Studies on Native Ribosomal Subunits from Rat Liver. Evidence for Activities Associated with Native 40S Subunits that Affect the Interaction with Acetylphenylalanyl-tRNA, Methionyl-tRNA$_f$, and 60S Subunits†

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ABSTRACT: The binding of the initiator tRNA Met-tRNA$_f$, and of acetylphenylalanyl-tRNA, has been examined with rat liver 40S subunits derived from 80S ribosomes by dissociation with native 40S subunits sedimented from the postmicrosomal fraction and with native 40S subunits extracted with high salt-containing solutions. Binding of Met-tRNA$_f$ and acetylphenylalanyl-tRNA to derived and to salt-extracted native 40S subunits is observed in the presence of the appropriate polynucleotide template and a highly purified binding factor obtainefrom the soluble fraction of rat liver homogenates (R.L. IF-1). Native 40S subunits bind acetylphenylalanyl-tRNA in a reaction that requires poly(U) but not exogenous binding factor; however, Met-tRNA$_f$ is not bound to native subunits, even when supplemented with the soluble binding factor, or under conditions where factor-independent, high Mg^{2+}-stimulated binding is observed with the derived and the salt-washed native 40S subunits. The extract obtained from native 40S subunits promotes the binding of acetylphenylalanyl-tRNA but not Met-tRNA$_f$ to derived and to salt-extracted native subunits. The addition of native 40S extract to incubations containing R.L. IF-1, Met-tRNA$_f$, and derived 40S subunits, inhibits the formation of 40S·Met-tRNA$_f$ complex. These data suggest that the binding activity that is specific for 40S subunits and initiator tRNA, and an activity that inhibits the interaction with Met-tRNA$_f$ specifically, are both associated with native 40S subunits, and can be extracted from them by treatment with high salt-containing solutions. Derived 40S subunits react quantitatively with 60S particles to form 80S ribosomes which do not bind acetylphenylalanyl-tRNA with binding factor R.L. IF-1. Native 40S subunits react only partly with 60S subunits; about half of the native 40S subunit population forms 80S ribosomes which do not subsequently bind acetylphenylalanyl-tRNA; the remaining native 40S subunits which do not react with 60S particles bind acetylphenylalanyl-tRNA but to a lesser extent. When preformed native 40S·acetylphenylalanyl-tRNA complex is incubated with 60S subunits, about half of the subunits form an 80S·acetylphenylalanyl-tRNA complex, while the rest remains as 40S·acetylphenylalanyl-tRNA. The addition of native 40S subunit salt extract to incubations containing preformed 80S ribosomes dissociates the particles to subunits. These data suggest that in addition to the initiator tRNA binding activity and the activity that inhibits Met-tRNA$_f$ interaction, part of the native 40S subunit population also contains an activity that dissociates 80S ribosomes.

An activity has been obtained from the supernatant of a number of cells, that stimulated the template-dependent, GTP-independent binding of analogues of initiator tRNA or Met-tRNA$_f$ to 40S subunits (Zasloff and Ochoa, 1971; Leader and Wool, 1972; Gasior and Moldave, 1972; McCroskey et al., 1972; Zasloff and Ochoa, 1973; Cimadevilla and Hardesty, 1975). Previous studies in this laboratory (Gasior and Moldave, 1972) indicated that the soluble factor isolated from rat liver stimulated the binding of acetylphenylalanyl-tRNA, phenylalanyl-tRNA, and *E. coli* fMet-tRNA$_f$ to 40S subunits derived from 80S ribosomes, in the presence of the appropriate polynucleotide template. With crude preparations of the binding factor, an interaction between homologous Met-tRNA$_f$ and 40S subunits could not be demonstrated; recent experiments described in detail elsewhere (McCuiston et al., 1975), however, indicated that highly purified preparations of the supernatant factor catalyzed the codon-dependent binding of Met-tRNA$_f$, as well as acetylphenylalanyl-tRNA and phenylalanyl-tRNA but not Met-tRNA$_m$, to derived 40S subunits.

Recent studies with extracts prepared from native ribosomal subunits presented evidence for a factor that catalyzed the GTP-dependent, mRNA-independent binding of Met-tRNA$_f$ to 40S subunits in Ehrlich ascites cells (Smith and Henshaw, 1975), and for a factor that catalyzed the dissociation of ribosomes to subunits in Ehrlich ascites cells (Ayuso-Parilla et al., 1973) and reticulocytes (Lubsen and Davis, 1974). This communication describes the reaction of free, native 40S subunits, and of derived 40S subunits from rat liver with aminoacylated tRNAs to form particle–substrate complexes in the presence of template, and with 60S subunits to form 80S ribisomes. Evidence is presented for a functional heterogeneity in the subunit population, and for the presence in native subunits of activities that catalyze the binding of initiator tRNA, the dissociation of ribosomes, and of an activity that affects the 40S subunit·Met-tRNA$_f$ complex.

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Materials and Methods

Preparation of Aminoacyl-tRNAs, Binding Factor, Ribosomes and Derived Ribosomal Subunits. The preparation of isotopically labeled [^{3}H]phenylalanyl-tRNA (Siler and Moldave, 1969), acetyl[^{3}H]phenylalanyl-tRNA (Haenni and Chapeville, 1966), [^{3}H]Met-tRNA$_f$, and [^{3}H]Met-tRNA$_m$ (Smith and Marcker, 1970; Schroer and Moldave, 1973) have been described in detail. The specific activity of the tRNA-bound acetylphenylalanine was 4300 cpm/pmol (1600–3000 cpm/μg of aminoacyl-tRNA) and that of the tRNA-bound methionine was 3450 cpm/pmol (1500–2900 cpm/μg of aminoacyl-tRNA). Acetylmethionyl-tRNA$_f$ was prepared by acetylating the labeled Met-tRNA$_f$ with acetic anhydride as described for phenylalanyl-tRNA (Haenni and Chapeville, 1966). ApUpG was prepared by the method of Leder (1967) or purchased from Miles Laboratories.

