All Factors Required for Protein Synthesis are Retained on Heparin Bound to Sepharose

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1. Postmitochondrial supernatants of rabbit reticulocyte lysates were chromatographed on heparin bound to Sepharose 4B, and the fraction retained on affinity columns was separated by subsequent gel filtration on Sepharose 4B into three fractions, two of them active in protein synthesis. 2. The heavier fraction sedimented at 40S and contained more than 10% RNA. This consisted predominantly of a 12S component, with smaller amounts of the 9S and 4S RNA species. The lighter fraction (18–20S) was composed of proteins with less than 1% RNA. 3. Different enzymic activities were associated with these fractions. 4. In the presence of both fractions, efficient translation took place on combined ribosomal subunits of rat liver with added cofactors. Globin messenger ribonucleoprotein stimulated this translation 5–6-fold. 5. Relatively large complexes of all factors required for protein synthesis are apparently isolated from reticulocytes by affinity chromatography on heparin–Sepharose 4B. Such complexes may occur naturally in the cytoplasm of mammalian cells.

Peptide-initiation factors required for eukaryotic protein synthesis are mostly isolated from ribosomal high-salt wash fraction (Filipowicz *et al.*, 1976; Staehelin *et al.*, 1975).

A fundamentally new approach to the isolation of these factors has established that heparin is a potent inhibitor of initiation of protein synthesis in a Krebs-ascites-cell cell-free system (Waldman & Goldstein, 1973a). This inhibition seemed to be specific for heparin, since other glycosaminoglycans tested were inactive (Waldman & Goldstein, 1973b). This inhibitory effect of heparin on protein synthesis is distinct from the ability of this substance to act as an anti-coagulant (Waldman *et al.*, 1974). On the basis of these results, Waldman *et al.* (1975) developed a technique for the isolation of peptide-initiation factors using affinity chromatography on heparin bound to Sepharose 4B. They concluded that the interaction with heparin is specific for initiation factors, since the passage of postmitochondrial supernatants through the affinity column did not affect the activity of such preparations in artificial template-dependent elongation.

Results in the present paper provide evidence that, under certain conditions, not only peptide-initiation factors, but essentially all protein factors required for cell-free protein synthesis are retained on heparin–Sepharose 4B. In the presence of factors isolated in this way, efficient translation of natural message takes place on ribosomal subunits.

Abbreviation used: EF 1, peptide-elongation factor 1.

Materials and Methods

Animals

Wistar rats (120–150g) and New Zealand rabbits (2.5–3.0kg) were bred in this laboratory and fed on a pelleted standard diet. Reticulocytosis was induced in rabbits by the administration of phenylhydrazine according to the schedule of Crystal *et al.* (1974). Blood was withdrawn by cardiac puncture into bottles containing a solution of heparin (50 units/ml of blood) and was chilled immediately in ice.

Chemicals and radiochemicals

ATP, GTP, phosphoenolpyruvate (Ag/Ba salt) and pyruvate kinase were obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Natural L-amino acids were products of Nutritional Biochemicals Corp., Cleveland, OH, U.S.A. Heparin was purchased from Spofa, Prague, Czechoslovakia, and was bound to CNBr–Sepharose 4B (Pharmacia, Uppsala, Sweden) by the method of Waldman *et al.* (1975). BD-cellulose (benzoylated DEAE-cellulose) was obtained from Serva, Heidelberg, West Germany.

L-[4,5-³H]Leucine (58 Ci/mmol) and L-[4-³H]-phenylalanine (12 Ci/mmol) were products of The Radiochemical Centre, Amersham, Bucks., U.K. L-[³⁵S]Methionine (391 Ci/mmol) was obtained from NEN Chemicals, Dreieichenhain, West Germany. L-[U-¹⁴C]Phenylalanine (315 mCi/mmol) was a product of the Institute for Research, Production and Uses of Radioisotopes, Prague, Czechoslovakia.

L-[³H]Phenylalanyl-tRNA was prepared by charging tRNA from *Escherichia coli* B (Calbiochem, Los Angeles, CA, U.S.A.) as described by Hradec *et al.* (1974). Rat liver tRNA was isolated by the method of Rogg *et al.* (1969). Initiator tRNA^{Met} was separated by BD-cellulose chromatography and charged with L-[³⁵S]methionine as described by Stanley (1972).

