Binding of Met-tRNA$_f$ to Native and Derived 40S Ribosomal Subunits†

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ABSTRACT: Our previous work has shown that the native 40S ribosomal subunits (those found free in the cell sap) but not polyribosomal 40S subunits have additional associated proteins that are removed by 0.5 M KCl. In this communication we present evidence that in the Ehrlich cell one of the native subunit associated proteins is the mammalian initiation factor that forms a Met-tRNA$_f$–factor–GTP complex, and is required for the binding of Met-tRNA$_f$ to the 40S subunit. Initial examination of the KCl wash of the Ehrlich cell total ribosomal pellet revealed a factor which (1) shifted the elution of Met-tRNA$_f$ and of GTP from the included to the excluded volume on Sephadex G-100 chromatography, (2) stimulated the binding of Met-tRNA$_f$ to Millipore filters, and (3) stimulated the binding of Met-tRNA$_f$ to salt-washed 40S subunits. These activities were dependent upon or enhanced by GTP; were inhibited by GDP; were much greater for Met-tRNA$_f$ than for Met-tRNA$_m$ or for lysyl-tRNA; and were concentrated in the KCl ribosomal wash and were not detected in the cell soluble fraction. Met-tRNA$_f$ bound in conjunction with a specific amount of KCl wash protein, to form a distinctive particle of buoyant density 1.40 g cm^{-3} in CsCl, identical in density to one form of the native 40S subunit. Native 40S subunits, but no other subunits, contained a factor which was eluted by 0.5 M KCl and which (1) stimulated the binding of Met-tRNA$_f$ to Millipore filters, and (2) stimulated the binding of Met-tRNA$_f$ to salt-washed 40S subunits. The factor appeared to be localized on the native 40S subunit of density 1.40 g cm^{-3}.

We have recently shown that mammalian native 40S subunits possess additional proteins that are not associated with the 40S ribosomal subunits derived from polyribosomes (Henshaw *et al.*, 1973; Hirsch *et al.*, 1973). The native 40S subunits (40S$_N$)[1] were found to be predominantly of two forms, one of buoyant density 1.49 g cm^{-3} (40S$_{N\text{-}H}$) and the other of buoyant density 1.40 g cm^{-3} (40S$_{N\text{-}L}$), containing respectively 9 × 10^4 and 7.5 × 10^5 daltons of protein in addition to the proteins of the ribosome-derived 40S subunit (40S$_D$) (buoyant density 1.51 g cm^{-3}). Although the evidence was circumstantial we suggested that some of these additional proteins on the native particles may be initiation factors (Ayuso-Parilla *et al.*, 1973a,b). Consistent with this suggestion was the finding that when derived 40S subunits were incubated with a crude preparation of reticulocyte initiation factors, proteins from the preparation bound to the subunits to produce particles identical in density with the native 40S subunits. In this paper we report evidence that one of the factors associated with the subunit of density 1.40 g cm^{-3} is a mammalian initiation factor required for the binding of Met-tRNA$_f$ to the 40S subunit.

Mammalian initiation factors have recently been partially purified from high salt washes of ribosomal pellets. One of these factors, referred to variously as factor C (Levin *et al.*, 1973), IF-E$_2$ (Schreier and Staehelin, 1973a), IF-1 (Chen *et al.*, 1972; Dettman and Stanley, 1973), and MP (Merrick *et al.*, 1974), appears to have many characteristics in common with the bacterial initiation factor 2, a factor required for the binding of Met-tRNA to the smaller ribosomal subunit. That is, (1) the factor forms a complex with GTP and Met-tRNA$_f$; (2) GDP competes with GTP and prevents binding of Met-tRNA$_f$ to the complex; (3) the factor–GTP–Met-tRNA$_f$ complex binds to the mammalian 40S subunit, and it is presumably by virtue of this activity that the protein is an initiation factor. In addition the mammalian factor is adsorbed onto cellulose nitrate filters (Chen *et al.*, 1972; Dettman and Stanley, 1973; Levin *et al.*, 1973; Schreier and Staehelin, 1973a; Merrick *et al.*, 1974). This information has provided criteria by which we have been able to test whether the IF-2-like factor is one of the proteins associated with the 40S$_N$ subunits.

Experimental Procedure

Materials

Chemicals. L-[^{35}S]Methionine was purchased from Amersham-Searle, uncharged rat liver tRNA and *Escherichia coli* MRE 600 cells from General Biochemicals, and poly(A,U,G) (1:1:1) from Miles Laboratories, Inc.

Methods

Cell Lines. Ehrlich ascites tumor cells were grown in suspension culture with Eagle's Minimal Essential spinner culture medium and 10% calf serum as previously described (van Venrooij *et al.*, 1970).

Preparation of E. coli MRE 600 Aminoacyl-tRNA Synthetase Enzymes. A crude enzyme fraction containing aminoacyl-tRNA synthetases was prepared from *E. coli* MRE 600 essentially according to the protocol of RajBhandary

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[1] Abbreviations used are: 40S$_N$, native 40S ribosomal subunit; 40S$_{N\text{-}H}$, native 40S ribosomal subunit of buoyant density 1.49 ± 0.02 g cm^{-3}; 40S$_{N\text{-}L}$, native 40S ribosomal subunit of buoyant density 1.40 ± 0.02 g cm^{-3}; 40S$_D$, 0.5 M KCl derived subunits or intraribosomal subunits of buoyant density 1.51 ± 0.02 g cm^{-3}; 60S$_N$, native 60S subunit; EF-1 and EF-2, mammalian elongation factors 1 and 2; IF-2, bacterial initiation factor 2; TEA, triethanolamine.

and Ghosh (RajBhandary and Ghosh, 1969). We confirmed that this preparation aminoacylates tRNA^{fMet} but not tRNA$^{Met}_m$ (Gupta *et al.*, 1970), as the incorporation of [^{35}S] methionine into hot acid-precipitable material was undetectable (less than 50 cpm above background) when 80,000 cpm of the [^{35}S]Met-tRNA$_f$ preparation was incubated in an elongating system which was able to incorporate 17,000 cpm of free [^{35}S]methionine added to the incubation.