The soluble binding factor specific for 40S subunits (R.L. IF-1) was purified from rat liver supernatant by fractionation with ammonium sulfate and chromatography on calcium phosphate gel, DEAE-Sephadex, and carboxymethylcellulose (McCuiston et al., 1975). Acrylamide gel electrophoresis of the purified preparation revealed one major band which accounted for over 80% of the total protein, and the molecular weight was estimated to be about 70000 on the basis of its elution from Sephadex G-200. The optimum Mg^{2+} concentration for the binding reaction was lowered by R.L. IF-1 to between 6 and 8 mM, and the optimal temperatures were between 10 and 15° for Met-tRNA$_f$ and 37° for acetylphenylalanyl-tRNA.

Rat liver ribosomes were prepared from microsomes, purified by centrifugation through discontinuous sucrose gradients containing 0.5 M NH_4Cl (Skogerson and Moldave, 1967, 1968), stripped of endogenous peptidyl-tRNA with puromycin (Gasior and Moldave, 1972), and dissociated into subunits with 0.88 M KCl (Martin and Wool, 1968; Gasior and Moldave, 1972). The procedure for the removal of peptidyl-tRNA with puromycin was modified to include EF-2 (50 μg/ml, eluted from hydroxylapatite columns; Moldave et al., 1971), GTP (0.2 mM), and lower concentrations of puromycin (0.02 mM). The dissociated subunits were resolved by ultracentrifugation for 17.5 hr in a linear-with-radius 20–45% sucrose gradient using a Beckman Ti-15 zonal rotor; the solutions used for the gradient zonal centrifugation contained 0.88 M KCl, 0.05 M Tris-HCl (pH 7.6), 12.5 mM $MgCl_2$, and 6 mM mercaptoethanol; the temperature of centrifugation was 10°. The resolved 40S and 60S subunits were concentrated by ultracentrifugation; they were designated as derived 40S (d40S) and derived 60S (d60S) subunits. Evidence that d40S subunits were free of 60S particles has been obtained (Thompson and Moldave, 1974); d60S subunits were necessary for poly(phenylalanine) synthesis and gel electrophoresis of RNA extracts did not reveal 28S RNA.

Preparation of Free, Native Subunits. The postmicrosomal supernatant fraction (Moldave et al., 1971) was used for the preparation of free, native subunits. Approximately 1.5 l. of the supernatant was centrifuged at about 80000g for 16 hr at 2°. The sedimented pellets were resuspended in 100 ml of medium C solution (50 mM Tris-HCl [pH 7.3 at 20°], 4 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, and 0.35 M sucrose) and centrifuged at 130000g for 4.5 hr at 2°. The material sedimented from this centrifugation was resuspended in medium C, at a concentration of about 200 A_{260} units/ml and could be stored frozen at this step. For resolution of the subunits, a linear-with-radius 20–45% sucrose gradient (containing 10 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 70 mM KCl, and 7 mM mercaptoethanol) was prepared with a high capacity gradient pump (Beckman) and the Ti-15 zonal rotor was allowed to run at 3000 rpm for several hours until the temperature reached 2°. Approximately 4000 A_{260} units of the resuspended pellets was layered on the gradient and centrifugation was carried out at 31000 rpm for 20 hr. At the end of the centrifugation period, the rotor was unloaded from the edge, with distilled water, using the high capacity pump; the eluate was continuously scanned at A_{254} and fractions were collected. The gradient fractions containing 40S and 60S subunits free of membranes or polysomes were pooled, individually, and sedimented by centrifugation at 80000g for 16 hr. Each subunit preparation was then resuspended in medium C at a concentration of about 150 A_{260} units/ml and stored frozen at −70° in aliquots. Sucrose gradient centrifugation analysis of the postmitochondrial supernatant revealed that the native subunits accounted for less than 10% of the total ribosome population; the free 80S monosome peak represented approximately 4% of the total. All of the subunit preparations were checked for purity by centrifugation through analytical linear (10–30%) sucrose gradients; the 40S preparation, designated as native 40S (n40S) subunits, was less than 10% contaminated with 60S subunits, as determined by analytical sucrose gradient centrifugation and by gel electrophoresis of extracted RNA. There was no evidence of d40S subunit contamination in the n40S fraction; CsCl density gradient centrifugation analysis, to be described in detail subsequently, revealed particles with densities characteristic of n40S subunits; particles with densities corresponding to d40S subunits were not detected. Further, rat liver ribosomes and polysomes do not appear to dissociate spontaneously to subunits under the conditions used to prepare native subunits, and require treatment with puromycin, high salt concentrations, and temperatures somewhat higher than 2°.

Salt-extracted 40S ("washed" n40S) subunits were prepared from free, native subunits by the addition of KCl and $MgCl_2$ to a final concentration of 0.88 M and 12.5 mM, respectively, to suspensions of n40S subunits in medium C. The suspension was centrifuged at 165000g for 4 hr at 2°, to sediment the "washed" n40S subunits; the particles were resuspended and centrifuged through a discontinuous 0.5–1.0 M sucrose gradient (Skogerson and Moldave, 1967, 1968) containing 0.88 M KCl and 12.5 mM $MgCl_2$ in medium C. The first supernatant was dialyzed overnight at 5° against a solution containing 10 mM Tris-HCl buffer (pH 7.5), 5% glycerol, 0.2 M (or occasionally 0.05 M) KCl, and 1 mM dithiothreitol, then concentrated by immersing the bag in several changes of Sephadex G-25, coarse, or by vacuum dialysis, to obtain the "n40S extract".

