more than 2500-fold) from rat liver cytosol. It has a mol.wt. of 265 000 and is composed of four subunits of identical molecular weight. 2. This factor directs the binding of methionyl-tRNA^{fMet} and to a lesser extent also of *N*-acetylphenylalanyl-tRNA, but not of methionyl-tRNA^{Met} or phenylalanyl-tRNA, to the smaller ribosomal subunit at high concentrations of GTP (8–10 mM) with an optimum at pH 4.0. As evidenced by sucrose-density-gradient centrifugation, initiator tRNA becomes bound to the 40S subunit or to 80S ribosomes. 3. A deacylase activity specific for methionyl-tRNA^{fMet} is associated with the pure factor. The factor significantly stimulates the translation of natural message in systems containing polyribosomes and both purified peptide-elongation factors. 4. The factor binds initiator tRNA or GTP to form unstable binary complexes and forms a ternary complex with methionyl-tRNA^{fMet} and GTP. This complex is relatively stable. 5. In the absence of any cofactors the factor forms a stable complex with 40S and 80S ribosomes. This preformed ribosomal complex binds efficiently initiator tRNA at pH 7.5 and low concentrations of GTP (1–2 mM). The ternary complex of the factor with methionyl-tRNA^{fMet} and GTP may be liberated from this ribosomal complex. 6. A protein factor capable of promoting the binding and simultaneously the deacylation of initiator tRNA may apparently have a regulatory function in physiological gene translation by removing an excess of methionyl-tRNA^{fMet} not required for translation.

In eukaryotic cells at least two types of peptide-initiation factors apparently exist which promote the binding of initiator tRNA to the smaller ribosomal subunit.

The first type represented by the MP factor of Merrick's group (Safer *et al.*, 1975) requires GTP, but the reaction is template-independent. A ternary complex of the factor with GTP and initiator tRNA appears to be an intermediate. Isolation of such a factor from different eukaryotic cells has been described by several authors (Chen *et al.*, 1972; Dettman & Stanley, 1972; Cashion & Stanley, 1974; Herrera *et al.*, 1977; Schreier *et al.*, 1977).

The other pathway of binding methionyl-tRNA^{fMet} to the 40S ribosomal subunit requires template but not GTP. This reaction apparently does not involve the formation of intermediary complexes and takes place at relatively low concentrations of Mg²⁺. Protein factors catalysing this reaction, which are similar to Merrick's M-1 factor (Merrick & Anderson, 1975), have also been described by several authors (Gasior & Moldave, 1972; Leader & Wool, 1972; Zasloff &

* Present address: Second Institute of Medical Chemistry and Biochemistry, Charles University Faculty of General Medicine, 12000 Prague 2, Czechoslovakia.

Ochoa, 1973; Grummt, 1974; Cimadevilla & Hardesty, 1975).

Protein factors directing the binding of initiator tRNA to the smaller ribosomal subunit by different pathways are apparently present side by side in single eukaryotic cells. Gupta's group (Majumdar *et al.*, 1977) has partially purified three protein factors involved in this binding reaction in rabbit reticulocytes. Herrera *et al.* (1977) have provided evidence that three binding factors specific for the natural initiator tRNA are present in rat liver cells. Adams *et al.* (1975) have concluded that two different and rather independent pathways of the binding of methionyl-tRNA^{fMet} to ribosomes apparently exist in rabbit reticulocytes. It seems that the search for initiation factors promoting the binding of initiator tRNA to 40S ribosomal subunits has not yet been completed, and further reports on the isolation of factors required for the binding reaction and possibly also on their different mechanisms are to be expected.

The present paper describes the purification to homogeneity of a protein promoting the binding of initiator tRNA to the 40S ribosomal subunit. This factor is different from others reported in the respect that it shows, even in its homogeneous form, a

deacylase activity specific for methionyl-tRNA^{fMet}. It forms intermediary binary and ternary complexes, and evidence is provided that this factor is involved in protein synthesis. Preliminary reports on a partial purification of this protein have been published by Hradec *et al.* (1974b) and by Hradec (1975).

Materials and Methods

Animals

Wistar rats of both sexes (120–150 g) bred in this laboratory were fed on standard pelleted diet.

Chemicals and radiochemicals

GTP (sodium salt) was a product of Koch–Light Laboratories, Colnbrook, Bucks., U.K., and tRNA from *Escherichia coli* B was obtained from Calbiochem, San Diego, CA, U.S.A. Adenylyl(3′-5′)-uridylyl(3′-5′)guanosine (ApUpG) was purchased from Boehringer, Mannheim, West Germany. tRNA from rat liver was isolated as described by Rogg *et al.* (1969). L-[³⁵S]Methionine (100 Ci/mmol) and L-[³H]phenylalanine (12.5 Ci/mmol), [³H]GTP (5.28 Ci/mmol) and [³²P]GTP (10.3 Ci/mmol) were products of The Radiochemical Centre, Amersham, Bucks., U.K. Rat liver tRNA was charged with L-[³⁵S]methionine in the presence of partially purified aminoacyl-tRNA synthetases from *E. coli* B as described by Matthaai *et al.* (1966). This preparation is designated as 'unfractionated methionyl-tRNA^{fMet}'. If required, both tRNA^{fMet} species were separated by BD(benzoylated)-cellulose chromatography by the procedure of Stanley (1972). tRNA^{fMet} was then charged with L-[³⁵S]methionine in the same way as unfractionated methionyl-tRNA^{fMet}. tRNA^{fMet} was charged with the same amino acid in the presence of partially purified aminoacyl-tRNA synthetases from rat liver essentially as described by Stanley (1972). The preparation of L-[³H]phenylalanyl-tRNA from *E. coli* B by using synthetases from the same source has been described by Hradec *et al.* (1971). *N*-Acetylphenylalanyl-tRNA and *N*-acetylmethionyl-tRNA were prepared by the acetylation of [³H]phenylalanyl-tRNA and [³⁵S]methionyl-tRNA^{fMet} as described by Haenni & Chapeville (1966).