Preparation of [^{35}S]Met-tRNA$_f$, [^{35}S]Met-tRNA$_m$, and Lysyl-tRNA. For most experiments [^{35}S]Met-tRNA$_f$ was prepared by charging stripped rat liver tRNA (4 mg) with L-[^{35}S]methionine (100–165 Ci/mmol) using the coli synthetase preparation, as described by Takeishi *et al.* (1968) and Gupta *et al.* (1971). The final phenol-purified preparation was diluted to 0.5–1 mg/ml of tRNA and usually contained 4–5 × 10^7 cpm/ml when prepared soon after the purchase of L-[^{35}S]methionine. Concentration of Met-tRNA$_f$ was calculated from the specific activity of the [^{35}S]methionine, and specific activity was corrected weekly for ^{35}S decay. ^{35}S was counted at 84% efficiency.

In the experiments where [^{35}S]Met-tRNA$_f$ and [^{35}S]Met-tRNA$_m$ of the same specific activity were compared, rat liver tRNA was charged with [^{35}S]methionine in separate parallel incubations under conditions described above. In one incubation the coli synthetase preparation was used, to produce Met-tRNA$_f$; and in the other Ehrlich cell S-100 fraction was used to produce predominantly Met-tRNA$_m$. After phenol extraction the preparations were further purified by chromatography in parallel on columns of benzoylated DEAE-cellulose (Gillam *et al.*, 1967; Smith and Marcker, 1970), under the conditions of Samuel *et al.* (1973), using stepwise elution. After elution of Met-tRNA$_f$ at 0.55 M NaCl, the column was washed extensively with 0.6 M NaCl before elution of Met-tRNA$_m$ with 0.75 M NaCl.

Unlabeled lysyl-tRNA was prepared as described previously (Smith *et al.*, 1973), except that only L-lysine was used in the charging mixture. We estimated the concentration of lysyl-tRNA by measuring in a parallel charging experiment the incorporation of radioactive L-lysine of known specific activity, and assuming identical charging efficiency. In 50 μg of total tRNA there were 96 pmol (4%) of lysyl-tRNA.

Preparation of Ribosomal Subunits. Native 40S ribosomal subunits were prepared from the Ehrlich cell cytoplasmic extract using Mg^{2+} precipitation as previously described (Hirsch *et al.*, 1973). Briefly, Mg^{2+}-precipitated total ribosomes were layered on 28-ml 20–40% sucrose gradients and were centrifuged for 17 hr at 28,000 rpm in a Spinco SW 25.1 rotor. The two forms of the native 40S subunit sediment slightly differently on sucrose gradients, forming a double absorbance peak, which was ordinarily collected in total as the 40S$_N$ subunit preparation. Preparations containing predominantly the 40S$_{N\text{-}L}$ or 40S$_{N\text{-}H}$ form were obtained by isolating separately the leading and trailing portions of the double peak, as described previously (Hirsch *et al.*, 1973). 40S$_D$ ribosomal subunits were derived by 0.5 M KCl treatment from 80S run-off monomeric ribosomes that had accumulated in Ehrlich cells treated with 15 mM NaF 30 min prior to harvesting, as previously described (Smith *et al.*, 1973).

Preparation of Ehrlich Cell KCl Ribosomal Washes and the S-100 (Soluble Protein) Fraction. Ehrlich cell cytoplasmic extracts were prepared as described previously (Hirsch *et al.*, 1973) except that cells were ruptured by Dounce homogenization rather than by detergent treatment. Ribosomes were sedimented by centrifugation at 232,000*g* (average) for 2.5 hr. The upper 4/5 of the supernatant portion was saved as the S-100 fraction, and the ribosomal pellet was treated with KCl to elute the initiation factors. The experiments were initially performed with an initiation factor preparation obtained as described by Shafritz and Anderson (1970), except that elution was with 1 M KCl, without further purification. In experiments when this preparation was used, it is referred to as "crude 1 M KCl ribosomal wash."

Many of the experiments have been repeated using a preparation eluted from the ribosomal pellet with 0.5 M KCl and further purified by stepwise elution from a column of DEAE-cellulose as described by Schreier and Staehelin (1973b) for their "IF fraction A," except that DEAE-cellulose elution was from 0.05 M KCl to 0.3 M instead of 0.12–0.3 M. The preparation was dialyzed against medium containing 10% v/v glycerol, 20 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, 0.2 mM EDTA, and 0.12 M KCl (Schreier and Staehelin, 1973b). This preparation is referred to as the "KCl ribosomal wash." Results using the two different preparations were qualitatively identical, but we have generally shown the data using the more purified preparation, as binding was higher under given conditions, presumably because of the higher concentration of the relevant factor and because of the removal of most of the methionyl-tRNA deacylase which is present in the crude preparation.

Millipore Binding Assays. [^{35}S]Met-tRNA$_f$ binding to Millipore filters was assayed in duplicate 0.5-ml incubations that contained, unless noted otherwise, 50 mM TEA-HCl pH 7.2 (20°), 3 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 100 mM KCl, 0.2 mM GTP, and [^{35}S]Met-tRNA$_f$ and KCl ribosomal wash as indicated. The mixtures were incubated at 28° for 15 min. The reactions were terminated by rapidly chilling to 0° and adding 5 ml of ice-cold 50 mM TEA-HCl (pH 7.2) (20°)–0.1 M KCl–3 mM $Mg(OAc)_2$–0.2 mM dithiothreitol. Immediately after dilution the solutions were poured slowly through Millipore filters, which had been washed immediately before use with 2 ml of a 0.02% solution of bovine serum albumin in the same medium. Filters were then washed five times with 2 ml of the above buffer. The filters were dried and counted in a liquid scintillation counter. Background binding in the absence of any KCl wash protein was somewhat variable, but variability was minimized by the albumin prewash, and by use of the same [^{35}S]Met-tRNA$_f$ preparation in a series of experiments.

