Protein Synthesis in the Cotyledons of *Pisum sativum* L.

PROTEIN FACTORS INVOLVED IN THE BINDING OF PHENYLALANYL-TRANSFER RIBONUCLEIC ACID TO RIBOSOMES*

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1. Proteinaceous factors contained in a 0.5M-KCl extract of ribosomes from pea cotyledons form a ternary complex at 0°C with [¹⁴C]phenylalanyl-tRNA and poly(U). The complex is measured by its quantitative retention on Millipore filters. 2. Complex-assembly is optimal at 5mM-Mg²⁺ and is independent of GTP and ribosomes. 3. The addition of ribosomes is required to stabilize the complex at 34°C. The complex binds to a puromycin-sensitive site on the ribosome. 4. Soluble factors from the 250000g supernatant of pea cotyledon form a Millipore-retainable complex dependent on GTP and ribosomes. 5. Complex-formation by soluble factors has a Mg²⁺ optimum of 10–12mM and forms a puromycin-insensitive complex with ribosomes. 6. The function of the ribosomal protein factors and the supernatant fraction in initiation of protein synthesis is discussed.

Miller & Schweet (1968) demonstrated that the poly(U)-dependent incorporation of phenylalanine by ribosomal preparations from rabbit reticulocytes could be prevented if the ribosomes were washed with 0.5M-KCl. Incorporating activity was restored if proteins from the KCl wash were added back to the washed ribosomes. The ribosomal-wash proteins were also required for natural protein synthesis in a cell-free system using endogenous haemoglobin mRNA. Shafritz *et al.* (1970) and Prichard *et al.* (1970) have subsequently demonstrated that the ribosomal wash contains three proteinaceous components; two of these are required in addition to supernatant components for poly(U)-directed phenylalanine incorporation and the third factor is required in addition to the first two for utilization of natural mRNA.

These ribosomal-wash proteins function in phenylalanine incorporation by catalysing the poly(U)-directed binding of phenylalanyl-tRNA to ribosomes in the reticulocyte system. They are distinct from other proteinaceous factors usually found in the supernatant component that are required for polypeptide-chain elongation (Shafritz & Anderson, 1970).

More recent investigations indicate that the ribosomal proteinaceous factors also catalyse the formation of the initiation complex required for protein synthesis (Crystal & Anderson, 1972; Shafritz & Anderson, 1970). Generally, formation of this complex involves participation of ribosomal subunits,

mRNA, initiator methionyl-tRNA, GTP and the ribosomal-wash proteinaceous factors, which have been designated IF-1, IF-2 and IF-3 (Lucas-Lenard & Lipmann, 1971; Crystal & Anderson, 1972; Caskey *et al.*, 1972).

In addition it has been demonstrated in animal tissue that the ribosomal proteinaceous factors required for the binding of initiator methionyl-tRNA to the ribosomes can be replaced by soluble proteins isolated from the cytosol (postribosomal supernatant) (Gasior & Moldave, 1972; Leader *et al.*, 1972; Zasloff & Ochoa, 1971). In contrast with the formation of the initiator complex involving ribosomal proteins, the soluble- (cytosol)-enzyme-catalysed binding does not require the obligate participation of GTP (Gasior & Moldave, 1972; Zasloff & Ochoa, 1973). Additionally Mazumder (1972) has shown that GTP is not necessary for the binding of initiator methionyl-tRNA to ribosomes in *Escherichia coli* systems using ribosomal proteinaceous factors. A detailed appraisal of initiator-complex-formation in animal systems indicates that although GTP stimulates the process, considerable complex-formation can take place in its absence (Kerwar *et al.*, 1970; Heywood & Thompson, 1971; Shafritz & Anderson, 1970). The requirement for mRNA for formation of the initiation complex has also been questioned (Levin *et al.*, 1973).

Marcus (1970) and Seal *et al.* (1972), using the soluble proteinaceous factors from the postribosomal supernatant of wheat embryos, have demonstrated the formation of an initiation complex requiring ribosomes, initiator methionyl-tRNA and tobacco

* This is no. 3 of the series. No. 2 is Wells & Beevers (1973).