Isolations

Rat liver homogenate and the postmitochondrial supernatant of the same tissue were isolated as described by Hradec *et al.* (1971). This fraction was used as the starting material for the isolation of the initiation factor. For the preparation of ribosomal subunits, postmitochondrial supernatants were passed through columns of heparin bound to

Sephacrose 4B (Hradec & Dušek, 1978). The fraction not retained on columns was adjusted by adding 10% (w/v) sodium deoxycholate to a final concentration of 1%, and centrifuged at 310 000g for 60 min at 4°C in a 70Ti rotor in a Beckman L 5-65 ultracentrifuge. Polyribosomes were converted into 80S ribosomes by treatment with puromycin, and both ribosomal subunits were separated by centrifugation in the Ti-14 zonal rotor as described by Hradec *et al.* (1978). Partial purification of both peptide-elongation factors has been described in the same paper. Polyribosomes for experiments on peptide elongation were purified by sedimentation through discontinuous sucrose gradients with subsequent washings as described by Hradec *et al.* (1971). Sucrose-density-gradient centrifugation was performed as described by Hradec *et al.* (1974a).

Incubation procedures

Incubation mixtures for the standard binding assay contained, in a final volume of 0.1 ml: 20 mM-Tris/HCl buffer, pH 7.5 (or 20 mM-potassium acetate buffer, pH 4.0, where indicated), 5 mM-MgCl₂, 7 mM-2-mercaptoethanol, 10 mM-GTP, 2 pmol of L-[³⁵S]-methionyl-tRNA^{fMet} (or the unfractionated preparation, where indicated), 0.1 A₂₆₀ unit of 40S ribosomal subunits, and quantities of the initiation factor as indicated in the legends for Figs. 2 and 3. Mixtures were incubated at 37°C for 2 min. Incubation mixtures for the assay of the formation of intermediary complexes contained, in a final volume of 0.1 ml: 20 mM-Tris/HCl buffer, pH 7.5 (or 20 mM-potassium acetate buffer, pH 4.0, where indicated), 3 mM-MgCl₂, and quantities of GTP, [³⁵S]methionyl-tRNA^{fMet} and of the factor as indicated for individual experiments. Mixtures were incubated at 37°C for 10 min unless otherwise stated. The binding reactions were terminated by the addition of ice-cold washing buffer of the same ionic composition as incubation mixtures, suspensions were filtered through cellulose nitrate filters (Synpor, Spolana, Uhřetíněves, Czechoslovakia) and filters were washed with the same buffer. After drying, the radioactivity retained on filters was assayed as described by Hradec (1972). A complex of the factor with ribosomes was formed in incubation mixtures containing, in a final volume of 1.0 ml: 20 mM-Tris/HCl buffer, pH 7.5, 3 mM-MgCl₂, 120 mM-KCl, 1.5 A₂₆₀ units of 40S subunits (or 10 A₂₆₀ units of 80S ribosomes) and quantities of the factor as indicated in the legend for Fig. 10. They were incubated at 37°C for 10 min and chilled in ice afterwards. The reaction mixtures were then layered on to discontinuous gradients of 30% (v/v) glycerol and 10% (v/v) glycerol buffered in the same way as the reaction mixtures. Centrifugation was done in the 8×11 ml rotor of a VAC 601 ultracentrifuge (H. Janetzki, Engelsdorf/Leipzig, German Democratic

Republic) at 150000g and 4°C for 15 h. Ribosomal pellets were rinsed with water and suspended in the same buffer as that used for their preincubation. The binding activity of these ribosomal complexes of the factor was assayed in incubation mixtures containing, in a final volume of 0.1 ml: 20 mM-Tris/HCl buffer, pH 7.5, 3 mM-MgCl₂, 120 mM-KCl, 2 mM-GTP, 2 pmol of [³⁵S]methionyl-tRNA^{fMet} and 0.2 A₂₆₀ unit of 40S ribosomal subunits (or 1.0 A₂₆₀ unit of 80S ribosomes) preincubated with the factor. Unless otherwise stated, the mixtures were incubated at 37°C for 2 min and filtered through cellulose nitrate filters as described by Hradec *et al.* (1978). Incubation mixtures for the assay of methionyl-tRNA^{fMet} deacylase activity and their processing have been described by Hradec (1975). Incubation mixtures for peptide elongation dependent on the natural message contained in a final volume of 0.1 ml: 50 mM-Tris/HCl buffer, pH 7.5, 5 mM-MgCl₂, 60 mM-KCl, 7 mM-2-mercaptoethanol, 6 µg of elongation factor 1 and 22 µg of elongation factor 2 protein, 120 µg of polyribosomes, 25 pmol of [³H]phenylalanyl-tRNA and quantities of the initiation factor as indicated in the legend for Fig. 6. They were incubated at 37°C for 30 min. Further processing of the samples and assay for radioactivity have been described by Hradec *et al.* (1971).