CsCl Equilibrium Density Gradient Analysis. Incubation mixtures (1 ml) that contained approximately 0.3–0.5 A_{260} unit of 40S ribosomal subunits, 40 μg of poly(A,U,G), and [^{35}S)Met-tRNA$_f$ and KCl ribosomal wash as indicated were incubated in reaction mixtures identical in ionic conditions with those described above for the Millipore filter binding assays. The reactions were terminated by chilling to 0° and by adding 4 ml of buffer containing 10 mM morpholinopropanesulfonic acid, 0.5 mg/ml of Brij, and 4% formaldehyde (pH 7.2) to fix the subunits for the CsCl density gradient analysis. The solutions were kept at 0° for 30 min before overlaying onto 5 ml of CsCl (density 1.51 g cm^{-3}) made up in the same buffer. The gradients were formed by spinning at 39,000 rpm for 17 hr and 4° in a Spinco SW 41 rotor. The gradients were monitored for absorbance at 260 nm as previously described (Morton and Hirsch, 1970). Fractions were collected and the density of selected parts of

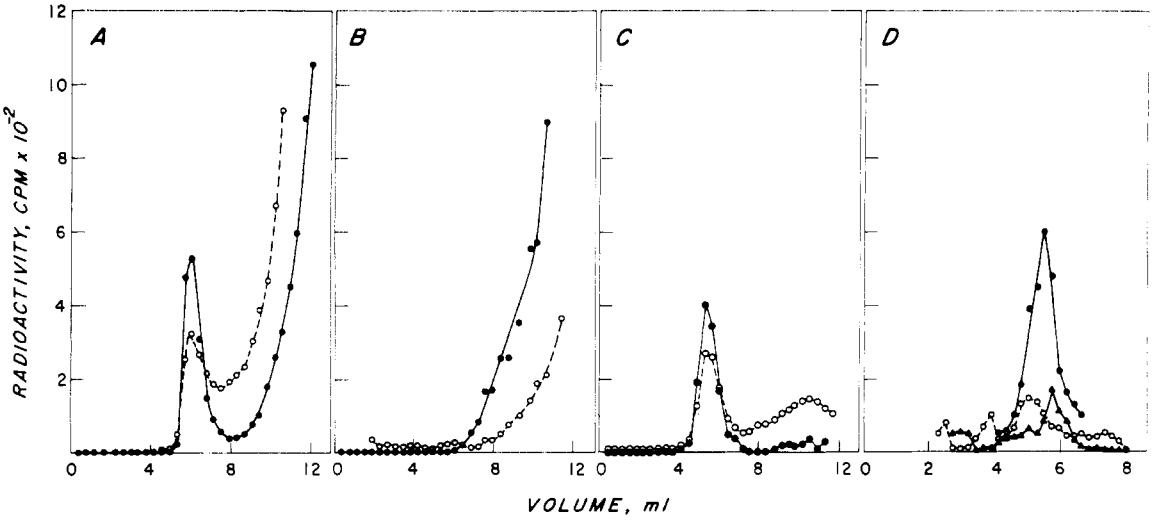

FIGURE 1: Isolation of factor-GTP-Met-tRNA$_f$ ternary complex by Sephadex G-100 column chromatography. In panels A, B, and C, crude 1 M KCl ribosomal wash was incubated with [^{35}S]Met-tRNA$_f$ and [^{3}H]GTP, 5 μM, and analyzed by Sephadex G-100 chromatography, as described under Methods. (●) [^{35}S]Met-tRNA$_f$; (○) [^{3}H]GTP. (A) Complete reaction mixture. Fractions (250 μl) were counted directly, as described under Methods. (B) Minus KCl ribosomal wash. Fractions (250 μl) were counted directly. (C) Complete reaction mixture. Fractions (250 μl) were collected on Millipore filters, washed twice with 5 ml of elution buffer, dried, and counted in a toluene-based scintillation mixture. In panel D, in place of [^{3}H]GTP reaction mixtures contained: (●) 0.2 mM GTP; (○) no GTP. (▲) 0.2 mM GTP + 0.4 mM GDP. Portions (100 μl) of each fraction were collected on Millipore filters, as described in panel C above. Note that, as smaller portions of each fraction were counted in panel D, the counts should be multiplied by 2.5 to be comparable to panels A, B, and C. Specific activities were: [^{3}H]GTP, 1.5 Ci/mmol, [^{35}S]Met-tRNA$_f$, 95 Ci/mmol.

Table I: Effect of KCl Ribosomal Wash, Met-tRNA$_f$, and Ribosomal Subunits on the Binding of [^{35}S]Met-tRNA$_f$ to Millipore Filters.[a]

KCl Wash (μg)	[^{35}S]Met-tRNA$_f$ (pmoles)	[^{35}S]Met-tRNA$_f$ Bound to Filters (fmoles)
0	1.5	12
0	3.1	18
31	1.5	88
62	1.5	145
62	3.1	286
62	4.6	408
93	1.5	195
31	3.1	155
31	3.1 + 40$_D$ + 60$_D$	141
31	3.1 + 40$_D$ + poly(A, U, G)	139

[a] The 0.5-ml reaction mixtures were incubated and analyzed as described under Methods. The specific activity of the [^{35}S]Met-tRNA$_f$ was 132 Ci/mmol. Where indicated 0.25 A_{260} unit of 40s$_D$ subunits, 0.4 A_{260} unit of 60s$_D$ subunits, and 40 μg of poly(A,U,G) were included in the incubations.

the gradients was determined by weighing known aliquots of solution. To each fraction 1 drop of 0.5% bovine serum albumin and 2 ml of ice-cold 5% Cl_3CCOOH was added. The precipitates were collected on Millipore filters which were washed with 2 ml of ice-cold 5% Cl_3CCOOH. The filters were dried and counted in a Beckman scintillation counter.

Isolation of the Factor-GTP-Met-tRNA$_f$ Complex by Sephadex G-100 Column Chromatography. Incubation mixtures (0.2 ml) that contained, unless noted otherwise, 50 mM TEA-HCl (pH 7.4) (20°), 3 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 100 mM KCl, 5 μM [8-^{3}H]GTP (1.5 μCi) or 0.2 mM unlabeled GTP, as indicated, and about 4 × 10^5 cpm of [^{35}S]Met-tRNA$_f$ were incubated at 28° for 15 min with 100μg of crude 1 M KCl ribosomal wash. The reactions were terminated by rapidly chilling to 0° and layering onto a Sephadex G-100 column (1.2 × 17 cm) previously equilibrated with 50 mM TEA-HCl (pH 7.4) (0°)-3 mM $Mg(OAc)_2$-1 mM dithiothreitol-100 mM KCl. Fractions were eluted with the same buffer, placed in a dioxane scintillation mixture, and counted in a Beckman liquid scintillation counter.