Binding Assays. Binding of aminoacylated tRNAs to 40S subunits was assayed essentially as described previously (Gasior and Moldave, 1972). Approximately 16 pmol of derived 40S, native 40S, or "washed" native 40S subunits was incubated with about 1 μg of purified R.L. IF-1, and 30 μg of acetylphenylalanyl-tRNA (plus 10μg of poly(uridylate)) or 10 μg of Met-tRNA$_f$ (plus 0.1 A_{260} unit of ApUpG) in a solution containing 30 mM morpholinopropanesulfonate (pH 7.2), 4 mM $MgCl_2$, 60 mM NH_4Cl, and 2 mM dithiothreitol. Some incubations also contained 16 or 32 pmol of derived 60S subunits. The total volume was 0.1 ml and,

Table I: Effect of Soluble and Ribonucleoprotein Extracts on the Binding of Acetyl[^{3}H]phenylalanyl-tRNA to Derived, Native, and Salt-Extracted Native 40S Subunits.[a]

Incubation Components	pmol of Ac-Phe-tRNA Bound		
	d40S	n40S	"Washed" n40S
AcPhe-tRNA	0.06	0.14	0.07
AcPhe-tRNA + poly(U)	0.31	3.37	0.30
AcPhe-tRNA + poly(U) + R.L. IF-1	2.93	4.19	1.64
AcPhe-tRNA + poly(U) + n40S extract	2.41		1.40
AcPhe-tRNA + poly(U) + n60S extract	0.20		

[a] Reaction mixtures containing various 40S subunit preparations and radioactive acetylphenylalanyl-tRNA were incubated with and without poly(U) and R.L. IF-1, as described above in Materials and Methods; some incubations contained 10 μg of protein extracted from n40S or n60S subunits. At the end of the incubation period, particle-bound radioactivity was determined by filtration through Millipore membranes.

Table II: Effect of Binding Factor R.L. IF-1 on the Binding of [^{3}H]Met-tRNA$_f$ to Derived, Native, and Salt-Extracted Native 40S Subunits.[a]

Incubation Components	pmol of Met-tRNA$_f$ Bound		
	d40S	n40S	"Washed" n40S
Met-tRNA$_f$	0.05	0.16	0.05
Met-tRNA$_f$ + ApUpG	0.15	0.19	0.08
Met-tRNA$_f$ + ApUpG + R.L. IF-1	0.86	0.25	0.93

[a] Reaction mixtures containing various 40S subunit preparations and radioactive methionyl-tRNA$_f$ were incubated with and without ApUpG and R.L. IF-1, as described above in Materials and Methods. Particle-bound radioactivity was determined by the Millipore filtration procedure.

unless otherwise specified, the incubations were carried out for 20 min at 37° for acetylphenylalanyl-tRNA, or for 15 min at 10 or 15° for Met-tRNA$_f$.

When the reaction mixtures were analyzed by the Millipore filtration method, 3 ml of cold incubation fluid was added at the end of the incubation period, the solutions were filtered through Millipore membranes, and the radioactivity was determined with a scintillation counter. When the reaction mixtures were analyzed by gradient centrifugation, incubations twice the volume and components listed above were layered on 12 ml of linear 10–30% sucrose gradients containing 30 m*M* morpholinopropanesulfonate (pH 7.2), 4 m*M* $MgCl_2$, 60 m*M* NH_4Cl, and 1 m*M* dithiothreitol. Centrifugation, in an SW 41 (Spinco) rotor, was carried out at 40000 rpm for 3.5 hr. The gradients were analyzed automatically with a scanning recording spectrophotometer. In some cases, 0.4-ml fractions were collected for radioactivity; gradient fractions were assayed for ^{3}H with a scintillation counter, after the addition of 5 ml of Aquasol (New England Nuclear) containing 5% H_2O. The optical density data are presented in the figures in the form of the spectrophotometric scans obtained from the recording apparatus; the radioactivity obtained by analysis of individual gradient fractions is superimposed over the continuous spectrophotometric pattern.

Some of the reactions were analyzed for acid-insoluble material. One milliliter of cold (2°) 10% trichloroacetic acid was added and after 30 min at 2°, the suspensions were filtered through glass-fiber filters; the filters were washed three times with 5 ml each of 5% trichloroacetic acid, dried, and counted in a scintillation counter.

Results

The binding of acetylphenylalanyl-tRNA to the three 40S subunit preparations obtained is shown in Table I. Compared to incubations in the absence of R.L. IF-1 and poly(uridylate) (lines 1 and 2), the addition of IF-1 and poly(U) to incubations containing derived 40S subunits (d40S) stimulated markedly the binding of the acylaminoacyl-tRNA to the particle (line 3); the results obtained with poly(U) alone were variable, the values were usually lower than that shown here. Binding of acetylphenylalanyl-tRNA to d40S subunits was also stimulated by the addition of an extract prepared from native 40S (n40S) subunits with high salt-containing solutions (line 4) but not similar extracts prepared from native 60S subunits (line 5). When the binding experiments were carried out with n40S subunits, isolated from the postmicrosomal supernatant, the addition of poly(U) was necessary to obtain extensive binding of acetylphenylalanyl-tRNA (line 2); the addition of R.L. IF-1 (line 3) or of the n40S extract, not shown here, had no effect. In contrast, the particles obtained from n40S subunits after treatment with high salt-containing solutions ("washed" n40S) responded like d40S subunits to polynucleotide template and binding protein; both poly(U) and either IF-1 or n40S extract were required for extensive binding of acetylphenylalanyl-tRNA.

Similar experiments to those described with acetylphenylalanyl-tRNA in Table I were also carried out with Met-tRNA$_f$ (Table II). The addition of ApUpG (line 2) did not significantly affect the binding of Met-tRNA$_f$ to any of the three types of subunits used. When R.L. IF-1 was also present (line 3), binding of this aminoacyl-tRNA to the d40S and the "washed" n40S subunits was markedly stimulated, but the n40S subunits did not bind Met-tRNA$_f$ even in the presence of added factor. Where binding did occur with active particles, the amount of Met-tRNA$_f$ bound was dependent on the concentration of R.L. IF-1 in the reaction.

The results presented above indicated that the interaction of both acetylphenylalanyl-tRNA and Met-tRNA$_f$ with d40S subunits was catalyzed by the purified binding factor from the soluble fraction. To determine whether the binding activity associated with n40S subunits required an N-blocked aminoacyl-tRNA, as in the case of acetylphenylalanyl-tRNA, Met-tRNA$_f$ was acetylated and used as a substrate in the 40S-binding reaction (Table III). Whereas in the presence of trinucleotide template and purified binding factor (line 3), significant binding to d40S subunits was obtained, the reaction with n40S subunits was not stimulated. These findings suggested that R.L. IF-1 also recognized acetylated Met-tRNA$_f$ as a substrate for this 40S-specific reaction, and that native 40S subunits were unable to bind the aminoacylated or the acylaminoacylated form of tRNA^{fMet}.