Polyacrylamide-gel electrophoresis

Analyses were performed in cylindrical gels by using the discontinuous gel and buffer system of Ornstein (1965); proteins were stained with Coomassie Brilliant Blue R-250 as described by Weber & Osborn (1969). Electrophoresis in the presence of sodium dodecyl sulphate was performed essentially as described by Weber & Osborn (1969). As molecular-weight standards catalase (240000), bovine serum albumin (67000), egg albumin (45000) and chymotrypsinogen (25000) (Serva Feinbiochemica, Heidelberg, West Germany) were used. Gels were scanned in visible light by using the G II photometer of Carl Zeiss, Jena, German Democratic Republic.

Preparative electrophoresis was performed in slabs of polyacrylamide (80 mm wide, 100 mm long, thickness 30 mm) in an apparatus of our own construction (O. Kříž, unpublished work). Essentially the discontinuous system of gels and buffers described by Ornstein (1965) was used. Up to 4 ml samples containing 10–15 mg of protein, 5% (v/v) glycerol and 0.02% (w/v) of Bromophenol Blue were applied to the stacking gel. For the sharpening of the sample zone, electrophoresis was run at 60 V for 10 min. After that, the electrophoretic separation was performed at 80 V and 180 mA for 230 min at 5°C. After the separation, 5 mm-wide vertical stripes were cut from the middle portion and margins of the gel slab and stained as described for analytical electro-

phoresis. In this way the band containing the factor was localized at a distance of 13 mm from the upper edge of the spacer gel. For the elution of proteins essentially the procedure of Ziola & Scraba (1976) was followed. A transverse slot was cut in the slab 15 mm from the upper edge, and this hole was filled with a thick suspension of hydroxyapatite (Serva Feinbiochemica). Electrophoretic elution of proteins into hydroxyapatite was performed at 100 V and 200 mA for 90 min. The factor was eluted from hydroxyapatite with 500 mM-potassium phosphate buffer, pH 7.0.

For the determination of the molecular weight of the native factor protein, a column (diameter 15 mm, length 900 mm) of Sephadex G-200 equilibrated against 20 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-KCl was used. A constant flow rate of 4.1 ml/h was maintained by a peristaltic pump. Ferritin (364000), catalase (240000), bovine serum albumin (67000) and egg albumin (45000) (Serva Feinbiochemica) were used as standard proteins. The absorbance of the eluate was continuously recorded at 254 nm.

Results

Purification of the factor

All operations were performed at 0–4°C. Standard buffer contained 10 mM-Tris/HCl buffer, pH 7.5, 5 mM-KCl and 7 mM-2-mercaptoethanol. A summary of the purification is given in Table 1.

Step 1: isolation of rat liver cytosol. Postmitochondrial supernatant was centrifuged at 110000g for 2 h and the upper two-thirds of the supernatant were carefully separated.

Step 2: precipitation with (NH₄)₂SO₄. Finely powdered (NH₄)₂SO₄ was slowly added to the cytosol with constant stirring to 35% saturation (209 mg/ml). After all salt had been dissolved, the suspension was stirred for an additional 60 min. The suspension was centrifuged at 12000g (rotor 10 × 85 ml, H. Janetzki VAC 20 centrifuge), the precipitate was discarded and the supernatant was adjusted to 70% saturation with (NH₄)₂SO₄ (238 mg/ml). After centrifugation under the same conditions, the precipitate was dissolved in the standard buffer and dialysed overnight against the same buffer.

Step 3: gel filtration on Sepharose 4B. After centrifugation of the material from step 2 at 12000g for 10 min, the supernatant was applied to a column of Sepharose 4B (diameter 100 mm, length 920 mm; K 100/100 column of Pharmacia, Uppsala, Sweden) equilibrated against the standard buffer. The column was eluted with the same buffer at a flow rate of 240 ml/h (maintained by a peristaltic pump). The A₂₆₀ of the eluate was continuously recorded, and

Table 1. *Purification of the factor from 200g of rat liver*

A unit of activity is defined as 1 pmol of [35 S]methionyl-tRNA^{Met} bound to the 40S ribosomal subunit under standard assay conditions (see the Materials and Methods section); specific activity is expressed as units/mg of protein.

Step	Total volume (ml)	Total protein (mg)	Activity (units)	Specific activity	Yield (%)
1. Cytosol	425	36125	20254	0.55	100
2. 35–70% (NH ₄) ₂ SO ₄ saturation	120	19920	19649	0.98	97
3. Gel filtration on Sepharose 4B	24	1020	13681	13.41	67
4. First DEAE-cellulose chromatography	11	112	9519	84.96	46
5. Second DEAE-cellulose chromatography	9.5	47.5	8348	176.73	41
6. Preparative electrophoresis	4	3.3	4554	1380.00	22

fractions (23 ml each) were collected. The elution pattern from Sepharose 4B was described by Hradec *et al.* (1974b). Fractions showing binding activity in the standard assay were pooled, proteins were precipitated with (NH₄)₂SO₄ at 70% saturation (472 mg/ml), and precipitate was dissolved in the standard buffer and dialysed against the same buffer overnight.