Results

(1) Presence of IF-2-Like Activity in the Ehrlich Cell KCl Ribosomal Wash. FORMATION OF FACTOR-GTP-Met-tRNA$_f$ COMPLEX. Figure 1A demonstrates that [^{3}H]GTP and [^{35}S]Met-tRNA$_f$ formed complexes with high molecular weight material when incubated with the crude 1 M KCl ribosomal wash fraction. Peaks of GTP and Met-tRNA$_f$ emerged in the excluded volume during analysis of the reaction mixture on a Sephadex G-100 column (Figure 1A), while both compounds eluted entirely in the included fractions when incubated in the absence of KCl wash (Figure 1B). The complexes formed also adsorbed onto Millipore filters (Figure 1C). Figure 1D shows that formation of Millipore filter-adsorbable Met-tRNA$_f$-protein complexes was greatly stimulated by 200 mM GTP and was inhibited by GDP. (The GTP concentration in panels A-C was very low (5 μM) to conserve labeled GTP.) As we have not purified the factor, it cannot be stated definitely that GTP and Met-tRNA$_f$ are complexing with the same protein but the GTP requirement for Met-tRNA$_f$ binding and the inhibition of binding by GDP are evidence for this, and as the factor-GTP-Met-tRNA$_f$ complex has been demonstrated with purified factor by others, we assume this is the case in our experiments. Stoichiometry of the reaction cannot be estimated from our experiments, as neither GTP nor Met-tRNA$_f$ was present in saturating amount, and

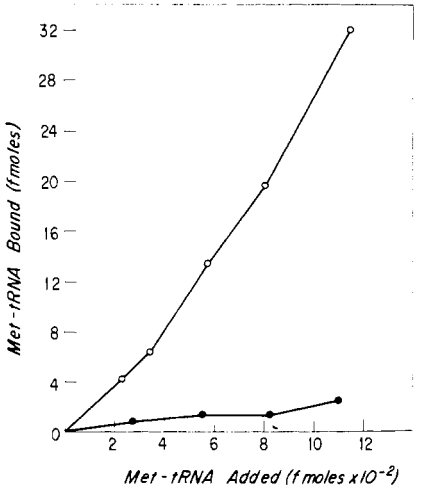

FIGURE 2. Comparison of binding of [^{35}S]Met-tRNA$_f$ and [^{35}S]Met-tRNA$_m$ to Millipore filters in the presence of KCl ribosomal wash proteins. Reaction mixtures containing 34 μg of KCl ribosomal wash protein were incubated and processed as described under Methods. (○) [^{35}S]Met-tRNA$_f$ binding; (●) [^{35}S]Met-tRNA$_m$ binding. Specific activity of [^{35}S]Met-tRNA was 90 Ci/mmol.

there are other proteins in the KCl wash which bind GTP.

The ability of the complex to adsorb to filters provides a convenient assay for complex formation, and we have used this property to delineate requirements for the reaction. Except for a small background, binding of Met-tRNA$_f$ to Millipore filters was dependent upon the presence of the KCl ribosomal wash, and was roughly linear with Met-tRNA$_f$ concentration over the range tested (Table I). The KCl ribosomal wash was found to be free of 40S subunits (results not shown) and the addition of poly(A,U,G) and 0.5 M KCl-washed 40S subunits to the incubation did not further stimulate [^{35}S]Met-tRNA$_f$ binding (Table I). Thus it is a factor-GTP-Met-tRNA$_f$ complex rather than a factor-GTP-Met-tRNA$_f$-mRNA-40S subunit complex which is binding to the filter. Binding was stimulated maximally by 0.1 mM GTP, and was markedly inhibited by GDP (Table II). (Binding in the absence of GTP varied among preparations and was unusually high with the KCl wash shown here.) [^{35}S]Met-tRNA$_f$ and [^{35}S]Met-tRNA$_m$ of identical specific activity were prepared from the same [^{35}S]methionine and were isolated and compared for factor-stimulated binding (Figure 2). A high preference for Met-tRNA$_f$ is demonstrated at these concentrations. We also tested the ability of unlabeled lysyl-tRNA to compete with the binding of Met-tRNA$_f$ (Table III). Uncharged tRNA decreased the binding of Met-tRNA$_f$ somewhat at high concentrations. However, lysyl-tRNA had little additional effect even when 110 pmol was incubated with 1 pmol of [^{35}S]Met-tRNA$_f$ in the presence of the KCl ribosomal wash.

The factor that stimulates binding was concentrated in the KCl ribosomal wash fraction, and little binding could be demonstrated in the Ehrlich cell soluble fraction (S-100) (Table IV). Table IV also shows that the failure of binding with the S-100 fraction was not due to the presence of large amounts of a methionyl-tRNA deacylase (Morrisey and Hardesty, 1972; Gupta and Aerni, 1973), as there was little deacylase in the S-100 fraction. For comparison the crude 1 M KCl ribosomal wash is shown. In this preparation binding was readily demonstrated despite the presence of large amounts of deacylase. Of course, this does not exclude the possibility that the IF2-like factor is present in the S-100 fraction but is masked by the presence of some other inhibitor.

Table II: Effect of GTP and GDP on the Binding of Met-tRNA$_f$ to Millipore Filters.[a]

Incubation		[^{35}S]Met-tRNA$_f$ Bound to Filters (fmol)
GTP (mM)	GDP (mM)	
0	0	71
0.05	0	160
0.1	0	169
0.2	0	150
0.2	0.2	70
0.05	0.2	54
0	0 minus KCl wash	17
0.2	0 minus KCl wash	16
0.2	0.2 minus KCl wash	17

[a] Mixtures were incubated and analyzed as described under Methods. They contained 31 μg of KCl ribosomal wash protein and 3.1 pmol of [^{35}S]Met-tRNA$_f$, specific activity 132 Ci/mmol.