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mosaic virus RNA as messenger RNA. Their observations differ from animal systems using soluble protein factors in that both ATP and GTP are required for initiation-complex-formation. The soluble proteins were divided into two components both of which were required in the binding process (Seal *et al.*, 1972). Lin & Key (1973) in another plant system have utilized soluble proteinaceous factors to study the poly(U)-directed binding of phenylalanyl-tRNA to ribosomes. The binding of phenylalanyl-tRNA to ribosomes was dependent on GTP and had a high Mg^{2+} optimum (10mM). Since elongation factors have the capacity to catalyse the binding of aminoacyl-tRNA to ribosomes at high Mg^{2+} concentrations these results suggest that binding was accomplished by elongation factors rather than by initiation factors (Shafritz & Anderson, 1970).

As part of our investigations into protein synthesis in the pea cotyledon, we have demonstrated the dependence of poly(U)-directed binding of phenylalanyl-tRNA to ribosomes on proteinaceous factors derived from ribosomes by salt-washing. Since this represents the first report of ribosomal proteins from plants catalysing this reaction the system has been characterized and is compared with the binding catalysed by soluble proteins. Additionally, the requirements for complex-formation are compared with those derived from bacterial and animal systems.

Materials and Methods

KCl-washed ribosomes from dry pea meal

Ribosomes were prepared by a modification of the procedures of Beevers & Poulson (1972). Dry pea meal (20g) was quick-frozen in liquid N_2 and extracted with a mortar and pestle in 5 vol. of buffer I [20mM-Tris-HCl (pH 7.6), 15mM-KCl, 5mM- $MgCl_2$, 5mM-2-mercaptoethanol] containing 0.25M-sucrose. The homogenate was squeezed through cheesecloth, filtered through Miracloth (Calbiochem, San Diego, Calif., U.S.A.) and centrifuged at 20000g (r_{max} , 10.63cm) for 15min. Ribosomes were prepared from the postmitochondrial supernatant by layering 10ml of the supernatant over 7.0ml of 1.6M-sucrose in buffer I and centrifuging at 78480g (r_{av} , 7.8cm) for 180min at 0°C. The surface of the ribosomal pellets was washed with buffer I, the pellets were then suspended in 0.5M-KCl buffer [20mM-Tris-HCl (pH 7.6), 0.5M-KCl, 5mM- $MgCl_2$, 5mM-2-mercaptoethanol] and stirred overnight (16–18h) at 4°C. Ribosomal subunits were pelleted by centrifugation at 159315g (r_{av} , 5.7cm) for 90min, and the KCl-wash supernatant was kept. The surface of the subunit pellet was washed and the pellet suspended at a concentration of 100 E_{260} units/ml in buffer I containing 40% (v/v) glycerol and stored at –20°C.

Supernatant enzyme and ribosome proteinaceous factors

The supernatant enzyme (S-250 protein) was prepared by centrifuging the postmitochondrial supernatant, prepared from dry pea meal as described above, at 269245g (r_{av} , 5.7cm) for 120min at 0°C to pellet the ribosomes. The upper two-thirds of the supernatant was fractionated with $(NH_4)_2SO_4$ between 45% and 70% saturation. The precipitate from the 70%-satd. $(NH_4)_2SO_4$ fractionation was dissolved in 10mM-Tris-HCl (pH 7.6)–5mM-2-mercaptoethanol–40% (v/v) glycerol and stored at –20°C. The preparation referred to as ribosomal protein factor represents the 0–70%-satd. $(NH_4)_2SO_4$ fraction from the 0.5M-KCl-wash supernatant of washed ribosomes. Protein precipitating at 70%-satd. $(NH_4)_2SO_4$ was pelleted by centrifugation and dissolved in 10mM-Tris-HCl (pH 7.6)–5mM-2-mercaptoethanol–40% glycerol and stored at –20°C.

Phenylalanyl-tRNA

Purified total transfer RNA was prepared from developing pea cotyledons by a modification of the method of Vanderhoef *et al.* (1970). Tissue was homogenized for 1min at top speed in a Waring blender in 2 tissue volumes of buffer [0.1M-Tris-HCl (pH 7.5)–0.1M-NaCl–5mM-2-mercaptoethanol] and 4 tissue volumes of water-saturated redistilled phenol. The homogenate was stirred for 30min (4°C) and centrifuged at 12000g for 10min. The aqueous layer was extracted twice with 3 vol. of ether (–20°C), then made 0.2M with respect to sodium acetate and the RNA was then precipitated with 2.5 vol. of 95% (v/v) ethanol. Total RNA, which precipitated during 2h of gentle stirring, was collected by centrifugation at 5000g for 5min and allowed to dry partially in air. The RNA pellet was suspended in 3.0M-sodium acetate (pH 6.0) and stored overnight at 4°C. The suspension was centrifuged (5000g for 5min) and the RNA from the supernatant was diluted with 1 vol. of water and then precipitated with 2.5 vol. of 95% ethanol. The RNA soluble in 3.0M-sodium acetate was further purified on DEAE-cellulose. The RNA was dissolved in column buffer [10mM-sodium acetate (pH 4.5)–10mM- $MgCl_2$ –0.3M-NaCl–0.5mM-2-mercaptoethanol] and adsorbed on a DEAE-cellulose column (1.7cm × 25cm) equilibrated in the same buffer. The column was washed with several volumes of column buffer and RNA was eluted by increasing the NaCl concentration of the buffer to 1.0M. The eluted RNA was precipitated with 2.5 vol. of 95% ethanol, collected by centrifugation and dissolved in 10mM- $MgCl_2$ –0.3M-NaCl.