Step 4: first DEAE-cellulose chromatography. Columns of Whatman DE-52 DEAE-cellulose (diameter 25 mm, length 300 mm) were loaded with step-3 protein, and non-adsorbed material was eluted with the standard buffer. The active fraction was then eluted with 100 mM-KCl in the standard buffer, concentrated as described for step 3 and dialysed overnight against 20 mM-potassium phosphate buffer, pH 8.2.

Step 5: second DEAE-cellulose chromatography. Step-4 proteins were separated on a column (diameter 15 mm, length 250 mm) of Whatman DE-52 DEAE-cellulose equilibrated against 20 mM-potassium phosphate buffer, pH 8.2. After elution of the non-adsorbed material, the column was eluted with a total volume of 500 ml of a linear gradient of 0–250 mM-KCl in the starting buffer. The factor was eluted between 50 and 80 mM-KCl. Fractions showing binding activity in the standard assay were pooled, concentrated by (NH₄)₂SO₄ precipitation and dialysed overnight against the standard buffer.

Step 6: preparative electrophoresis. This was performed as described in the Materials and Methods section, up to 10 mg of step-5 protein being separated on a single slab. After elution from hydroxyapatite, the solution was concentrated by ultrafiltration through an Amicon PM 30 filter (Amicon, Oosterhout, The Netherlands); it was then divided into small portions for one set of experiments, glycerol was added up to 10% (v/v), and the solution was quickly frozen and stored on solid CO₂. No losses of activity were noted during at least 3 months.

Molecular weight and purity of the factor

The step-6 factor showed a single band in polyacrylamide-gel electrophoresis in non-denaturing

Fig. 1. *Determination of the molecular weight of the factor by gel filtration on Sephadex G-200*

Experimental details are described in the Materials and Methods section.

conditions. In the presence of sodium dodecyl sulphate a single band corresponding to a mol.wt. of 63000 appeared in the gel (results not shown). Gel filtration on Sephadex G-200 revealed that the mol.wt. of the factor is $265000 \pm 5\%$ (Fig. 1). These results are consistent with the view that the native factor is composed of four subunits of identical molecular weight. The factor binds initiator tRNA and also *N*-acetylphenylalanyl-tRNA. It does not bind methionyl-tRNA^{Met} or phenylalanyl-tRNA (results not shown).

Some properties of the factor

The factor binds initiator tRNA and GTP to a ternary complex which is retained on cellulose nitrate filters. However, the formation of this complex requires much higher quantities of the factor and

Fig. 2. Dependence of the binding of initiator tRNA to 40S ribosomes (a) and deacylation of initiator tRNA (b) on the quantity of the factor added to the incubations

(a) Incubation mixtures for the standard binding assay contained all components described in the Materials and Methods section, 2 pmol of pure $[^{35}\text{S}]\text{methionyl-tRNA}^{\text{Met}}$ and quantities of the factor as indicated and were incubated at pH 4.0 (○) or 7.5 (●) for 2 min. (b) Incubation mixtures for the assay of deacylase activity were composed as described by Hradec (1975), contained 10 pmol of unfractionated $[^{35}\text{S}]\text{methionyl-tRNA}^{\text{Met}}$ and were incubated for 60 min at 37°C in the presence (▲) or absence (△) of 40S ribosomal subunits (0.1 A_{260} unit/incubation mixture).

significantly longer incubation times than those used for the standard binding assay with ribosomal subunits. The blank values for the ternary complex usually did not exceed 10% of the value obtained in the presence of 40S ribosomal subunits and were subtracted from these values.

Depending on its concentration, the factor binds methionyl-tRNA^{Met} to the 40S subunit and deacylates specifically the initiator tRNA. The deacylation is significantly inhibited by the addition of 40S ribosomal subunits (Fig. 2). It does not deacylate methionyl-tRNA^{Met} or phenylalanyl-tRNA (Hradec, 1975). The binding reaction proceeds very rapidly and is completed within 2 min. After this, a relatively rapid release of bound initiator tRNA occurs from the ribosome (Fig. 3b). However, binding of *N*-acetyl-phenylalanyl-tRNA under the same conditions requires at least 60 min of incubation. The binding reaction catalysed by the factor is absolutely dependent on the addition of GTP. The optimum concentration of this nucleotide is 8 mM, if incubations are performed at pH 4.0, and approx. 10 mM at pH 7.5 (Fig. 3a). The binding reaction is significantly stimulated by Mg^{2+} with somewhat different optima if

incubation is performed at pH 4.0 or 7.5 (Fig. 4a). KCl has a strong inhibitory effect on the binding of initiator tRNA to the smaller ribosomal subunit directed by the factor at either pH 4.0 or 7.5 (Fig. 4b). 2-Mercaptoethanol stimulates the reaction by approx. 20%, and ApUpG has no effect (results not shown).