Subcellular preparation

Rabbit reticulocytes were collected, washed and lysed as described by Crystal *et al.* (1974). The lysate was centrifuged at 30000g and 4°C for 15 min in a VAC 20 centrifuge (H. Janetzki, Engelsdorf/Leipzig, German Democratic Republic). The upper two-thirds of the supernatant was applied to a column (25 mm × 300 mm) of heparin-Sepharose 4B equilibrated with 50 mM-Tris/HCl buffer (pH 7.5)/5 mM-MgCl₂/25 mM-KCl and maintained at 4°C during chromatography. The non-adsorbed fraction was eluted with the same buffer. This fraction was then passed through a Sephadex G-25 column (25 mm × 1000 mm) at 4°C; the eluate, containing 50–70 mg of protein/ml, was frozen in small portions each suitable for one set of experiments and kept at –80°C. The fraction retained on heparin-Sepharose 4B was eluted with 20 mM-Tris/HCl buffer (pH 7.5)/1 M-KCl/0.1 mM-EDTA. This fraction was then concentrated and dialysed against 20 mM-Tris/HCl buffer (pH 7.5)/5 mM-MgCl₂/120 mM-KCl by using a PM-30 membrane (Amicon, Oosterhout, The Netherlands).

Polyribosomes from rat liver were isolated as described by Hradec *et al.* (1971) and ribosomal subunits from rat liver as reported by Hradec *et al.* (1974). Peptide-elongation factors were separated and purified by the method of Bermek & Matthaei (1970).

Globin messenger ribonucleoprotein was prepared from EDTA-treated polyribosomes from rabbit reticulocytes as described by Lebleu (1974).

Globin mRNA was isolated from this preparation by extraction with phenol/chloroform as described by Perry *et al.* (1972). The same method was used for the extraction of RNA from proteins retained on heparin-Sepharose columns, if required. Density-gradient analysis showed that messenger ribonucleoprotein contained only the 15S fraction, whereas the globin mRNA preparations contained the 9S component with a small amount of 12S RNA. Centrifugation in sucrose density gradients was performed as described by Hradec *et al.* (1974).

Incubations

Incubation mixtures for the assay of protein synthesis in postmitochondrial supernatants contained, in a final volume of 0.1 ml, 50 mM-Tris/HCl buffer, pH 7.5, 5 mM-MgCl₂, 100 mM-KCl, 1 mM-ATP,

5 mM-phosphoenolpyruvate, 4 μg of pyruvate kinase, 0.2 mM-GTP, 19 essential amino acids (25 nM each), 2.5 nM labelled amino acid (0.1 μCi) and 500–700 μg postmitochondrial-supernatant protein. Mixtures were incubated for 50 min at 37°C. Incubation mixtures for the assay of translation on ribosomal subunits contained, in a final volume of 0.1 ml, 50 mM-Tris/HCl buffer, pH 7.5, 2.5 mM-MgCl₂, 80 mM-KCl, 0.2 mM-ATP, 5 mM-phosphoenolpyruvate, 2 mg of pyruvate kinase/ml, 1 mM-GTP, 19 essential amino acids (25 nM each), 2.5 nM labelled amino acid added (0.1 μCi), 0.2 A₂₆₀ unit of 40S and 0.5 A₂₆₀ unit of 60S ribosomal subunits, and quantities of protein factors as indicated. Mixtures were incubated for 180 min at 37°C. Incubation mixtures for the assay of poly(U)-dependent phenylalanine polymerization were as described in Hradec *et al.* (1971). The binding of L-[³⁵S]methionyl-tRNA^{Met} to 40S ribosomal subunits was assayed in mixtures containing, in a total volume of 0.1 ml, 20 mM-Tris/HCl buffer, pH 7.5, 2.5 mM-MgCl₂, 10 mM-GTP, 0.2 A₂₆₀ unit of 40S ribosomal subunits and the quantities of protein fractions as indicated. These mixtures were incubated for 20 min at 37°C (J. Hradec, P. Pohlreich & O. Kříž, unpublished work). For the study of deacylation of initiator tRNA, incubation mixtures were as described by Hradec (1975). Mixtures for charging tRNA with L-[³H]leucine contained, in a final volume of 0.1 ml, 20 mM-Tris/HCl, pH 7.5, 10 mM-MgCl₂, 10 mM-ATP, 19 essential amino acids (25 nM each), 2.5 nM-L-leucine (0.1 μCi) and quantities of protein factors and rat liver tRNA as indicated.