The RNA was further fractionated by chromatography on benzoylated DEAE-cellulose by the method of Gilliam *et al.* (1967). DEAE-cellulose-purified RNA (4000–6000 E_{260} units) was adsorbed

on a benzoylated-DEAE-cellulose column (2.4 cm $\times$ 55 cm) equilibrated with 10 mM-MgCl₂-0.3 M-NaCl. The column was washed with 10 mM-MgCl₂-1.0 M-NaCl, until the E_{260} was less than 0.5 unit. This procedure eluted most of the RNA. The tRNA^{Phe} was then eluted with 10 mM-MgCl₂-1.2 M-NaCl-15% (v/v) ethanol. The tRNA^{Phe} was precipitated with 2.5 vol. of 95% ethanol and dissolved in distilled deionized water.

Aminoacylation

Aminoacylation of purified tRNA^{Phe} with [¹⁴C]-phenylalanine was carried out in 10 ml reaction mixtures. The reaction mixture contained 0.1 M-Tris-HCl (pH 7.6), 1 mM-ATP, 5 mM-MgCl₂, 50 μ Ci of [U-¹⁴C]phenylalanine (492 μ Ci/ μ mol; 900 c.p.m./pmol), 200 E_{260} units of purified tRNA^{Phe} and 500 μ g of aminoacyl-tRNA synthetase [prepared from developing pea cotyledons as described by Vanderhoef *et al.* (1970) except that 10 mM-Tris-HCl (pH 7.6) was used in place of Hepes buffer]. The mixture was incubated at 30°C for 15 min and the reaction stopped by adding 2 vol. of 10 mM-sodium acetate (pH 4.5)-10 mM-MgCl₂-0.3 M-NaCl. The reaction mixture was centrifuged to remove precipitated proteins and the resulting supernatant applied to a DEAE-cellulose column (0.9 cm $\times$ 17 cm) equilibrated with 10 mM-sodium acetate (pH 4.5)-10 mM-MgCl₂-0.3 M-NaCl. The column was washed with several volumes of buffer and the [¹⁴C]phenylalanyl-tRNA was eluted by raising the NaCl concentration of the buffer to 1.0 M. The [¹⁴C]phenylalanyl-tRNA was precipitated with ethanol. The collected precipitate was dissolved in water and stored at -20°C in 0.5 ml samples in 1.0 ml septum vials.

Phenylalanyl-tRNA binding and [¹⁴C]phenylalanyl-puromycin synthesis

Binding of [¹⁴C]phenylalanyl-tRNA was measured essentially as described by Nirenberg & Leder (1964). Reactions (details are given in the appropriate Tables) were stopped by the addition of 4 ml of an ice-cold dilution buffer containing 50 mM-Tris-HCl (pH 7.6), 100 mM-KCl and MgCl₂ corresponding to the concentration in the reaction mixture. This mixture was immediately collected on a nitrocellulose membrane (0.45 μ m; Millipore Filter Corp., Bedford, Mass., U.S.A.) and washed with 5 $\times$ 5 ml of dilution buffer. Care was taken not to allow any air to be drawn through the membrane. Only [¹⁴C]phenylalanyl-tRNA associated in a complex was retained.