Sucrose-density-gradient analysis showed that in the presence of the factor $[^{35}\text{S}]\text{methionyl-tRNA}^{\text{Met}}$ becomes bound to the smaller ribosomal subunit, and a complex slightly heavier than 40S is formed. With a comparable efficiency methionyl-tRNA^{Met} also becomes bound to 80S ribosomes, but not to 60S subunits (Fig. 5).

Addition of the factor to incubation mixtures containing polyribosomes and both peptide-elongation factors significantly enhances peptide synthesis directed by natural message present on polyribosomes (Fig. 6).

Binary complexes of the factor with initiator tRNA or GTP

If the factor had been incubated with methionyl-tRNA^{Met} at pH 4.0 and reaction mixtures were

Fig. 3. Effect of GTP on the binding of initiator tRNA to 40S ribosomal subunits promoted by the factor (a) and the time course of the binding reaction (b)

Mixtures, prepared and incubated as described in the Materials and Methods section, contained 1 μg of factor protein and were incubated at pH 4.0 (○) or 7.5 (●).

Fig. 4. Effect of MgCl_2 (a) and KCl (b) on the binding of initiator tRNA to 40S ribosomal subunits catalysed by the factor. Mixtures, prepared as described in the Materials and Methods section, contained 1 μg of factor protein and were incubated at pH 4.0 (○) or 7.5 (●).

Fig. 5. Sucrose-gradient analysis of ribosomes incubated with the factor and initiator tRNA

Incubation mixtures, prepared as described for the standard assay, contained $2\mu\text{g}$ of the factor, 10pmol of purified $[^{35}\text{S}]\text{methionyl-tRNA}^{\text{Met}}$ and $0.2 A_{260}$ unit of 40S ribosomal subunits (a), $0.5 A_{260}$ unit of 60S ribosomal subunits (b) or $0.2 A_{260}$ unit of 40S and $0.5 A_{260}$ unit of 60S subunits (c). The incubation mixtures were then layered on 10–30% (w/v) linear gradients of sucrose buffered as for the incubations and were centrifuged as described by Hradec *et al.* (1974a). Individual fractions (0.25ml) were diluted with ice-cold buffer of the same ionic composition, filtered through cellulose nitrate filters; the filters were washed with the same buffer and the radioactivity retained on them was counted. —, A_{260} record; ····, radioactivity.

Fig. 6. Effect of the factor on the translation of natural message on polyribosomes (a) and the time course of the reaction in the presence (□) and absence of the factor (■) (b)

Incubation mixtures were prepared and incubated at 37°C as described in the Materials and Methods section.

Fig. 7. Gel filtration of the binary complex of the factor with initiator tRNA (a) and with GTP (b)

Incubation mixtures of the ionic composition described in the Materials and Methods section contained 55 μg of the factor and 20 pmol of $[^3\text{S}]\text{methionyl-tRNA}^{\text{Met}}$ (2600 c.p.m./pmol) or the same quantity of the factor and 0.01 mM-GTP (400000 c.p.m. of $[^3\text{H}]\text{GTP}$ and 650000 c.p.m. of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$) in a final volume of 0.15 ml. After the incubation, mixtures were chilled in ice and applied to a column (diameter 10 mm, length 300 mm) of Sephadex G-200 equilibrated against a buffer of the same ionic composition as that of incubation mixtures, and kept at 5°C. The column was eluted with the same buffer, and the A_{280} of the eluate was continuously recorded. Fractions (1.3 ml each) were collected and 1.0 ml portions of each fraction were mixed with Bray's (1960) scintillation mixture and assayed for radioactivity. —, A_{280} ; □, ^3S ; ●, ^3H ; ○, ^{32}P .

filtered through cellulose nitrate filters, a small amount of radioactivity was constantly retained on filters. The radioactivity was directly proportional to the amount of factor present up to approx. 10 μg of factor protein/incubation mixture. This indicated that a complex is formed between the factor and initiator tRNA. Gel filtration of incubation mixtures on Sephadex G-200 revealed that a portion of initiator tRNA becomes associated with the factor and is eluted from the column in the same volume (Fig. 7a).

During the incubation at 37°C the factor forms a complex with GTP which is also retained on cellulose nitrate filters. This binding reaction shows a linear time course for approx. 20 min. The factor becomes saturated with this nucleotide at approx. 0.015 mM concentration. This reaction is slightly stimulated by Mg^{2+} (optimum concentration 2–3 mM), but is not affected by K^+ (results not shown). The binary complex of the factor with GTP is unstable. After preincubation of the factor with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$, this nucleotide can be exchanged for non-radioactive GTP and to a lesser extent also for GDP. However, GTP cannot be replaced by ATP. Gel filtration of incubation mixtures containing the factor and both $[^3\text{H}]\text{GTP}$ and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ revealed that a portion of the radioactivity becomes eluted with the factor. A

significant change in the $^3\text{H}/^{32}\text{P}$ ratio in free GTP indicated that a high proportion of the terminal phosphate of this nucleotide is liberated during the incubation (Fig. 7b).