Table III: The Effect of Lysyl-tRNA and Uncharged tRNA on the Binding of [^{35}S]Met-tRNA$_f$ to Millipore Filters.[a]

Incubation	[^{35}S]Met-tRNA Bound to Filters (fmoles)
Complete minus KCl ribosomal wash	17
Complete	161
+ 22 pmol of lysyl-tRNA (0.19 A_{260} unit of RNA)	138
+ 44 pmol of lysyl-tRNA (0.38 A_{260} unit of RNA)	123
+ 110 pmol of lysyl-tRNA (0.95 A_{260} unit of RNA)	99
+ 0.12 A_{260} unit uncharged tRNA	152
+ 0.24 A_{260} unit uncharged tRNA	141
+ 0.6 A_{260} unit uncharged tRNA	129
+ 1.2 A_{260} unit uncharged tRNA	124

[a] The incubations contained 130 μg of crude 1 M KCl ribosomal wash protein, and 1.1 pmol of [^{35}S]Met-tRNA$_f$, specific activity 151 Ci/mmol.

To test for the binding of the factor-GTP-Met-tRNA$_f$ complex to subunits, we incubated 40S$_D$ ribosomal subunits with [^{35}S]Met-tRNA$_f$, GTP, poly(A,U,G), and the KCl ribosomal wash for 15 min at 28°. After "fixation" with formaldehyde we analyzed the reaction mixtures on CsCl density gradients as described under Methods (Figure 3). The 40S$_D$ particles, that were originally of density 1.52 g cm^{-3} (Figure 3A), were converted to a mixture of particles sedimenting predominantly at densities of 1.49 and 1.40 g cm^{-3} (Figure 3B), indicating the binding of about 9×10^4 and 7.5×10^5 daltons of protein, respectively, to the subunits (Hirsch *et al.*, 1973). Moreover, a peak of radioactive Met-tRNA$_f$ was observed in association with the particle of density 1.40 g cm^{-3}, and only these particles were labeled.

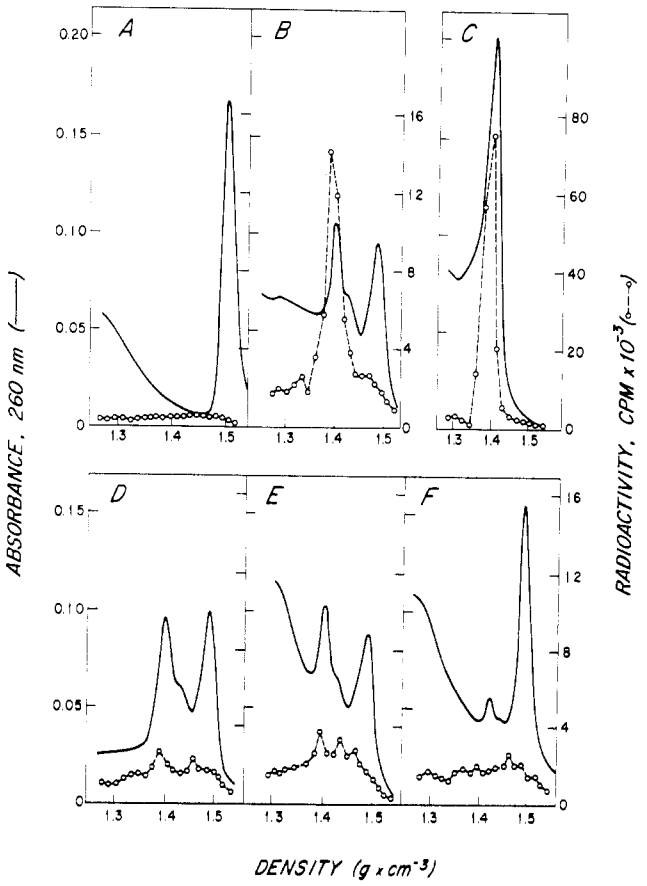

FIGURE 3: Binding of [^{35}S]Met-tRNA$_f$ to 40S$_D$ ribosomal subunits. Reaction mixtures (1 ml) contained 0.35 A_{260} units of 40S$_D$ subunits (about 25 pmol); 7.7 pmol of [^{35}S]Met-tRNA$_f$, specific activity 76 Ci/mmol (1 pmol = 140,000 cpm); and, except as noted, 93 μg of KCl ribosomal wash protein and 0.2 mM GTP. (A) No KCl ribosomal wash; (B) complete; (C) 248 μg of KCl ribosomal wash protein; (D) minus GTP; (E) plus 0.4 mM GDP (in addition to GTP); (F) 270 μg of S-100 protein instead of KCl ribosomal wash. The mixtures were incubated and were analyzed on CsCl gradients for absorbance (—), density, and radioactivity (O) as described under Methods.

The original density of the subunits was not altered in the absence of the KCl ribosomal wash, and neither did [^{35}S]Met-tRNA$_f$ bind to particles (Figure 3A). The appearance of the peak of [^{35}S]Met-tRNA$_f$ at density 1.40 g cm^{-3} with the 40S$_D$ ribosomes was dependent on the presence of the Met-tRNA$_f$ in the incubation mixture before "fixation" with formaldehyde (results not shown). With more KCl wash the subunits were converted almost entirely to the 1.40 g cm^{-3} form, and binding was increased (Figure 3C). Increasing the amount of Met-tRNA$_f$ also produced an increase in binding, and with 248-μg KCl ribosomal wash and 25 pmol of 40S$_D$ subunits, addition of 1.5 pmol of [^{35}S]Met-tRNA$_f$ led to binding of 0.30 pmol (not shown); 3.1 pmol yielded 0.51 pmol (not shown); and 7.7 pmol gave 1.25 pmol (Figure 3C). We have not attempted to saturate the system, but the data of Figure 3C indicate the binding of about 5 pmol of Met-tRNA$_f$ per 100 pmol of ribosomal subunits under very under-saturating conditions. Binding is stimulated by GTP (Figure 3D *vs.* 3B) and inhibited by GDP (Figure 3E). Met-tRNA$_f$ was not bound in the presence of Ehrlich cell sap (Figure 3F), which also did not convert the subunits of density 1.52 (Figure 3A) to 1.40, as the KCl wash did (Figure 3B). Figure 4 demonstrates that the factor-dependent binding is specific for the initiator tRNA, as Met-tRNA$_m$ did not bind under these conditions.