For the study of [¹⁴C]phenylalanyl-puromycin formation complete reaction mixtures containing the following components in a final volume of 0.5 ml, 50 mM-Tris-HCl, pH 7.6, 100 mM-KCl, 1 mM-2-mercaptoethanol, 22 pmol of [¹⁴C]phenylalanyl-tRNA

(900 c.p.m./pmol; 220 000 c.p.m./ E_{260} unit), 5 mM-MgCl₂, 50 μ g of poly(U), 0.6 E_{260} unit of salt-washed ribosomes and where indicated 40 μ g of S-250 protein or 80 μ g of ribosomal protein factors with or without 0.2 mM-GTP, were incubated at 0°C for 10 min. Subsequently, 50 μ l of 10 mM-puromycin hydrochloride, neutralized to pH 7.0 with NaOH, was added and incubated for 30 min at 0°C. The reaction was terminated by the addition of 1.0 ml of 0.1 M-sodium acetate buffer, pH 5.5, and the mixture extracted with 1.5 ml of ethyl acetate for 2 min with a vortex mixer (Mazumder, 1972). A portion (1.0 ml) of the ethyl acetate phase was removed and added to 12 ml of scintillation fluid [80 g of PPO (2,5-diphenyloxazole), 2 litres of toluene, 1 litre of Triton X-100] and counted for radioactivity in a Beckman LS-100 scintillation counter. The results are expressed per 1.5 ml of ethyl acetate phase.

Results

Binding of [¹⁴C]phenylalanyl-tRNA with supernatant factors

In the presence of S-250 proteins, prepared from the ribosomal supernatant fraction, it was found that the production of radioactivity retainable on nitrocellulose filter required washed ribosomes, poly(U), GTP and Mg²⁺ (Table 1).

Under conditions of the standard assay (0°C and 5 mM-MgCl₂) the binding reaction was proportional to the concentration of supernatant protein added up to 40 μ g (Fig. 1) and increased linearly with concentration of 0.5 M-KCl-washed ribosomes to about 0.6 E_{260} unit of ribosomes/reaction vol. of 0.5 ml (Fig. 2).

Although routine assays were conducted at 5 mM-MgCl₂, binding was enhanced by increasing Mg²⁺ concentrations. However, at the higher concentration of Mg²⁺ an increasing proportion of the binding was non-enzymic (Fig. 3). When adjustments were made for the non-enzymic binding the optimal concentration for the supernatant-protein-directed binding was 10-12 mM. This is similar to the value reported by Lin & Key (1973) using a similar supernatant system also derived from pea and by Shafritz & Anderson (1970) in a reticulocyte system using enzymes extracted from the postribosomal supernatant.

The binding reaction was completed in 10 min at 0°C. If the reaction mixture was made up to 5% (v/v) trichloroacetic acid and heated to 95°C before filtration no radioactivity was retained. This acid-lability of the Millipore-retainable radioactivity indicates that the radioactive component retained or bound was [¹⁴C]phenylalanyl-tRNA (Table 2). In contrast if the reaction in the presence of S-250 proteins was conducted at 34°C there was a tenfold

Table 1. *Requirements for the formation of a nitrocellulose-filter-retainable complex by using soluble S-250 proteins*

The complete incubation system contained the following components in a final volume of 0.5 ml: 50 mM-Tris-HCl (pH 7.6), 100 mM-KCl, 1 mM-2-mercaptoethanol, 22 pmol of [14 C]phenylalanyl-tRNA (900 c.p.m./pmol; 220 000 c.p.m./ E_{260} unit), 5 mM-MgCl₂, 0.2 mM-GTP, 50 μ g of poly(U), 0.6 E_{260} unit of salt-washed ribosomes and 40 μ g of S-250 protein; 0.5 mM-guanylyl 5'-methylendiphosphonate and 0.2 mM-ATP were added where indicated. The mixture was incubated at 0°C for 10 min. Complex formation was determined as described in the Materials and Methods section.

Incubation system	Omission	[14 C]Phenylalanyl-tRNA bound (c.p.m.)
Complete	—	1809
	Mg ²⁺	202
	GTP	238
	Poly(U)	240
	Ribosomes	234
	S-250 Protein	234
+ Guanylyl 5'-methylene-diphosphonate	GTP	194
+ ATP	GTP	202

Fig. 1. *Dependence of the binding assay on factor concentration*

Reaction mixture and assay conditions are as described in Table 1. The ribosomal protein factors and S-250 protein were added as indicated. Each value is corrected for the amount of radioactivity bound in the absence of factor. Δ , S-250 protein; $\circ$, ribosomal protein factors; $\square$, S-250 protein + ribosomal protein factors.

Fig. 2. *Dependence of the S-250-protein-binding reaction on ribosomal concentration*

Incubation conditions and components are as described in Table 1. KCl-washed ribosomes were added as indicated. Each value is corrected for the amount of radioactivity bound in the absence of factor.

increase in the radioactivity retained on the Millipore filter and this was not eliminated if the reaction mixtures were heated in trichloroacetic acid before filtration. This is consistent with the formation of poly[14 C]phenylalanine at elevated incubation temperatures.