Ternary complex of the factor with initiator tRNA and GTP

If the factor had been incubated with suitable quantities of initiator tRNA and GTP and incubation mixtures were filtered through cellulose nitrate filters, a major portion of added radioactivity remained on the filters. Gel filtration of incubation mixtures on Sephadex G-200 provided direct evidence for the formation of a ternary complex, since both methionyl-tRNA^{Met} and GTP were eluted in the same volume as the factor (Fig. 8). At optimum conditions initiator tRNA was bound to the factor in approx. stoichiometric proportions. The time course of the reaction indicated that the formation of the ternary complex proceeds for approx. 10 min at 37°C (Fig. 8). In the presence of GTP, model initiators (*N*-acetylphenylalanyl-tRNA or *N*-acetylmethionyl-tRNA^{Met}) become bound to the factor with a similar efficiency. However, at least 30 min of incubation at 37°C was required for this effect. The factor is not able to form a ternary complex with $[^3\text{H}]\text{phenyl-}$

Fig. 8. Gel filtration of the ternary complex of the factor with initiator tRNA and GTP (a) and the complex of the factor with 80S ribosomes (b)

Incubation mixtures used for the preparation of the ternary complex and gel filtration of the complex on Sephadex G-200 were described in the legend for Fig. 7. The complex of the factor with 80S ribosomes was prepared by preincubation of ribosomes with the factor, and this ribosomal complex was then incubated with initiator tRNA and GTP as described in the Materials and Methods section. Incubation mixtures were applied to columns (diameter 8 mm, length 150 mm) of Sepharose 4B equilibrated against a buffer of the same ionic composition as that of incubation mixtures. The column kept at 5°C was eluted with the same buffer and the A_{254} was continuously recorded. Fractions (0.8 ml each) were collected. Each fraction was diluted with ice-cold elution buffer, filtered through cellulose nitrate filters, and the radioactivity retained on filters was counted. —, A_{254} ; □, ^{35}S ; ○, ^{32}P .

Fig. 9. Time course of the ternary-complex formation (a) and the effect of GTP on the reaction (b)

Incubation mixtures were prepared as described in the Materials and Methods section and contained 10 μg of the factor, 2 pmol of $[^{35}\text{S}]\text{methionyl-tRNA}^{\text{Met}}$ and 10 mM-GTP (a) or quantities of the nucleotide as indicated (b) and were incubated at pH 4.0 (Δ) or 7.5 ($\blacktriangle$).

alanyl-tRNA or [^{35}S]methionyl-tRNA^{Met} (results not shown). The formation of the ternary complex was almost absolutely dependent on GTP, with an optimum concentration of 5–10 mM (Fig. 9). The reaction was also stimulated by Mg^{2+} in optimum concentrations of 2–3 mM, but K^+ did not have any significant effect on the ternary-complex formation (results not shown). Addition of deacylated tRNA^{fMet}, tRNA^{Met} or unfractionated tRNA had no effect on the ternary-complex formation.

The ternary complex of the factor is apparently relatively stable. If a 20-fold excess of unlabelled methionyl-tRNA^{fMet} or GTP were added into incubation mixtures of the factor preincubated with labelled initiator tRNA and GTP, and mixtures were incubated for further 15 min at 37°C, no exchange of radioactivity was found between the ternary complex and its individual components.

Binding of the factor to ribosomes

If the factor had been preincubated with 40S ribosomal subunits or 80S ribosomes in the absence of initiator tRNA and GTP and ribosomes were pelleted afterwards, ribosomes treated in this way showed in the presence of GTP a significant binding of initiator tRNA, although this activity was almost completely absent from control ribosomes not pre-

treated with the factor. The 60S ribosomal subunits did not bind initiator tRNA after a similar pretreatment with the factor. Gel filtration on Sepharose 4B provided direct evidence for the binding of the factor to ribosomes (Fig. 8). The radioactivity eluted in fractions corresponding by their elution volumes to the ternary complex of the factor was retained on cellulose nitrate filters. This indicates that during the incubation the ternary complex of the factor becomes liberated from the ribosome. The binding activity of subunits pretreated with the factor was directly proportional to the quantity of the factor added for preincubation (Fig. 10a). Optimum binding of the factor to the 40S subunit was obtained after preincubation for 10 min at 37°C, although some binding also occurred at 0°C (results not shown). Binding of the factor to the 40S subunit does not require GTP, but this nucleotide is essential for the subsequent binding of initiator tRNA to subunits preincubated with the factor. This binding is proportional to the quantity of preincubated 40S or 80S ribosomes added (Fig. 10b).

If the complex of 40S ribosomal subunits with the factor was incubated with GTP even in the absence of initiator tRNA, the nucleotide became bound to the ribosome, as evidenced by the retention of radioactive GTP on cellulose nitrate filters. Incubation of the 80S ribosomal complex of the factor with a mixture of [^3H]GTP and [$\gamma\text{-}^{32}\text{P}$]GTP indicated

Fig. 10. Complexes of the factor with 40S (○) and 80S (●) ribosomes

(a) Ribosomes were preincubated with amounts of the factor as indicated and tested for the binding of initiator tRNA as described in the Materials and Methods section. (b) Amounts of the ribosomal complexes of the factor as indicated were assayed for the binding of initiator tRNA as described in the Materials and Methods section.