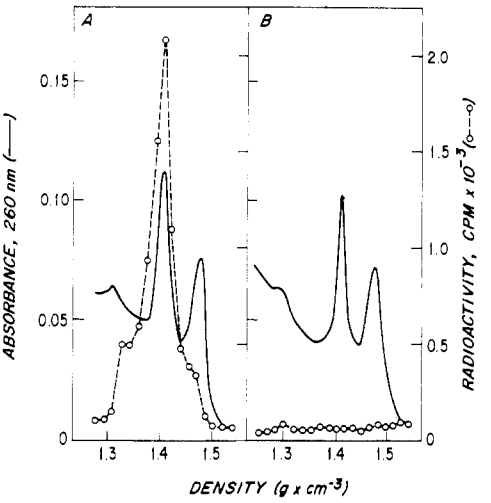

FIGURE 4: Comparison of binding of Met-tRNA$_f$ and Met-tRNA$_m$ to the 40S$_D$ subunit. Reaction mixtures containing 93 μg of KCl wash and 0.35 A_{260} unit of 40S$_D$ subunits were incubated and analyzed on CsCl gradients as described under Methods. (A) 0.45 pmol of [^{35}S]Met-tRNA$_f$; (B) 0.61 pmol of [^{35}S]Met-tRNA$_m$. Specific activity of [^{35}S]Met-tRNA was 85 Ci/mmol.

Table IV: Comparison of Stimulation of Met-tRNA$_f$ Binding to Millipore Filters and of Met-tRNA$_f$ Deacylation by the KCl Ribosomal Washes and by the S-100 Fraction.[a]

	[^{35}S]Met-tRNA$_f$ Bound to Filters (fmol)	[^{35}S]Met-tRNA$_f$ Hydrolyzed in 5 min (fmol)
Crude 1 M KCl ribosomal wash		
47 μg	157	18
94 μg	279	35
KCl ribosomal wash		
31 μg	179	
62 μg	308	6.4
S-100 fraction		
13 μg	22	
34 μg	26	
67 μg	22	
134 μg	19	4.1
Buffer	22	(7.8)

[a] Binding to filters was measured as described under Methods. Incubations contained 3.1 pmol of [^{35}S]Met-tRNA$_f$, specific activity 119 Ci/mmol. Deacylase activity was measured under the same ionic conditions by the loss of cold Cl_3CCOOH precipitable ^{35}S. Incubations initially contained 85 fmol of [^{35}S]Met-tRNA$_f$, and reported values are corrected for the spontaneous hydrolysis of 7.8 fmol.

Supportive evidence for the formation of a specific Met-tRNA$_f$–40S subunit complex was gained when we analyzed incubation mixtures on sucrose velocity gradients instead of CsCl density gradients (results not shown). [^{35}S]Met-tRNA$_f$ cosedimented with the 40S ribosomal subunit. Complex formation was dependent on the presence of the KCl ribosomal wash in the incubations. In the absence of subunits no label sedimented into the gradient. Attempts to demonstrate by CsCl density gradient analysis an mRNA dependency for the binding of the ternary complex to the 40S subunit have been unsuccessful, and although poly(A,U,G) was routinely included in the incubations, it was

Table V: Stimulation of Binding of [^{35}S]Met-tRNA$_f$ to Millipore Filters by the KCl Wash of Native 40S Subunits.[a]

	[^{35}S]Met-tRNA$_f$ Bound to Filters (fmol)
No KCl wash	12
KCl 40$_N$ wash, 3.3 μg	36
9.8 μg	105
KCl ribosomal wash, 31 μg	158

[a] 40S$_N$ subunits, approximately 24 A_{260} units, were isolated from multiple sucrose gradient analyses, as described under Methods, and were sedimented at 161,000g for 2.5 hr. Proteins were eluted with 0.5 M KCl under the conditions described by Schreier and Staehelin (1973b), and the supernatant fraction after centrifugation at 232,000g (average) for 2.5 hr was dialyzed against buffer D containing 0.12 M KCl and was used without further purification as the KCl 40$_N$ wash. Yield was 260 μg of protein. Incubations contained 3.1 pmol of [^{35}S]Met-tRNA$_f$, 132 Ci/mmol.

not necessary (results not shown). However, this finding does not mean that there is no mRNA requirement for binding of the ternary complex to the subunit, as there may be mRNA in the KCl wash.

(*2*) *Presence of IF2-Like Activity on the Native 40S Subunit.* The fact that the factor-GTP-Met-tRNA$_f$ ternary complex became bound to a subunit identical in density with the native 40S$_{N\text{-}L}$ subunit is consistent with our previous indirect evidence that the additional proteins on the 40S$_{N\text{-}L}$ subunit include initiation factors, and the evidence that binding to Millipore filters of [^{35}S]Met-tRNA$_f$ is stimulated by ribosomal wash proteins provided a means of testing the hypothesis further. As 0.5 M KCl reversibly removes the additional proteins from the 40S$_N$ subunits (Hirsch *et al.*, 1973), we prepared a crude 0.5 M KCl wash fraction from native 40S$_N$ subunits, as described in Table V. (Because of the small amount available this material was not further purified on DEAE-cellulose.) Table V shows that the KCl wash of the 40$_N$ subunit contains the factor which stimulates binding of Met-tRNA$_f$ to Millipore filters, in somewhat greater concentration, per microgram of protein, than the KCl ribosomal wash. Figure 5 demonstrates that the 40$_N$ KCl wash also converts 40$_D$ subunits to forms of density 1.40 and 1.49 g cm^{-3}, and stimulates the binding of Met-tRNA$_f$ to the 1.40 g cm^{-3} subunit.

Because the Met-tRNA$_f$–40S subunit complex which is formed *in vitro* during incubation of 40$_D$ subunits with KCl wash is identical in density with the native 40S$_{N\text{-}L}$ subunit (1.40 g cm^{-3}), we compared KCl washes of the 40S$_{N\text{-}L}$ and 40S$_{N\text{-}H}$ subunits. We were able to separate small amounts of material enriched for one form of the subunit and to prepare sufficient crude 0.5 M KCl wash from each to assess stimulation of binding to filters (Table VI). Binding activity was eluted primarily from the 40S$_{N\text{-}L}$ preparation. Although the binding activity was low relative to the KCl ribosomal wash included for comparison, the quantities of subunits used was very low. The small amount of activity present in the 40S$_{N\text{-}H}$ and 60S$_N$ washes may be due to the contamination of the fractions with 40S$_{N\text{-}L}$ particles when the subunits were isolated from the sucrose gradients.