The binding of [14 C]phenylalanyl-tRNA was dependent on the presence of GTP. In so far as the binding could be prevented by guanylyl 5'-methylene-diphosphonate (Table 1), the hydrolysis of GTP appears to be an integral part of the binding catalysed by S-250 protein.

These characteristics of the binding reaction catalysed by the supernatant protein are analogous to those involving aminoacyl transferases (Legocki & Marcus, 1970; Shafritz & Anderson, 1970).

Binding of [14 C]phenylalanyl-tRNA with ribosomal protein factors

Initial studies investigating the ribosomal-protein-factor-catalysed binding of [14 C]phenylalanyl-tRNA were based on experiments that had established the requirements for the S-250-protein-mediated reaction. By using this approach it was found that nitrocellulose filter-retainable radioactivity was produced when ribosomal protein factors were incubated with ribosomal subunits, GTP, Mg²⁺, poly(U) and [14 C]phenylalanyl-tRNA (Table 3). However, a systematic elimination of components indicated that the production of a complex capable of being retained by the filters was dependent only on the presence of ribosomal protein factors, poly(U), Mg²⁺ and [14 C]phenylalanyl-tRNA. Ribosomal subunits and

Table 2. Influence of enzyme source (S-250 protein or ribosomal protein factors) and reaction temperature on the sensitivity of the binding complex to discharge by hot 5% trichloroacetic acid

The complete incubation system and conditions were used as described in Tables 1 and 3 except for the incubation at 34°C. Millipore-bound radioactivity was determined as described in the Materials and Methods section. Sensitivity to trichloroacetic acid was determined by stopping the reaction with 3 ml of cold 5% trichloroacetic acid, and after a 10 min incubation at 0°C the mixture was heated at 95°C for 20 min. After cooling of the acidified reaction mixture to 0°C for 10 min, the hot-trichloroacetic acid-insoluble radioactivity was collected on nitrocellulose filters, washed with additional cold 5% trichloroacetic acid and counted for radioactivity as described in the Materials and Methods section.

Reaction temperature	Radioactivity (c.p.m.)		
	Enzyme ribosomal protein factors		Enzyme S-250 protein
	Millipore-bound	Millipore-bound	Hot-trichloroacetic acid-precipitable
0°C	1066	1726	50
34°C	360	19 500	18 300

Table 3. Requirements for the formation of a nitrocellulose-filter-retainable complex by using ribosomal protein factors

Incubations were as described in Table 1 except that S-250 protein was replaced by 80 µg of ribosomal protein factors.

Incubation system	[¹⁴ C]Phenylalanyl-tRNA bound (c.p.m.)
Complete	990
— Ribosomes	1066
— GTP	1078
— Mg ²⁺	328
— Ribosomes, GTP	1162
— Ribosomes, GTP, ribosomal protein factors	247
— Ribosomes, GTP, poly(U)	276

GTP were not required for the formation of a retainable complex. It has been suggested that the binding of [¹⁴C]phenylalanyl-tRNA by ribosomal protein factors and its retention on the filter may be due to the presence of phenylalanyl-tRNA synthetase (Levin *et al.*, 1973). However, this seems unlikely since (a) the formation of the retained complex is poly(U) dependent and (b) the S-250 fraction, which is enriched in phenylalanyl-tRNA synthetase, does not form a retainable complex in the absence of ribosomes and GTP.

The formation of the complex capable of binding to the nitrocellulose filters catalysed by ribosomal protein factors was time-dependent at 0°C, and when studies of complex-formation were conducted over a 10 min period the reaction was dependent on Mg²⁺ and concentration of ribosomal protein factors. The optimal Mg²⁺ concentration for the formation of the nitrocellulose-filter-retainable complex catalysed by ribosomal protein factors in the presence of poly(U) and [¹⁴C]phenylalanyl-tRNA was 5 mM (Fig. 3). This is in contrast with a 10–12 mM optimal Mg²⁺ concentration required for the binding catalysed

Fig. 3. Effect of the concentration of MgCl₂ on the binding reaction with S-250 protein (Δ), ribosomal protein factor (○), or without enzyme (□)

Factor-catalysed activity has been corrected for non-enzymic binding. Conditions of standard assay are as described in Table 1 except for MgCl₂ concentration.

by the S-250 protein. The Mg²⁺ concentration required for optimal binding by the ribosomal protein factors is similar to the concentration reported by Shafritz & Anderson (1970) for the poly(U)-dependent binding of [¹⁴C]phenylalanyl-tRNA catalysed by proteins prepared from rabbit reticulocyte ribosomes. As the Mg²⁺ concentration was raised above 7 mM the ribosomal-protein-factor-promoted binding decreased (Fig. 3). The Mg²⁺ concentration for optimal binding was unaltered by the addition of ribosomes.