Derived 40S subunits were shown to react readily with Met-tRNA$_f$ in the absence of binding factor, in solutions containing relatively high levels of magnesium ions (Schroer and Moldave, 1973). In order to determine whether native 40S subunits were also capable of carrying out the

Table III: Effect of Template and Binding Factor R.L. IF-1 on the Binding of Acetyl[^{3}H] methionyl-tRNA$_f$ to Derived and Native 40S Subunits.

Incubation Additions	pmol of AcMet-tRNA$_f$ Bound	
	d40S	n40S
None	0.06	0.18
ApUpG	0.15	0.32
ApUpG + R.L. IF-1	0.87	0.31

[a] Reaction mixtures containing derived or native 40S subunit preparations and radioactive acetylmethionyl-tRNA$_f$ were incubated with and without ApUpG and R.L. IF-1 as described above in Materials and Methods. Particle-bound radioactivity was determined by the Millipore filtration procedure.

Table IV: Effect of Magnesium Ions on the Factor-Independent Interaction of [^{3}H]Met-tRNA$_f$ with Various Ribosomal 40S Subunits.[a]

Ribosomal Subunit	pmol of Met-tRNA$_f$ Bound	
	5 mM $MgCl_2$	20 mM $MgCl_2$
d40S	0.04	0.45
d40S + ApUpG	0.40	2.88
n40S	0.27	0.25
n40S + ApUpG	0.23	0.37
"Washed" n40S	0.04	0.29
"Washed" n40S + ApUpG	0.14	1.55

[a] Reaction mixtures containing various 40S subunit preparations and radioactive methionyl-tRNA$_f$ were incubated in 5 or 20 mM $MgCl_2$-containing solutions, with and without ApUpG; an exogenous source of initiator tRNA binding factor was not included in the incubation; other conditions were as described in the text. Particle-bound radioactivity was determined by the Millipore filtration procedure as described, except that the solutions used contained 5 or 20 mM $MgCl_2$ to correspond with incubation concentrations.

template-dependent, high Mg^{2+}-stimulated binding reaction, incubation mixtures containing derived, native, or "washed" native 40S subunits and low (5 mM) or high (20 mM) concentrations of $MgCl_2$ were analyzed (Table IV). The extent of binding to d40S and to salt-extracted n40S subunits was stimulated in the presence of template, at both $MgCl_2$ concentrations; indeed, extensive binding with both subunits was observed in incubations containing ApUpG and 20 mM $MgCl_2$. However, the addition of trinucleotide template had no effect on the reaction between Met-tRNA$_f$ and native 40S subunits, at 5 or at 20 mM $MgCl_2$ concentrations.

The results presented in Tables I–III suggested that a binding factor for initiator tRNA was present in n40S subunits as well as in the soluble fraction, and that an activity was present in n40S subunits that interfered with the interaction between 40S particles and Met-tRNA$_f$ but not acetylphenylalanyl-tRNA. Further, they suggested that both derived and native subunits were capable of reacting with acetylphenylalanyl-tRNA and with Met-tRNA$_f$, but that in order to bind Met-tRNA$_f$, the n40S subunits required extraction with high salt-containing solution. Evidence consistent with these suggestions was obtained by examining the effect of the extract obtained from native 40S subunits on the binding of acetylphenylalanyl-tRNA and Met-tRNA$_f$. The extent of binding of acetylphenylalanyl-tRNA was dependent on the concentration of extract protein in the incubation; in the absence of extract, binding to

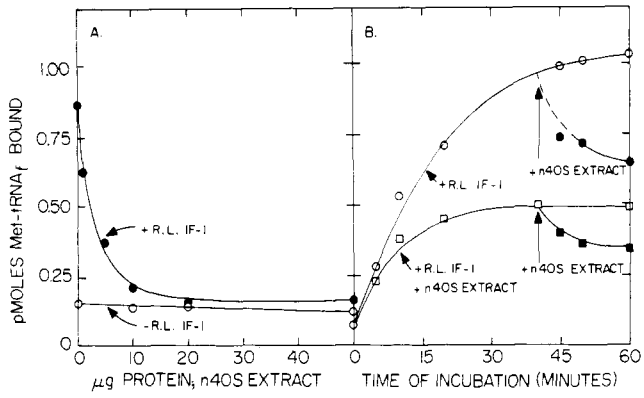

FIGURE 1: (A) The effect of varying concentrations of n40S extract protein on the binding of Met-tRNA$_f$ and on the purified soluble factor (R.L. IF-1)-dependent binding of Met-tRNA$_f$ to derived 40S subunits. Radioactive Met-tRNA$_f$, ApUpG, and d40S subunits were incubated with varying concentrations of n40S extract protein as such (○) or with 0.9 μg of R.L. IF-1 (●) for 40 min at 10°. Particle-bound radioactivity was determined by the Millipore filtration procedure. (B) The effect of n40S extract on the time-dependent binding of Met-tRNA$_f$ to derived 40S subunits. Radioactive Met-tRNA$_f$, R.L. IF-1, ApUpG, and d40S subunits were incubated without (○) or with (□) 5 μg of n40S extract protein; after 40 min of incubation, 5 μg of n40S extract protein was added to one set of incubations that had not received n40S extract (●) and to one set of incubations that contained n40S extract from the beginning (■); the four sets of incubations were then continued for an additional 20 min. Analyses were carried out by the Millipore filtration procedure.

d40S subunits was negligible, in contrast to incubations with extract. Thus, the binding activity that stimulated the interaction between initiator tRNA and 40S subunits was associated with n40S subunits and could be extracted by treatment with high salt.

Binding of Met-tRNA$_f$ to d40S subunits was not obtained when varying concentrations of n40S extract were added to the incubation (Figure 1A, open circles). Indeed, the binding of Met-tRNA$_f$ to d40S subunits, stimulated by the purified soluble factor R.L. IF-1, was markedly inhibited when n40S extract was also present (closed circles); the extent of inhibition was dependent on the concentration of n40S extract protein added. Figure 1B shows the rate of binding of Met-tRNA$_f$ obtained with factor R.L. IF-1 alone (open circles) and in the presence of 5 μg of n40S extract protein (open squares). When n40S extract was added to both sets of incubations after 40 min (closed symbols), there was a significant decrease in the amount of radioactive methionine associated with the particles, suggesting that the 40S·Met-tRNA$_f$ complex was dissociated by an activity in the n40S extract.