In view of the evidence for the presence of Met-tRNA$_f$-binding activity on the 40S$_{N\text{-}L}$ subunits, we incubated

Table VI: Stimulation of Binding of [^{35}S]Met-tRNA$_f$ to Millipore Filters by KCl Washes from Ribosomal Subunits.[a]

KCl Wash	[^{35}S]Met-tRNA$_f$ Bound to to Millipore Filter (fmol)
None	11
50 μl of 40S$_{N\text{-}H}$	14
100 μl of 40S$_{N\text{-}H}$	15
50 μl of 40S$_{N\text{-}L}$	19
100 μl of 40S$_{N\text{-}L}$	33
50 μl of 40S$_D$	11
100 μl of 40S$_D$	11
50 μl of 60$_N$	13
100 μl of 60$_N$	14
20 μl (62 μg of protein) of KCl ribosomal wash	250

[a] Subunits were isolated on sucrose gradients as described under Methods. Approximately 2 A_{260} units of each type of subunit were used to prepare crude 0.5 M KCl washes as described in Table V. The protein content of these washes was too low to measure. Incubations contained 3.1 pmol of [^{35}S]Met-tRNA$_f$, 100 Ci/mmol.

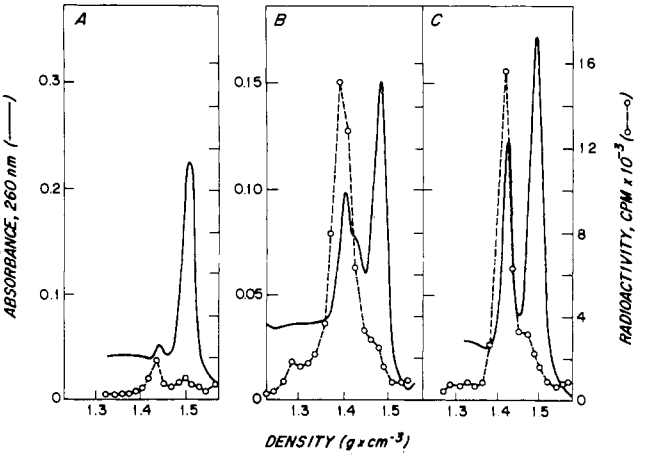

FIGURE 5: Stimulation of binding of Met-tRNA$_f$ to 40S$_D$ subunits by the KCl wash prepared from native 40S subunits. Reaction mixtures, containing 0.51 A_{260} unit of 40S$_D$ subunits and 7.7 pmol of [^{35}S]Met-tRNA$_f$, were incubated and analyzed as described under Methods. (A) No KCl ribosomal wash; (B) 93 μg of KCl ribosomal wash; (C) 26 μg of 40S$_N$ KCl wash.

[^{35}S]Met-tRNA$_f$ and GTP directly with 40S$_{N\text{-}L}$ subunits, expecting that the Met-tRNA$_f$ might bind in the absence of added factors. However, binding was undetectable (Figure 6A). (In this experiment a preparation enriched for 40S$_{N\text{-}L}$ subunits was used, but identical results were obtained with total 40S$_N$ preparations.) A possible explanation for this might be that the 40S$_N$ subunits contained a ternary complex which was too tightly bound to exchange with free GTP and Met-tRNA$_f$. To test this we first made the incubation with 40S$_{N\text{-}L}$ subunits 0.5 M with respect to KCl, to dissociate the subunit-associated proteins. The mixture was then dialyzed against a low salt buffer to facilitate protein reassociation to the subunit, and was "fixed" and examined on a CsCl gradient. A small amount of [^{35}S]Met-tRNA$_f$ now bound to the subunit and was again exclusively associ-

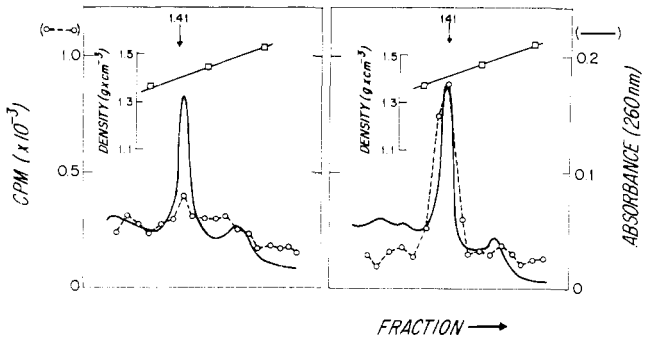

FIGURE 6: Binding of Met-tRNA$_f$ to the 40S$_{N-L}$ subunit without added KCl ribosomal wash. Reaction mixtures containing 0.5 A_{260} unit of 40S$_N$ subunits, enriched for 40S$_{N-L}$ subunits as described under Methods, and 4.3 pmol of [^{35}S]Met-tRNA$_f$ (125 Ci/mmol) were incubated as described below and were analyzed on CsCl gradients as described under Methods. (A) Incubated for 15 min at 28°, as usual; (B) made 0.5 M with respect to KCl and incubated 10 min at 20°; then dialyzed for 3 hr at 20° against buffer of composition identical with the standard reaction mixture (100 mM KCl, 50 mM TEA-HCl, 3 mM $Mg(OAc)_2$, 1 mM dithiothrietol, and 0.2 mM GTP).

ated with the particles of density 1.41 g cm^{-3} (Figure 6B). As no exogenous factors were added, the Met-tRNA$_f$-binding factor must have been present on the 40S$_N$ subunits.