Fig. 4. Effect of ribosomes on the stability of the ribosomal protein factor-dependent ternary complex

Incubation conditions and components are as described in Table 3, except that [^{14}C]phenylalanyl-tRNA was limiting (2 pmol; 900 c.p.m./pmol) in all reactions. In addition, the reaction was run to completion (20 min) at 0°C and then transferred to a 34°C water bath with the addition of 3.0 E_{260} units of ribosomes (O) or an equal volume of water (●). The reaction was scaled up fivefold. Samples (0.5 ml) were taken at the times indicated and the percentage of the ternary complex remaining was determined: 100% complex was 1686 c.p.m.

In contrast with the binding catalysed by S-250 protein, the formation of a nitrocellulose-filter-retainable complex in the presence of ribosomal protein factors was not stimulated by elevated temperatures; in fact, the Millipore-bound radioactivity decreased when the reaction mixture was incubated at 34°C (Table 2). The Millipore-bound radioactivity was completely eliminated by prior treatment of the reaction mixtures with 5% trichloroacetic acid at 95°C, indicating that the retainable radioactivity was due to [^{14}C]phenylalanyl-tRNA. The finding that less Millipore-bound radioactivity was produced from reaction mixtures incubated at 34°C than at 0°C indicated that the binding complex formed in the presence of poly(U), [^{14}C]phenylalanyl-tRNA and ribosomal protein factors was heat-labile or was not assembled at elevated temperatures. When reaction mixtures consisting of ribosomal protein factors, poly(U), [^{14}C]phenylalanyl-tRNA and Mg^{2+} were incubated at 0°C for 20 min and then incubated at 34°C in the presence of either 0.6 E_{260} unit of KCl-washed ribosomes or an equal volume of water, there was a rapid loss of Millipore-retainable radioactivity at the elevated temperatures in the reaction mixture without ribosomes. This loss of

Millipore-retainable radioactivity was prevented by the addition of KCl-washed ribosomes (Fig. 4).

These results with the ribosomal-protein-factor-mediated formation of a Millipore-retainable complex are interpreted as indicating the formation of a ternary complex with the composition ribosomal protein factor-poly(U)-[^{14}C]phenylalanyl-tRNA.

Ribosome binding

The observation that the complex was stabilized against high-temperature decay by the addition of KCl-washed ribosomes suggests that the ternary complex is able to bind to ribosomes.

This proposition was confirmed in experiments involving sucrose-density-gradient centrifugation of reaction mixtures in which ribosomal protein factors, poly(U) and [^{14}C]phenylalanyl-tRNA had been incubated in the presence of KCl-washed ribosomes. Ribosomes washed in 0.5 M-KCl dissociate into 40S and 60S subunits (Lin & Key, 1971; Wells & Beevers, 1973). However, if the salt-washed ribosomes are returned to low-salt buffers some subunit reassociation occurs, with the production of 80S ribosomes (Fig. 5). Similar reassociation is observed in preparations from animal tissues (Heywood & Thompson, 1971). In spite of this re-formation of monomeric ribosomes from the 40S and 60S subunits it was observed that when incubations were carried out at 0°C at 5 mM- MgCl_2 , radioactivity from [^{14}C]phenylalanyl-tRNA became associated only with the 40S subunit (Fig. 5a). If ribosomal protein factors were omitted from the reaction mixture no binding to the 40S subunit occurred (results not shown). It was also apparent (Fig. 5b) that GTP was not required for the binding of the complex to the 40S subunit.

When the supernatant S-250 protein was included in the initial incubation at 0°C along with the ribosomal protein factors and GTP, radioactivity was associated with the 40S and 80S regions of the density gradients (Fig. 5c). If reaction mixtures containing ribosomal protein factors, GTP, poly(U), KCl-washed ribosomes and [^{14}C]phenylalanyl-tRNA were incubated at 0°C and then transferred to 34°C with the addition of S-250 preparation there was a relocation of radioactivity from the 40S to the 80S region (Figs. 5a and 5d). In none of the experiments did radioactivity become associated with the 60S region.