Attempts were made to determine whether the inhibitory effect of n40S extract on Met-tRNA$_f$ binding was due to the deacylation of the substrate. Derived 40S subunits were incubated with labeled Met-tRNA$_f$ or acetylphenylalanyl-tRNA, and the appropriate template, under a variety of conditions, and the reactions were analyzed for 40S·aminoacyl-tRNA complex retained on Millipore filters and for the amount of aminoacyl-tRNA precipitated with acid (Table V). Examination of the particle-bound radioactivity data indicated that Met-tRNA$_f$ was bound only to d40S subunits with R.L. IF-1, and that this reaction was inhibited by n40S extract. Acetylphenylalanyl-tRNA was bound to d40S subunits if poly(U) and R.L. IF-1 or n40S extract were present. Recoveries of acid-insoluble Met-tRNA$_f$ from most of the incubations were within 15 or 20% of the

Table V: Effect of n40S Extract on the Binding of [^{3}H]Met-tRNA$_f$ and Acetyl[^{3}H]phenylalanyl-tRNA to 40S Subunits and on the Hydrolysis of the Aminoacyl-tRNA.

Incubation Additions[a]	pmol of [^{3}H]Amino Acid[b] Retained on Millipore	pmol of [^{3}H]Amino Acid[b] In Acid-Insoluble Fraction
A. Met-tRNA$_f$		
d40S subunits + ApUpG	0.16	3.50
d40S subunits + ApUpG + R.L. IF-1	0.76	3.32
d40S subunits + ApUpG + R.L. IF-1 + n40S extract	0.26	3.07
d40S subunits + ApUpG + n40S extract	0.14	3.06
B. AcPhe-tRNA		
d40S subunits + poly(U)	0.34	4.70
d40S subunits + poly(U) + R.L. IF-1	2.64	6.20
d40S subunits + poly(U) + n40S extract	2.12	5.08

[a] Incubations contained 16 pmol of d40S subunits, 10 μg of radioactive Met-tRNA$_f$ or 30 μg of radioactive AcPhe-tRNA, 20 μg of n40S extract (with Met-tRNA$_f$) or 50 μg (with AcPhe-tRNA), and ApUpG, poly(U) or R.L. IF-1 as described in the text: other incubation components and conditions as described above. A duplicate of each reaction mixture was analyzed at zero time for cold 10% trichloroacetic acid insoluble radioactivity; approximately 3.55 ± 0.53 pmol of radioactive methionine was recovered from solutions containing [^{3}H]Met-tRNA$_f$ and 6.50 ± 0.80 pmol of radioactive phenylalanine was recovered from solutions containing acetyl-[^{3}H]phenylalanyl-tRNA. [b] After 40 min of incubation at 10° (Met-tRNA$_f$) or 20 min at 37° (acetylphenylalanyl-tRNA), one set of incubations was analyzed for particle-bound radioactivity by the Millipore filtration procedure, and a duplicate set was analyzed for cold 10% trichloroacetic acid insoluble radioactivity.

Table VI: Effect of 60S Subunits on the Factor-Promoted Binding of Acetyl[^{3}H]phenylalanyl-tRNA to Derived and Native 40S Subunits.[a]

First Incubation Components	Second Incubation Additions	pmoles of AcPhe-tRNA Bound to Particles
d40S	None	0.08
d40S + poly(U)	None	0.30
d40S + poly(U) + R.L. IF-1	None	1.78
d40S + poly(U) + R.L. IF-1 + 60S	None	0.35
d40S + poly(U) + R.L. IF-1	60S	1.95
n40S	None	0.07
n40S + poly(U)	None	2.06
n40S + poly(U) + 60S	None	1.39
n40S + poly(U)	60S	2.19

[a] Derived or native 40S subunits were incubated with or without poly(U), R.L. IF-1, and derived 60S subunits for 20 min at 37° (first incubation) as described above. Derived 60S subunits were then added to one incubation containing d40S subunits and to one containing n40S subunits, and all samples were allowed to incubate for an additional 20 min at 37° (second incubation). The concentration of d60S subunits added was greater than that of the 40S subunits in the reaction. Particle-bound radioactivity was then determined by the Millipore filtration procedure.

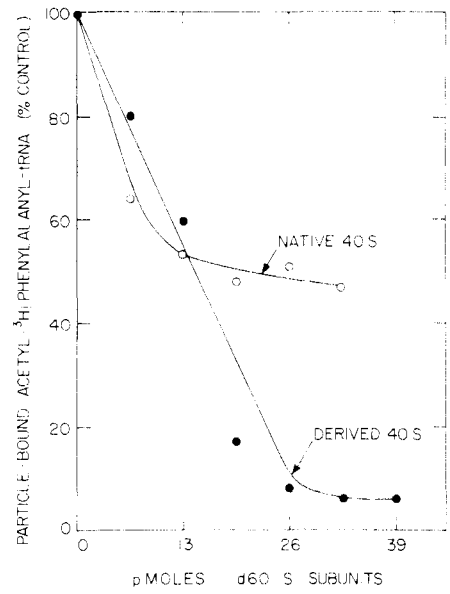

FIGURE 2: The effect of 60S subunits on the binding of acetylphenylalanyl-tRNA to derived (●) and native (○) 40S subunits. Incubations with native subunits contained poly(uridylic acid) (100% = 1.54 pmol of acetyl[^{3}H]phenylalanine), and those with derived subunits contained poly(U) and R.L. IF-1 (100% = 1.69 pmol of acetyl[^{3}H]phenylalanine).

amount present at zero time, before incubation. The recoveries obtained with acetylphenylalanyl-tRNA were within 25–30% of the control, although binding of this substrate was not inhibited by the extract. Some variability was obtained in several experiments designed to examine whether the n40S extract caused hydrolysis of Met-tRNA$_f$ in the presence of d40S subunits.