Discussion

The bacterial initiation factor IF-2 is recognized functionally by its ability to stimulate the binding of fMet-tRNA$_f$ to the smaller ribosomal subunit, which it does through the formation of an IF-2–GTP–fMet-tRNA$_f$ complex (Rudland *et al.*, 1971; Lockwood *et al.*, 1971). A mammalian initiation factor which has recently been purified by several laboratories appears to be analogous, as it forms a Met-tRNA$_f$–GTP–factor complex which binds to the 40S subunit (Chen *et al.*, 1972; Levin *et al.*, 1973; Schreier and Staehelin, 1973a; Dettman and Stanley, 1973; Merrick *et al.*, 1974). We detect a like activity in a KCl wash of Ehrlich cell ribosomes. Because we have not purified the factor we cannot state unequivocally that it is identical with the factor reported by others and cannot exclude proteins which nonspecifically enhance binding of Met-tRNA$_f$ to the 40S subunit. However, the specificity of the factor detected here is demonstrated by its ability to bind Met-tRNA$_f$ to the subunit in preference to other aminoacyl-tRNA species, including Met-tRNA$_m$; by its requirement for GTP and inhibition by GDP; by its participation in the formation of a unique subunit–factor complex of buoyant density 1.40 g cm^{-3} which contains Met-tRNA$_f$; and by its almost exclusive presence in the KCl wash fraction and absence from other cellular fractions. These characteristics exclude the possibility that it is an aminoacyl-tRNA synthetase, elongation factor 1, or a ribosomal structural protein that nonspecifically stimulates the binding of aminoacyl-tRNA to Millipore filters (Brot *et al.*, 1970; Gupta *et al.*, 1973) and strongly suggest that there exists a factor in the Ehrlich ribosomal wash which is similar to that purified from other mammalian cells, and which has a similar function to bacterial IF-2.

We assume that the factor stimulating Met-tRNA$_f$ binding to the 40S subunit is also the factor stimulating binding to Millipore filters, as the specificities are identical, and others have reported that the purified factor has both activities (cited above).

We have previously presented indirect evidence that the extra proteins associated with native 40S subunits include initiation factors (Ayuso-Parilla *et al.*, 1973a,b). This is supported more directly by the present demonstration that the subunits contain a Met-tRNA$_f$-binding activity (Figure 5; Table V). Consistent with those data, we have recently found that native 40S subunits within the cell include a subpopulation which contains bound Met-tRNA$_f$ (Smith and Henshaw, in preparation). The native 40S subunit–Met-tRNA$_f$ complexes band with the 40S$_{N-L}$ subunits at 1.40 g cm^{-3}, as do the complexes formed *in vitro* in the presence of the KCl ribosomal wash (Figure 3). The present evidence indicates that the factor which stimulates binding is also included in the 1.40 g cm^{-3} complex. We previously proposed that the 40S$_{N-H}$ subunits (1.49 g cm^{-3}) bind additional factors and become converted to 40S$_{N-L}$ particles in the ribosome cycle. The present evidence indicates that among the additional factors which bind to the 40S$_{N-H}$ subunits is the Met-tRNA$_f$–factor–GTP ternary complex.

Despite the fact that the 40S$_N$ subunits contain a factor which stimulates the binding of Met-tRNA$_f$, free Met-tRNA$_f$ did not bind well to the native 40S subunits when it was incubated with them under the usual binding conditions (Figure 6A). A small amount of [^{35}S]Met-tRNA$_f$ binding did occur under conditions in which subunit–factor complexes were dissociated by the presence of 0.5 M KCl and were allowed to reform by dialysis, in the presence of [^{35}S]Met-tRNA$_f$ (Figure 6B). The binding demonstrated under these conditions is probably a gross underestimate of the binding activity present, as the dialysis period allows for prolonged exposure to the methionyl-tRNA deacylase, and there may be substantial competition from endogenous Met-tRNA$_f$ as well. Preferential binding under these conditions suggests the possibility that the binding factor may exist on the 40S subunits in nonexchangeable complexes. However, many other hypotheses are equally tenable, and further studies are required to explain the absence of direct binding of Met-tRNA$_f$ to 40S$_N$ subunits.

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Large Peptides of Bovine and Guinea Pig Myelin Basic Proteins Produced by Limited Peptic Hydrolysis†

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ABSTRACT: Bovine and guinea pig myelin basic proteins were cleaved with pepsin at pH 3.0 or pH 6.0 (enzyme/substrate, 1:500, w/w), and the peptides were isolated and identified. At pH 3.0 cleavage of the bovine protein occurred principally at three sites: Phe-Phe (88-89), Phe-Phe (42-43), and Leu-Asp (36-37). Minor cleavages occurred at Leu-Ser (110-111), Phe-Ser (113-114), and Ile-Phe (152-153). A study of the time course of the hydrolysis showed that the reaction was biphasic; nearly all of the protein was cleaved at Phe-Phe (88-89) before significant cleavages at other sites occurred. At pH 6.0 cleavage of the bovine protein occurred almost exclusively at a single site, the Phe-Phe bond at position 88-89, resulting in bisection of the protein. Treatment of the guinea pig protein with pepsin under the same conditions resulted in the production of peptides which were identical with those of the bovine protein in chromatographic and electrophoretic properties and in N-terminal and C-terminal residues but which differed slightly in amino acid composition.

Myelin sheaths of the central nervous system of vertebrates contain a highly basic protein which accounts for about 30% of the total protein (Kies *et al.*, 1964; Autilio, 1966; Eng *et al.*, 1968). It induces an autoimmune disease, experimental allergic encephalomyelitis, when injected with Freund's complete adjuvant into a number of different species of animal, including guinea pig, rat, rabbit, and monkey (for a review, see Kies, 1973). Since the complete amino acid sequences of the bovine (Eylar *et al.*, 1971; Brostoff *et al.*, 1974), human (Carnegie, 1971), and the smaller of the two rat (Dunkley and Carnegie, 1974) myelin basic proteins have been determined, this protein provides an excellent model for the study of the relationship between primary structure and immunological activity.

Sequence studies by Eylar *et al.* (1971) and Carnegie (1971) have shown that the myelin basic protein can be extensively digested by a variety of proteolytic enzymes. At pH 3.0 and 37° with an enzyme/substrate ratio of 1:50 (w/w), pepsin cleaves the protein into at least 17 peptides, the largest of which consists of 46 residues (Eylar *et al.*, 1971). In order to obtain additional, relatively large fragments of the basic protein as part of a systematic exploration of immunologically active regions, we have cleaved the protein at a limited number of peptide bonds by decreasing the pepsin/substrate ratio to 1:500. The present report describes the preparation, purification, and characterization of the several relatively large peptides produced in high yield by limited peptic cleavage of the bovine and guinea pig myelin basic proteins.

Materials and Methods

Myelin Basic Proteins. Bovine and guinea pig myelin basic proteins, isolated from quick-frozen brain by the procedure of Deibler *et al.* (1972), were further purified and

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