Puromycin-sensitivity of radioactivity bound to ribosomes

The observed differences in requirements for binding mediated by ribosomal protein factors and S-250 proteins suggested that the two components promoted binding at two different sites on the ribosome. This concept was supported by the finding (Fig. 1) that ribosomal protein factors and the S-250 proteins

Fig. 5. Sucrose-density-gradient profiles of initiation complexes

Formation of the ternary complex and binding to ribosomes were performed in consecutive incubation steps; all contained 50 mM-Tris-HCl (pH 7.6), 100 mM-KCl, 5 mM-MgCl₂, 1 mM-2-mercaptoethanol, 0.6 E_{260} unit of KCl-washed ribosomes and 50 μ g of poly(U). (a) Step 1: additions for ternary-complex-formation in 100 μ l were 22 pmol of [¹⁴C]phenylalanyl-tRNA (900 c.p.m./pmol), 80 μ g of ribosomal protein factors; incubation was at 0°C for 10 min. (b) Step 2: as step 1 but 0.2 mM-GTP omitted; incubation was for 10 min at 0°C. (c) Step 3: as step 1 but 40 μ g of S-250 protein added; incubation was for 10 min at 0°C. (d) Step 4: as step 1, but mixture transferred, after 10 min at 0°C, to 34°C for an additional 10 min. The sedimentation profile of each incubation stage was determined in 5.4 ml sucrose gradients (15–45%, w/w) in the same buffer-salt mixtures used in the corresponding incubations. Centrifugation was at 189 000 g (r_{av} . 8.35 cm) for 4.3 h at 0°C. The E_{254} of the gradients was monitored in an ISCO fractionator and 0.3 ml fractions were collected at a flow rate of 0.3 ml/min. Each fraction of each gradient was diluted in the corresponding buffer, collected on nitrocellulose membranes and counted for radioactivity as described in the Materials and Methods section. ----, [¹⁴C]Phenylalanyl-tRNA; —, E_{260} .

promoted binding co-operatively at 0°C and is further confirmed by the sucrose-density-gradient information, which indicates that ribosomal protein factors stimulated binding only to the 40S subunits whereas in the presence of S-250 proteins and ribosomal protein factors label became associated both with the 40S subunit and monomeric ribosomes.

Additional indications that the ribosomal protein factor and S-250 protein were catalysing binding at different ribosomal sites were obtained from studies of the sensitivity of the bound radioactivity to release

by puromycin. Some 85% of the radioactivity bound in the presence of ribosomal protein factors was capable of complexing with puromycin (Table 4). The formation of the [¹⁴C]phenylalanyl-puromycin complex was not dependent on GTP. In contrast only 18% of the radioactivity bound in the presence of S-250 proteins at 0°C complexed with puromycin. When incubations were conducted at 34°C in the presence of ribosomal protein factors, S-250 proteins and GTP, the polyphenylalanine produced was quantitatively released by puromycin.

Table 4. Puromycin-reactivity of the complex formed in the presence of ribosomal subunits

Incubation conditions and components are described in Table 1 except that after the 10min incubation at 0°C, 50 μ l of 10mM-puromycin (adjusted to pH 7.0 with NaOH) was added to one set of samples, and another identical set received 50 μ l of water. After further incubation for 30min at 0°C, the amount of [¹⁴C]phenylalanyl-tRNA bound and its puromycin-reactivity were measured as described in the Materials and Methods section.

Enzyme	GTP (mM)	[¹⁴ C]Phenylalanyl-tRNA bound (c.p.m.)	[¹⁴ C]Phenylalanyl-puromycin formed (c.p.m.)	Puromycin reactivity (%)
Ribosomal protein factor	None	2094	1780	85
Ribosomal protein factor	0.2	1948	1730	89
S-250 protein	None	32	—	—
S-250 protein	0.2	916	168	18
Ribosomal protein factor+S-250 protein	0.2	3042	2663	88

Thus on the basis of the ultracentrifugation and puromycin studies it appeared that the binding of [¹⁴C]phenylalanyl-tRNA, catalysed by ribosomal protein factor, was to a puromycin-sensitive site on the 40S ribosomal subunit. The S-250 protein factors appeared to catalyse binding at 0°C into puromycin-insensitive sites.