Previous results with 40S-specific binding factor (Gasior and Moldave, 1972) indicated that the addition of derived 60S subunits to a binding reaction mixture containing d40S subunits and poly(U) inhibited significantly the binding of acetylphenylalanyl-tRNA. If 60S subunits were added after binding had been allowed to occur, significant amounts of acylaminoacyl-tRNA were retained on the particle. The effect of 60S subunits on native and derived 40S subunits was compared (Table VI). Poly(U) stimulated the interaction between acetylphenylalanyl-tRNA and d40S subunits about threefold (line 2) while supplementation with R.L. IF-1 increased binding about 20-fold (line 3), as compared to incubations without poly(U) and IF-1 (line 1). The addition of d60S particles at the beginning of the binding reaction (first incubation, line 4) inhibited the binding stimulated by IF-1 almost completely. In contrast, the addition of 60S subunits to the second incubation after binding had been allowed to occur (line 5), did not decrease the amount of radioactive acetylphenylalanyl-tRNA recovered with the ribonucleoprotein particle. Native subunits, as stated above, did not require R.L. IF-1 and binding of acetylphenylalanyl-tRNA was increased 25- to 30-fold by addition of poly(U) alone (line 7), as compared to reactions in the absence of template (line 6). The addition of derived 60S particles at the beginning of the first incubation with n40S subunits (line 8) inhibited binding of acetylphenylalanyl-tRNA about 35% in this experiment, while the addition of derived 60S subunits to the second incubation, after binding (line 9), had no effect on the amount of aminoacylated-tRNA that remained associated with the particles.

The effect of varying concentrations of 60S subunits on the interaction between acetylphenylalanyl-tRNA and 40S subunits is shown in Figure 2. The amount of particle-bound acetylphenylalanyl-tRNA decreased in proportion to the concentration of 60S subunits that were present in incubations with derived (closed circles) or native (open circles)

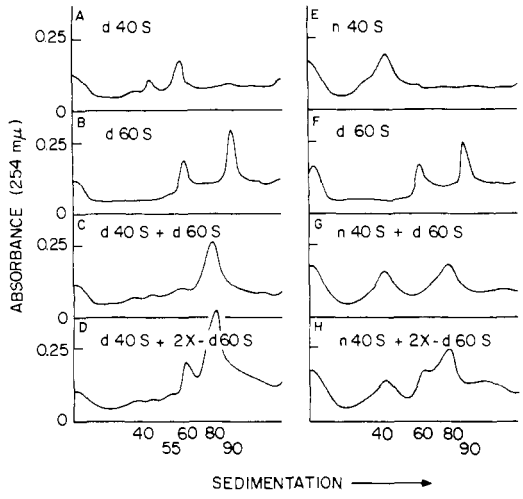

FIGURE 3: Sucrose gradient centrifugation patterns of derived 40S subunits, native 40S subunits, derived 60S subunits, and combinations of 40S with 60S subunits. (A) Derived 40S subunits; (B) derived 60S subunits; (C) equivalent concentrations of derived 40S and derived 60S subunits; (D) derived 40S subunits plus 60S subunits in which the concentration of the 60S particles was twice that of the 40S subunits; (E) native 40S subunits; (F) derived 60S subunits; (G) equivalent concentrations of native 40S and derived 60S subunits; (H) native 40S subunits plus derived 60S subunits in which the concentration of the 60S particles was twice that of the 40S subunits.

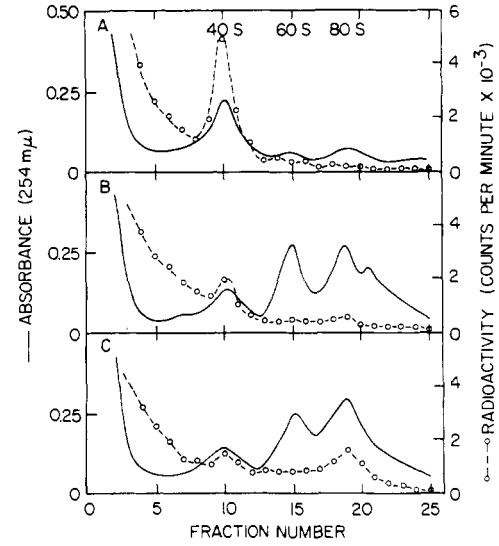

FIGURE 4: Gradient centrifugation analysis of binding reactions containing native 40S subunits and radioactive acetylphenylalanyl-tRNA, with and without 60S subunits. (A) Native 40S subunits incubated with poly(U) and radioactive acetylphenylalanyl-tRNA; (B) native 40S subunits incubated with poly(U), radioactive acetylphenylalanyl-tRNA, and derived 60S subunits; (C) 60S particles were added to the incubation 20 min after the initiation of the binding reaction with 40S subunits, poly(U), and radioactive acetylphenylalanyl-tRNA, and the incubation was continued for 20 min.

40S subunits. However, the binding of acylaminoacyl-tRNA to d40S subunits was almost completely inhibited, while binding to native subunits was only decreased about 50% even at relatively high concentrations of 60S particles. Maximal inhibition, in this experiment, was obtained when the ratio of d60S to d40S subunits was about 1.6–1.0. It was suggested previously (Gasior and Moldave, 1972) that the effect of 60S subunits on d40S particles could be due to the formation of 80S ribosomes which were not responsive to the factor-dependent binding of acetylphenylalanyl-tRNA. One possible explanation of the results obtained with the n40S subunits in Figure 2 was that the preparation consisted of at least two stages or populations of particles; both were capable of binding acylaminoacyl-tRNA in the presence of template and did not require exogenous binding factor, but one type was capable of interacting with 60S subunits to form 80S ribosomes and was therefore prevented from binding acetylphenylalanyl-tRNA, while the other was unaffected by 60S particles. To examine this possibility, the interaction between 60S and 40S subunits, and the formation of 80S ribosomes, was investigated with derived and with native subunit preparations. Small and large ribosomal subunits were centrifuged through linear (10–30%) sucrose density gradients as such or together (Figure 3); the sedimentation values listed along the horizontal axes are only approximations. Under the centrifugation conditions used, d40S (Figure 3A) revealed two fractions, one sedimenting at about "40S" and the other at about "55S", while the derived 60S preparation (Figure 3B) had a fraction sedimenting at "60S" and another at "90S"; the higher *s* values for each particle represent dimers (Martin et al., 1971; Nonomura et al., 1971). When approximately equivalent concentrations of d40S and d60S particles were incubated at 37° for 20 min prior to centrifugation, most of the ribonucleoprotein appeared in the gradient as a single peak at the 80S position (Figure 3C). When twice the number of d60S subunits as in Figure 3C were added to the incubation, density gradient fractionation also revealed the appearance of surplus 60S particles (Figure 3D). Native 40S subunits exhibited one main, broad peak, at about 40S, with some minor components (Figure 3E). The derived 60S preparation used for this experiment (Figure 3F) is similar to that described in part B of this figure. When molar equivalents of n40S and derived 60S particles were incubated together and then centrifuged, two particles were obtained with sedimentation values of approximately 40S and 80S (Figure 3G); thus, some of the 40S subunits appeared to remain in the 40S region, the rest of the 40S subunits and all of the added 60S particles seemed to be associated with 80S ribosomes. When the concentration of 60S subunits in the experiment was doubled (Figure 3H), some n40S subunits still remained, unconverted to 80S, although 60S particles were present in excess as evidenced by the appearance of an additional peak in the 60S area.