Assay of radioactivity

Portions of incubation mixtures (75 μl) in which peptide-bond formation was assayed were applied to discs of Whatman GF 83 filters, and the filters were washed as described by Hradec *et al.* (1971). Mixtures for the assay of aminoacyl-tRNA formation were treated in the same way, omitting only washing with hot trichloroacetic acid. Mixtures for the binding reactions were chilled in ice and filtered through cellulose nitrate filters (Spolana, Uhřetíněves, Czechoslovakia). Filters were then washed with ice-cold buffers prepared as described by Hradec *et al.* (1971). Glass-fibre and cellulose nitrate filters were put into scintillation vials containing toluene-based scintillation mixtures (Hradec *et al.*, 1971). Radioactivity was counted in a Nuclear-Chicago mark II liquid-scintillation spectrometer with an efficiency of 40% for ³H. Protein and RNA were determined by the method of Warburg & Christian (1942).

Results

Gel filtration of proteins retained on heparin-Sepharose

As checked by the translation of globin mRNA in the presence of the fraction not retained on the

affinity column, the columns of heparin-Sepharose used in the present experiments could be loaded with approx. 150 ml of postmitochondrial supernatants of reticulocyte lysates containing up to approx. 8 g of protein. Under these conditions 0.8–1.0% of the total protein remained bound to the affinity material. Chromatography on Sepharose 4B of the fraction retained on heparin-Sepharose resulted in its separation into three well-defined fractions (Fig. 1). This separation was very reproducible, although the proportion of individual fractions varied considerably with different preparations. In some batches, only traces of fraction A were present.

Fraction A appearing at the void volume of Sepharose columns was composed apparently of heavy aggregates resulting probably as artifacts from the isolation procedure. This fraction was already pelleted by low-speed centrifugation and showed no protein-synthesis activity. As revealed by density-gradient centrifugation, fraction B consisted predominantly of a 40S component. Besides that, small quantities of a 4S fraction were also present together with traces of a 15S component (Fig. 2a). Fraction B contained considerable quantities of RNA; the amount, calculated on the

basis of the A_{260}/A_{280} ratio, was higher than 10%. If RNA was extracted with phenol/chloroform and subjected to density-gradient centrifugation, the most abundant RNA species sedimented at 12S. Small amounts of a 9S and a 4S component were also present (Fig. 2b).

Sucrose-density-gradient centrifugation of fraction C demonstrated the presence of a single 18–20S component (Fig. 2c).

Activity of separated fractions

L-Leucyl-tRNA complex was also formed in the presence of fraction B if no exogenous tRNA was added. Addition of rat liver tRNA resulted in a slight stimulation of the leucyl-tRNA formation. A 5–7-fold enhancement of the response to added tRNA was obtained, however, if globin messenger ribonucleoprotein or globin mRNA was present in incubation mixtures (Fig. 3).

Fraction C was free of the aminoacyl-tRNA-synthesizing activity; it was, however, capable of peptide elongation. This activity could be demonstrated only if polyribosomes were present in the incubation mixtures (Fig. 4). No poly(U)-dependent phenylalanine polymerization occurred in mixtures containing both ribosomal subunits in suitable proportion instead of polyribosomes. Addition of poly(U) in usual quantities (Hradec *et al.*, 1971) to mixtures containing polyribosomes resulted in a significant inhibition of peptide elongation in the presence of fraction C.

In the presence of fraction C, moreover, binding of initiator tRNA to 40S ribosomal subunits as well as deacylation of Met-tRNA_i was demonstrated (Fig. 5).

Translation of natural message in the presence of proteins retained on heparin-Sepharose

After the incubation of crude proteins retained on heparin-Sepharose 4B with ribosomal subunits, low-molecular-weight cofactors and either L-[³H]-leucine or L-[³H]phenylalanine no radioactivity was found in the hot-trichloroacetic acid-precipitable portion of the mixture. However, in agreement with others (Waldman *et al.*, 1975), the same fraction was fully active in restoring the protein-synthesizing activity of postmitochondrial supernatants of rabbit reticulocytes passed through the affinity columns of heparin-Sepharose (results not shown).

In the presence of separated fractions B or C only negligible incorporation of radioactivity into protein could be demonstrated. However, if both fractions B and C were added in suitable proportions, an efficient translation of added natural message took place. The translation was not absolutely

Fig. 1. Gel filtration on Sepharose 4B of the fraction retained on heparin-Sepharose

A column of Sepharose 4B (diameter 25 mm, length 950 mm) in 20 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-MgCl₂ and 120 mM-KCl was loaded with 85 mg of protein retained on the affinity column and eluted with the same buffer at a flow rate of 45 ml/h. The effluent was continuously monitored at 260 nm and fractions (11 ml) were collected. Fractions under individual peaks were pooled and concentrated by ultrafiltration through an Amicon PM-10 membrane.