Discussion

The poly(U)-dependent binding of [¹⁴C]phenylalanyl-tRNA catalysed by ribosomal protein factors shows close similarities to that described by Shafritz & Anderson (1970) involving purified proteinaceous factors from rabbit reticulocyte ribosomes. According to Shafritz & Anderson (1970) and Crystal & Anderson (1972), these same factors function in peptide-chain initiation, by using AUG or natural mRNA and initiator methionyl-tRNA. However, the requirements for poly(U)-directed binding of [¹⁴C]phenylalanyl-tRNA by ribosomal protein factors differ in some respects from those established for the formation of an initiation complex with methionyl-tRNA and AUG in animal tissues. The most recent evidence of Levin *et al.* (1973) has demonstrated that binding of methionyl-tRNA can occur without mRNA, but there is a strict dependence on GTP. In the binding of phenylalanyl-tRNA to ribosomes from plants, catalysed by ribosomal protein factors, there is no requirement for GTP but a strict dependence on poly(U). This lack of a requirement for GTP is similar to the previously described formation of an initiation complex in animal systems by using soluble (postribosomal supernatant) enzymes (Gasior & Moldave, 1972; Zasloff & Ochoa, 1973) and is similar to the report by Mazumder (1972) of the formation of an initiation complex in bacterial systems using ribosomal proteins which show no GTP dependence. In agreement with the studies using natural mRNA or AUG and methionyl-tRNA (Dubnoff *et al.*, 1972;

Crystal & Anderson, 1972; Levin *et al.*, 1973; Weeks *et al.*, 1972; Heywood & Thompson, 1971) in animal, bacterial and plant systems the poly(U)-directed binding of [¹⁴C]phenylalanyl-tRNA in the presence of ribosomal protein factors and washed ribosomes occurs on the small subunit.

The S-250 protein fraction from peas catalysed different reactions from the partially purified soluble fractions from wheat seeds (Seal *et al.*, 1972). The soluble, postribosomal supernatant, proteins from wheat seeds, in the presence of tobacco mosaic virus-RNA, can catalyse the formation of an initiation complex with methionyl-tRNA on the 40S subunit (Weeks *et al.*, 1972). It appears that the S-250 protein fraction from the dry pea seed catalyses reactions more typical of aminoacyltransferases and peptide-chain elongation (Legocki & Marcus, 1970; Shafritz & Anderson, 1970). Significantly the soluble protein from dry wheat seeds contains both protein-chain initiation factors and transfer (elongation) factors (Seal *et al.*, 1972; Legocki & Marcus, 1970). In the dry pea preparations the initiation components, as measured by the poly(U)-mediated phenylalanyl-tRNA binding at low Mg²⁺ concentration and low temperature, appear to be confined to the ribosomes.

Seal *et al.* (1972) indicate that the soluble proteins from wheat seeds functional in protein-chain initiation were unable to catalyse poly(U)-directed phenylalanyl-tRNA binding to ribosomes. This clearly contrasts with our present investigation. Although there may be inherent differences between the two proteins, it is possible that part of the difference may be accounted for by the fact that Seal *et al.* (1972) used deoxycholate-washed ribosomes. We have observed that KCl washes or ribosomal subunits prepared from ribosomes extracted in the presence of deoxycholate are non-functional in poly(U)-directed phenylalanine binding involving ribosomal protein factors. In contrast the S-250-protein-catalysed binding was unaffected by deoxycholate extraction.

Although work is continuing to characterize the initiation process in peas using natural mRNA or AUG and methionyl-tRNA, current evidence with the poly(U)-[¹⁴C]phenylalanyl-tRNA system suggests the following sequence of reactions:

- (1) Ribosomal protein factors+poly(U)+[¹⁴C]phenylalanyl-tRNA →
(ribosomal protein factors-poly(U)-[¹⁴C]phenylalanyl-tRNA)
- (2) (Ribosomal protein factors-poly(U)-[¹⁴C]phenylalanyl-tRNA)+40S →
(40S-ribosomal protein factors-poly(U)-[¹⁴C]phenylalanyl-tRNA)
- (3) (40S-Ribosomal protein factors-poly(U)-[¹⁴C]phenylalanyl-tRNA)+60S $\xrightarrow[\text{GTP}]{\text{S-250}}$
(80S-poly(U)-[¹⁴C]phenylalanyl-tRNA)+ribosomal protein factors+GDP

The participation of 60S subunits in reaction (3) is at present speculative and is based chiefly on published evidence that similar reactions have been proposed during the initiation reaction using AUG, initiator methionyl-tRNA and ribosomal subunits.

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