The data in Figure 3 indicated that some of the native 40S subunits were converted to 80S ribosomes in the presence of d60S subunits, and that some n40S particles were not capable of forming 80S ribosomes under these conditions. This finding was in contrast to the observation made with d40S subunits which were completely converted to 80S ribosomes, and was consistent with the partial inhibition by 60S subunits of acetylphenylalanyl-tRNA binding to n40S particles described in Figure 2. The suggestion that the n40S population capable of forming 80S ribosomes and the resistant population were both able to bind acetylphenylalanyl-tRNA, as interpreted from the results in Figure 2, was examined by carrying out sucrose gradient analysis of the binding reaction products (Figure 4). When native 40S particles were incubated with acetyl[^{3}H]phenylalanyl-tRNA and poly(U), centrifugation revealed mainly 40S subunits, and all of the labeled acetylphenylalanyl-tRNA was associated with them (Figure 4A). The addition of an amount of 60S subunits in excess of 40S particles, at the beginning of the binding reaction (Figure 4B), revealed a decrease in both the amount of material that sedimented at 40S and in

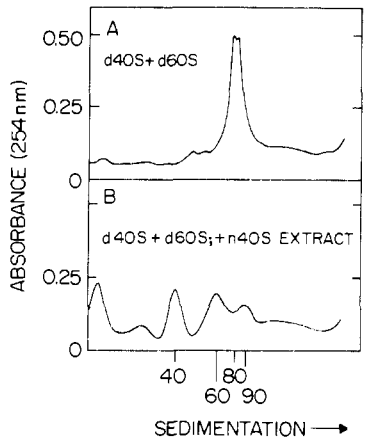

FIGURE 5: The effect of n40S extract on the gradient centrifugation pattern of d40S plus d60S subunits. Equivalent concentrations of d40S and d60S subunits were incubated at 37° for 10 min, to form 80S ribosomes. The reaction mixtures were incubated in the absence (A) and presence (B) of 180 μg of n40S extract protein, for an additional 10 min at 37° prior to gradient centrifugation.

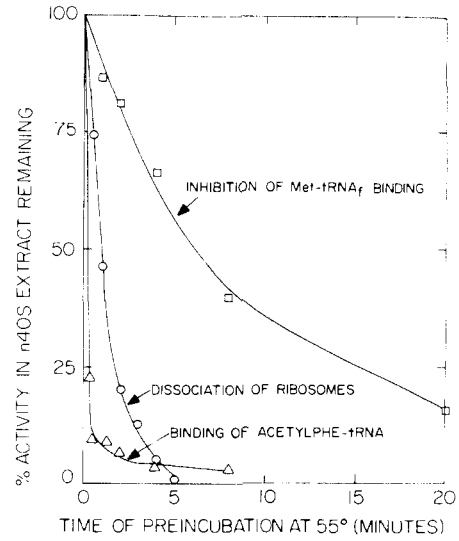

FIGURE 6: The effect of time of incubation of n40S extract at 55° on the inactivation of the acetylphenylalanyl-tRNA-40S subunit binding activity (△), the ribosome dissociation activity (○), and the activity that interferes with the binding of Met-tRNA$_f$ to d40S subunits (□). Binding activity was assayed in incubations containing radioactive acetylphenylalanyl-tRNA, poly(U), d40S subunits, and 20 μg of control n40S extract (zero time), or of n40S extract that had been maintained for varying times at 55°; after 20 min at 37°, particle-bound radioactivity was determined by the Millipore filtration procedure. Ribosome dissociating activity was assayed in incubations containing 24 pmol of 40S and 60S subunits which had previously been incubated for 10 min at 37° to form 80S ribosomes, and 180 μg of control n40S extract (zero time) or of n40S extract maintained for varying periods of time at 55°; after 10 min of incubation, the reaction mixtures were analyzed on sucrose gradients and the amount of dissociation was calculated by determining the areas under the peak of 80S ribosomes remaining and the areas under the peaks of subunits formed. The Met-tRNA$_f$ binding inhibitor was assayed in incubations containing radioactive Met-tRNA$_f$, ApUpG, binding factor R.L. IF-1, d40S subunits, and 20 μg of control n40S extract (zero time) or of n40S extract maintained for varying periods of time at 55°; after 20 min of incubation at 10°, particle-bound radioactivity was determined by the Millipore filtration procedure.

the amount of radioactivity associated with it; a substantial amount of 40S particles was converted to ribosomes as indicated by the peak sedimenting at 80S and slightly ahead of it, while the presence of surplus amounts of 60S subunits was confirmed by the amount of material in the 60S region. When the binding reaction between n40S subunits, radioactive acetylphenylalanyl-tRNA, and poly(U) was allowed to occur at 37° for 20 min, prior to the addition of d60S subunits (Figure 3C), centrifugation revealed that radioactivity was associated with both the 40S and the 80S particles; thus, part of the 40S population reacted with 60S subunits to form 80S ribosomes which were subsequently unable to bind acetylphenylalanyl-tRNA, or were able to bind acetylphenylalanyl-tRNA and then react with 60S subunits to form an 80S·acetylphenylalanyl-tRNA complex. The binding activity of the 40S subunits capable of reacting with 60S subunits to form ribosomes appears to be slightly higher than that of the 40S particles that did not reassociate.