Fig. 2. Sucrose-gradient analysis of fraction B (a), RNA extracted from fraction B (b) and of fraction C (c). Approx. 1.2 A_{260} units of these materials in 100 μ l of 20 mM-Tris/HCl buffer, pH 7.5, containing 5 mM- $MgCl_2$ and 120 mM-KCl were layered on 4.9 ml of a linear gradient of 5–20% (w/w) sucrose in the same buffer. Gradients were run at 40000 rev./min and 25°C for 3.5 h in a type 3170 ultracentrifuge (MOM, Budapest, Hungary) as described by Hradec *et al.* (1974). Standards of 4S, 18S and 28S RNA and of 40S and 60S ribosomal subunits were run under the same conditions to determine the S values.

Fig. 3. L-Leucyl-tRNA synthetase activity of fraction B. Incubation mixtures were composed as described in the Materials and Methods section and contained either no messenger ribonucleoprotein (●) or 3 μ g of globin messenger ribonucleoprotein (○), 10–20 μ g of fraction-B protein and quantities of rat liver tRNA as indicated.

Fig. 4. Peptide-elongation activity of fraction C with polyribosomes. Incubation mixtures contained all components described in the Materials and Methods section [without poly(U)] and quantities of fraction-C protein as indicated.

Fig. 5. Binding of L-[³⁵S]Met-tRNA_f to 40S ribosomal subunits (a) and deacylation of initiator tRNA (b) in the presence of fraction C

Mixtures for the binding assay are described in the Materials and Methods section and those for the assay of deacylase activity were described by Hradec (1975). Both types of mixtures contained 6.3 pmol of initiator tRNA.

dependent on added message, but was stimulated by it by 3–4-fold. Globin messenger ribonucleoprotein or mRNA used in these experiments could be fully replaced by the addition of RNA extracted from fraction B.

The increase in hot-trichloroacetic acid-precipitable radioactivity was proportional to the incubation period for at least 5 h of incubation. If free labelled amino acid (L-[³H]leucine) was added, a linear time course of incorporation was found from the beginning of the incubation. On the other hand, the addition of L-[³H]phenylalanyl-tRNA resulted in a lag phase of 30–40 min, after which the linear incorporation took place. In experiments in which free [¹⁴C]phenylalanine and [³H]phenylalanyl-tRNA were added together at the beginning of the incubation, an incorporation linear with time without any lag phase was found with ¹⁴C, but virtually no incorporation of ³H was found during the first 30 min of incubation. After this delay, a linear incorporation of ³H was again found (Fig. 6).

Under saturating conditions of the labelled amino acid, its utilization was very efficient, with an average rate of approx. 400 pmol of L-[³H]leucine incorporated into protein/h.

Translation of natural message proceeded optimally at low Mg²⁺ (approx. 2.5 mM) concentrations and globin messenger ribonucleoprotein was translated more efficiently than globin mRNA. Translation was enhanced by approx. 30% by the addition of tRNA.

Fig. 6. Time course of translation of globin messenger ribonucleoprotein on ribosomal subunits

Incubation mixtures contained all components described in the Materials and Methods section, 2 μg of globin messenger ribonucleoprotein, 5.1 μg of fraction B and 58 μg of fraction-C protein, 28 nmol of L-[¹⁴C]phenylalanine, 15 pmol of L-[³H]phenylalanyl-tRNA and 30 μg of rat liver tRNA, and were incubated at 37°C for the time indicated. ○, ¹⁴C radioactivity; ●, ³H radioactivity.

The system required ATP, phosphoenolpyruvate, pyruvate kinase and GTP as essential cofactors. In the absence of both ATP and GTP the incorporation was

decreased to approx. 5% of the original value. Omission of ATP and the ATP-generating system inhibited protein synthesis by 85%. Omission of GTP inhibited the system by approx. 70% and omission of ATP by approx. 45%.

Discussion

All protein factors and RNA species required for protein synthesis are apparently present in the fraction of reticulocyte postmitochondrial supernatants retained on heparin-Sepharose 4B. The presence of aminoacyl-tRNA synthetases, peptide-initiation factors and peptide-elongation factors has been directly demonstrated in our experiments. It remains an open question whether this fraction also contains peptide-termination factors. Formation of a L-leucyl-tRNA complex in the presence of fraction B without any addition of exogenous tRNA indicates that the 4S RNA present in this fraction is identical with tRNA. This fraction apparently also contains mRNA, as evidenced by the fact that the system described here rather efficiently incorporates amino acids into protein in the absence of added message.