Fig. 11. Binding of methionyl-tRNA^{Met} and GTP on 80S ribosomes preincubated with the factor

The complex of the factor with 80S ribosomes was prepared as described in the Materials and Methods section. Incubation mixtures for the binding assay contained in a final volume of 0.1 ml all components described in the same section, 15 pmol of [³⁵S]methionyl-tRNA^{Met}, [³H]GTP (250000 c.p.m.), [γ -³²P]GTP (260000 c.p.m.) and 1.2 A₂₆₀ units of 80S ribosomes preincubated with the factor. Gel filtration on Sepharose 4B was performed as described in the legend for Fig. 4; collected fractions were mixed with Bray's (1960) scintillation mixture and counted for radioactivity. —, A₂₅₄; □, ³⁵S; ●, ³H; ○, ³²P.

that the nucleotide becomes split on the ribosome, since the quantity of [³²P]GTP retained on filters was low compared with the amount of [³H]GTP. Direct evidence for the binding of GTP to 80S ribosomes preincubated with the factor was provided by gel filtration on Sepharose 4B. After gel filtration of incubation mixtures containing the 80S ribosomal complexes of the factor, initiator tRNA and GTP, both methionyl-tRNA^{Met} and GTP were eluted in the same volume as ribosomes. A significant change in the ³H/³²P ratio in fractions corresponding to the ribosomal complex of the factor indicated that hydrolysis of GTP occurred on 80S ribosomes (Fig. 11). Since the [³H]GTP was eluted in the same fraction as ribosomes, it seems reasonable to suppose that after the splitting of GTP, GDP remained bound to 80S ribosomes.

Discussion

If the formation of a ternary complex with initiator tRNA and GTP is considered as a function characteristic for initiation factors promoting the binding of

initiator tRNA to ribosomes in the presence of GTP, but without requirements for a template, then factor isolated in our laboratory belongs undoubtedly to this group of factors. A designation eIF-2 was proposed for these factors at the International Symposium on Protein Synthesis held in Bethesda in 1976 (Schreier *et al.*, 1977). However, even when these factors isolated by different authors have common basic functional properties, they apparently differ widely in their chemical structure. This is documented by the fact that molecular-weight estimations for these different factors range from 90000 (Safer *et al.*, 1975) to 145000 (Herrera *et al.*, 1977). However, our factor is different from all these proteins because both the binding and the deacylating activity are associated with a single protein purified to homogeneity. Morrissey & Hardesty (1972) and McCuiston *et al.* (1976) have described partial purification of a deacylase specific for methionyl-tRNA^{Met} on 40S ribosomal subunits. In agreement with these results, the deacylating activity of our partially purified factor was enhanced by the smaller ribosomal subunit (Hradec, 1975). However, the

deacylase activity of the present pure factor was significantly inhibited by the addition of 40S ribosomal subunits. It seems probable that the deacylase activity stimulated by the 40S subunit present in less-purified preparations of the factor (Hradec, 1975) was removed during subsequent purification steps and is therefore absent from the pure factor. Nygard & Hultin (1977) have described a partial purification of a methionyl-tRNA^{fMet} deacylase which is inhibited by GTP and the initiator tRNA is protected from its action if bound to the 40S ribosomal subunit. Both these properties are very similar to the deacylase activity of our factor. Unfortunately, Nygard & Hultin (1977) did not report whether their purified enzyme is also capable of binding initiator tRNA.

Certain conditions of our standard assay, in particular the acid reaction, may seem artificial, although it cannot be fully excluded that an instantaneous acid reaction may exist in a cellular compartment adjacent to the ribosomal binding site for initiator tRNA. Both unusual properties of the factor, i.e. the requirement for high GTP concentrations and an acid pH optimum, represent conditions inhibitory for the deacylase activity, as found with partially purified preparations of the factor (Hradec, 1975). This may explain why these conditions are necessary for maximum binding of initiator tRNA to the ribosomal subunit. Furthermore, it should be taken into consideration that the binding reaction assayed in our experiments is itself highly artificial and does not occur in the cell. Physiologically, the reaction is not stopped at the stage of this complex, but immediately proceeds to the 80S initiation-complex formation and subsequent steps of protein synthesis (Trachsel *et al.*, 1977). This may explain why this artificial reaction has non-physiological requirements.

If ribosomes had been washed with high concentrations of KCl (Schreier & Staehelin, 1973), the factor could be isolated from this wash fraction. However, yields were much lower than if the isolation was performed from cytosol (P. Pohlreich & J. Hradec, unpublished work). The factor is apparently rather firmly bound to ribosomes, since relatively high blank values were obtained in the binding assay with 40S subunits subjected to washings with high concentrations of KCl. Zero blank values were obtained only if postmitochondrial supernatants used for the isolation of ribosomes had been passed through heparin-Sepharose 4B. This retention on heparin-Sepharose seems to be characteristic of peptide initiation factors (Waldman *et al.*, 1975; van der Mast *et al.*, 1977).

The significance of the complex between the factor and the smaller ribosomal subunit for the binding of initiator tRNA in physiological conditions is not quite clear at the moment. In contrast with the free factor, the ribosomal complex of the factor readily binds, in the presence of GTP, initiator tRNA at a

physiological pH and at low concentrations of the nucleotide. It cannot be excluded that normally the factor exists bound to ribosomes and that the formation of the ternary complex occurs at the ribosome and not with the free factor in the cytosol. Since during the gel filtration of the factor bound to ribosomes the ternary complex of the factor and not the free factor has been liberated from ribosomes, the formation of the ternary complex on ribosome seems to be probable. It thus seems possible that the complex of the factor with ribosomes represents the normal situation within the cell and is a prerequisite necessary for the binding of initiator tRNA promoted by the factor. This opinion is supported by the finding that the deacylase activity of the factor is inhibited by ribosomal subunits. If such an explanation is valid, then the free factor may regulate the supply of initiator tRNA, and the factor bound to ribosomes would be required for the binding of this substrate to ribosomes.