One explanation for the failure of part of the n40S population to react with 60S subunits to form 80S ribosomes, was that they contained an activity that inhibited the formation of ribosomes. Consistent with this interpretation is the data presented in Figure 5, which describes the effect of n40S extract on preformed 80S ribosomes. Figure 5A shows the gradient centrifugation pattern obtained when d40S and d60S particles were incubated as such prior to sedimentation; only 80S ribosomes were detected. Figure 5B presents the results of an experiment in which n40S extract was added after the formation of 80S ribosomes had been allowed to occur, and the incubation was continued briefly prior to centrifugation; 80S ribosomes were not obtained under these conditions and only ribosomal subunits plus a small amount of the faster sedimenting material were evident in the gradient.

The data presented above indicated that several activities were present on native 40S subunits and in extracts prepared from them with high salt-containing solutions. Evidence suggesting that the activities responsible for binding of initiator tRNA to 40S subunits, inhibition of Met-tRNA$_f$ binding, and dissociation of ribosomes were distinct, is summarized in Figure 6 which describes the rates of inactivation of the various activities in the extract at 55°. The activity which interfered with the formation of 40S·Met-tRNA$_f$ complex (squares) had a half-life at this temperature of about 6 min, and the ribosome dissociation factor (circles) had a half-life of about 1 min. The acetylphenylalanyl-tRNA binding activity (triangles) was the most labile, and was almost completely inactivated in tubes immersed in a 55° water bath for 30 sec.

Discussion

Studies in this laboratory (Gasior and Moldave, 1972) revealed a factor in the supernatant of rat liver homogenates which catalyzed the GTP-independent binding of acetylphenylalanyl-tRNA to derived 40S subunits, in the presence of poly(U). It has been found recently (McCuiston et al., 1975) that this soluble binding factor (R.L. IF-1), when purified, also stimulates the binding of Met-tRNA$_f$ to d40S subunits, with ApUpG; the Met-tRNA$_f$ binding reaction is not observed with crude cytoplasmic fractions. The studies described here indicate that the postmicrosomal fraction also contains a population of ribonucleoprotein particles consisting partly of free 40S and 60S subunits, at relatively low temperature (2°) and monovalent cation (70 m*M* KCl) concentration, and that the native 40S subunits contain several particle-associated activities.

A number of findings are consistent with the suggestion that a binding and an inhibitory activity are associated with n40S subunits. The high salt extract obtained from n40S

subunits stimulates the binding of acetylphenylalanyl-tRNA to d40S subunits but not the binding of Met-tRNA$_f$ The extract inhibits the R.L. IF-1 promoted binding of radioactive Met-tRNA$_f$ to d40S subunits and when added to preformed d40S·Met-tRNA$_f$·AUG complex, the extract releases radioactivity from the complex. The deacylation of free and particle-bound Met-tRNA$_f$ by a specific hydrolase has been reported (Morrisey and Hardesty, 1972; Gupta and Aerni, 1973; Cimadevilla et al., 1974) and evidence for a hydrolase specific for 40S-bound Met-tRNA$_f$, in the soluble fraction of rat liver cytoplasm, has also been obtained in this laboratory. However, it is not clear whether the inhibition of Met-tRNA$_f$ binding to d40S subunits, or the dissociation of the d40S·Met-tRNA$_f$ complex that is seen in the presence of n40S extract is due to the deacylation of the aminoacyl-tRNA. The inhibition does not appear to be due to the complete hydrolysis of available substrate, since considerable amounts of Met-tRNA$_f$ remain under conditions where complete inhibition of binding is observed; also, it does not appear to be due to ribonuclease activity, since acetylphenylalanyl-tRNA binding is not inhibited. Experiments designed to examine the mechanism by which inhibition occurs, and to establish whether or not a hydrolase is present in the n40S extract which may be specific for the 40S-bound substrate, are in progress.

Another explanation for the failure of n40S subunits to bind Met-tRNA$_f$ (other than the presence of a specific inhibitor) is that the binding of this substrate could differ from that of acetylphenylalanyl-tRNA, and that it is inhibited by tRNA or Met-tRNA$_f$ on the n40S subunits. Analysis of RNA extracts of 40S subunits (native or derived) by gel electrophoresis reveals the presence of some 4S RNA. However, several observations argue against the role of tRNA or Met-tRNA$_f$ in the inhibition observed. The n40S extract prevents the otherwise normal and extensive binding of Met-tRNA$_f$ to derived 40S subunits promoted by R.L. IF-1. The n40S extract also acts on preformed d40S·Met-tRNA$_f$ to dissociate this complex. The labile nature of the inhibitory activity at 55° would suggest that the effect is due to a protein in the n40S extract. "Washed" n40S subunits are capable of binding Met-tRNA$_f$ in the presence of R.L. IF-1, although washing is carried out under conditions where the tRNA or aminoacylated tRNA is usually not removed.

The addition of d60S subunits to incubations containing d40S subunits, binding factor, template, and acetylphenylalanyl-tRNA or Met-tRNA$_f$ completely inhibits the reaction due to the removal of 40S subunits in the form of 80S ribosomes. An activity is present on some of the n40S subunits which prevents their interaction with 60S subunits; the finding that n40S extract dissociates preformed 80S ribosomes to subunits is consistent with such an interpretation.

Studies reported here and others in this laboratory suggest that an initiator tRNA-binding factor and an activity (or activities) that interfere with the interaction between Met-tRNA$_f$ and 40S subunits are found not only in association with n40S subunits but are present in the soluble fraction of the cytoplasm, and suggest functional roles. In addition, part of the n40S subunit population also contains an activity that dissociates 80S ribosomes to ribosomal subunits. Comparison of the temperature-dependent inactivation of the three particle-associated activities would suggest that the reactions are catalyzed by distinct proteins. Purification of the various activities, and studies to determine whether the particle-bound and soluble factors are identical, are in progress.

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