The 12S RNA present in this fraction is found constantly in preparations of globin mRNA and its function is largely unknown (Lebleu, 1974). Since globin messenger ribonucleoprotein or mRNA could be replaced by this RNA species in our experiments, it seems reasonable to assume that it acts as a natural template. Only a future analysis of the product of translation can give a definitive answer.

Only free amino acid and not aminoacyl-tRNA is utilized for translation in our system. This explains the delayed incorporation of phenylalanine from phenylalanyl-tRNA. Aminoacyl-tRNA is obviously first deacylated and the free amino acid is then utilized for the synthesis of a new aminoacyl-tRNA complex catalysed by synthetases present in fraction B. It cannot be excluded that aminoacyl-tRNA of bacterial origin used in our experiments cannot serve as a substrate for translation in a mammalian system. This explanation does not seem very probable, in view of the fact that aminoacyl-tRNA from *E. coli* is a very efficient substrate for peptide-elongation factors of different mammalian cells (Hradec, 1970).

Waldman *et al.* (1975) have reported that the portion of reticulocyte postmitochondrial supernatant not retained on heparin-Sepharose 4B retains its activity in peptide elongation. In agreement with these results, we have found that poly(U)-dependent phenylalanine polymerization is not impaired by the passage of postmitochondrial supernatants through columns of heparin-Sepharose 4B (J. Hradec & Z. Dušek, unpublished work). However, our fraction C is also very active in catalysing peptide elongation. The sedimentation behaviour of this fraction

indicates that elongation factors are present here as rather large complexes. Multiple forms of EF 1 have been repeatedly reported during the past years. They occur in calf brain (Legocki *et al.*, 1974) and also in rabbit reticulocytes (Kemper *et al.*, 1976). All these multiple forms seem to have a comparable functional activity and efficiently translate synthetic polynucleotides (Kemper *et al.*, 1976). On the contrary, peptide-elongation activity present in our fraction C is unable to translate artificial templates, although it is very active with polyribosomes. It thus seems unlikely that our fraction C can be compared with large complexes of EF 1 present *in vivo* in cytoplasm. Moreover, peptide-initiation factors are also present in this fraction, as evidenced by its binding activity for initiator tRNA. Since cytoplasmic peptide-elongation factors are apparently not retained on heparin-Sepharose, as suggested by our results and those of Waldman *et al.* (1975), elongation factors in fraction C obviously form a complex with initiation factors. This can explain their retention on affinity columns, since only peptide-initiation factors appear to react specifically with heparin (Waldman *et al.*, 1974).

No translation takes place on ribosomal subunits in the presence of the crude fraction retained on heparin-Sepharose 4B, whereas fractions separated from this material by subsequent gel filtration on Sepharose 4B are, if combined, very active in this respect. This fact seems to indicate that an inhibitor of protein synthesis is present in the crude fraction and is eliminated by subsequent gel chromatography. The isolation of such a factor in reticulocyte lysates has been reported by Gross (1976). This translational repressor protein blocks the formation of an initiation complex between initiator tRNA and the 40S subunit (Legon *et al.*, 1973) and prevents combination of both ribosomal subunits with deacylation of ribosome-bound Met-tRNA_i (Balkow *et al.*, 1973). A similar inhibitor has been reported to be present in Friend leukaemia cells (Pinphanichakarn *et al.*, 1977). It cannot be excluded that such a factor is present in our preparation and inhibits peptide-initiation factors in the active fractions.

Fraction B represents apparently a large complex of informational macromolecules with aminoacyl-tRNA synthetases. In addition to leucyl-tRNA synthetase, ligases for several other amino acids are also present (J. Hradec, O. Kříž & Z. Dušek, unpublished work). Evidence has accumulated that the aminoacyl-tRNA synthetases occur in multiple forms of relatively large complexes in cytoplasm and are partially rather firmly bound to ribosomes. Bandyopadhyay & Deutscher (1971) reported the presence of such complexes and their results have been confirmed by several authors (Vennegoor & Bloemendal, 1972; Ussery *et al.*, 1977). It cannot be excluded that fraction B has some relation to these complexes.

Nevertheless, the surprising fact that the aminoacyl-tRNA-synthesizing activity of fraction B is significantly stimulated by mRNA has not been reported so far.

Relatively large complexes of essentially all factors required for protein synthesis are thus isolated by affinity chromatography on heparin-Sepharose 4B. The possibility that they may be artifacts resulting from the isolation technique is not very probable, since a mild and rapid procedure was used for the preparation. It cannot be excluded, however, that such a complex occurs naturally in mammalian cells and may play some role in regulating protein synthesis, as suggested by others (Ussery *et al.*, 1977).

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