It cannot be decided at the moment whether the binding or deacylation of initiator tRNA is the primary function of the factor described here. However, its significant stimulating effect on natural template-dependent peptide elongation seems to indicate that it is involved in the sequence of events leading to peptide synthesis. There was not an absolute need for this factor in our present experiments. This may be explained by the fact that polyribosomes and even elongation factor 1 prepared by our present procedures were not completely free of this factor and probably also of other peptide-initiation factors. Unfortunately, in the present experiments a complete set of purified peptide-initiation factors were not available, which would allow a more detailed study of the involvement of our factor in peptide initiation in general.

The careful technical assistance of Mrs. J. Žemlová, Mrs. V. Růžicková and Mrs. E. Novotná is gratefully acknowledged.

References

- Adams, S. L., Safer, B., Anderson, W. F. & Merrick, W. C. (1975) *J. Biol. Chem.* **250**, 9083–9089
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Cashion, L. M. & Stanley, W. M. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 436–440
- Chen, Y. C., Woodley, C. L., Bose, K. K. & Gupta, N. K. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1–9
- Cimadevilla, J. M. & Hardesty, B. (1975) *J. Biol. Chem.* **250**, 4389–4397
- Dettman, G. & Stanley, W. M. (1972) *Biochim. Biophys. Acta* **287**, 124–133
- Gasior, E. & Moldave, K. (1972) *J. Mol. Biol.* **66**, 391–402
- Grummt, F. (1974) *Eur. J. Biochem.* **43**, 337–342
- Haenni, A. L. & Chapeville, F. (1966) *Biochim. Biophys. Acta* **114**, 135–148

- Herrera, F., Sadnik, I., Gough, G. & Moldave, K. (1977) *Biochemistry* **16**, 4664–4672
- Hradec, J. (1972) *Biochem. J.* **126**, 923–931
- Hradec, J. (1975) *Nucleic Acids Res.* **2**, 2119–2129
- Hradec, J. & Dušek, Z. (1978) *Biochem. J.* **172**, 1–7
- Hradec, J., Dušek, Z., Bermek, E. & Matthaei, H. (1971) *Biochem. J.* **123**, 959–966
- Hradec, J., Dušek, Z. & Mach, O. (1974a) *Biochem. J.* **138**, 147–154
- Hradec, J., Pohlreich, P., Dušek, Z. & Grozdanovič, J. (1974b) *Acta Biol. Med. Germ.* **33**, 905–912
- Hradec, J., Tuháčková, Z. & Dušek, Z. (1978) *Biochem. J.* **172**, 9–13
- Leader, D. P. & Wool, I. G. (1972) *Biochim. Biophys. Acta* **262**, 360–370
- Majumdar, A., Roy, R., Das, A., Dasgupta, A. & Gupta, N. K. (1977) *Biochem. Biophys. Res. Commun.* **78**, 161–169
- Matthaei, H., Heller, G., Voigt, H. P., Neth, R., Schöh, G. & Kubler, H. (1966) *Genet. Elements: FEBS Symp.* **233–250**
- McCuiston, J., Parker, R. & Moldave, K. (1976) *Arch. Biochem. Biophys.* **172**, 387–398
- Merrick, W. C. & Anderson, W. F. (1975) *J. Biol. Chem.* **250**, 1197–1206
- Morrissey, J. & Hardesty, B. (1972) *Arch. Biochem. Biophys.* **152**, 385–397
- Nygard, O. & Hultin, T. (1977) *Eur. J. Biochem.* **72**, 537–542
- Ornstein, L. (1965) *Ann. N.Y. Acad. Sci.* **121**, 321–349
- Rogg, H., Wehrli, W. & Staehelin, M. (1969) *Biochim. Biophys. Acta* **195**, 13–15
- Safer, B., Anderson, W. F. & Merrick, W. C. (1975) *J. Biol. Chem.* **250**, 9067–9075
- Schreier, M. H. & Staehelin, T. (1973) *J. Mol. Biol.* **73**, 329–349
- Schreier, M. H., Erni, B. & Staehelin, T. (1977) *J. Mol. Biol.* **116**, 727–753
- Stanley, W. M. (1972) *Anal. Biochem.* **48**, 202–216
- Trachsel, H., Erni, B., Schreier, M. H. & Staehelin, T. (1977) *J. Mol. Biol.* **116**, 755–767
- van der Mast, C., Thomas, A., Goumans, H., Ames, H. & Voorma, H. O. (1977) *Eur. J. Biochem.* **75**, 455–464
- Waldman, A. A., Marx, G. & Goldstein, J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2353–2356
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Zasloff, M. & Ochoa, S. (1973) *J. Mol. Biol.* **73**, 65–76
- Ziola, B. R. & Scraba, D. G. (1976) *Anal. Biochem.* **72**, 366–371