Binding of Met-tRNA\textsubscript{f} to Native 40 S Ribosomal Subunits in Ehrlich Ascites Tumor Cells*

KELVIN E. SMITHZ AND EDGAR C. HENSHAW§

From the Departments of Medicine, Harvard Medical School, and Beth Israel Hospital, Boston, Massachusetts 02215

Two forms of native 40 S ribosomal subunits, distinguishable by their buoyant densities, are recovered from Ehrlich ascites cells. In this communication, we describe experiments designed to test whether Met-tRNA\textsubscript{f} is associated with either form. Our results indicate that (a) in the cell, Met-tRNA\textsubscript{f} is bound to the native 40 S subunit, and in particular, to the subunit of density 1.40 g x cm\textsuperscript{-3}; (b) under the growth conditions used, less than 10% of the low density native subunits have bound Met-tRNA\textsubscript{f}; (c) the majority of the Met-tRNA\textsubscript{f} containing native 40 S subunits, isolated from sucrose gradient analyses of cell extracts, join with 60 S subunits in vitro to form 80 S monosomes only if additional ribosomal wash factors are provided; and (d) the 80 S monosomes so formed are completed initiation complexes that can form peptide bonds. These results support the hypothesis that various forms of the native 40 S subunit represent different stages in the formation of a complete initiation complex.

A Met-tRNA\textsubscript{f}-40 S subunit complex is thought to be an essential intermediate in the initiation of polypeptide chain synthesis in mammalian cells. The formation of such complexes in vitro has frequently been described in highly purified systems, and has recently been demonstrated in reticulocyte lysates (1, 2), but their existence in vivo has not been documented. However, the possibility that they might be present, along with free subunits, was suggested by our earlier finding that in cytoplasmic extracts prepared from Ehrlich ascites tumor cells, the native 40 S subunits (i.e., those existing as separate particles rather than as a component of ribosomes) occur predominantly in two forms, of buoyant density 1.49 ± 0.02 and 1.40 ± 0.02 g x cm\textsuperscript{-3} in CsCl; these contain, respectively, 9 x 10\textsuperscript{5} and 7.5 x 10\textsuperscript{5} daltons of exchangeable protein in addition to the usual 40 S subunit proteins (3, 4). Our evidence further suggested that these proteins might include initiation factors (5), including one (6-11) that resembles bacterial IF-2 in forming a Met-tRNA\textsubscript{f}-factor-GTP complex that binds to the 40 S subunit: this factor was found only on the subunit of density 1.40 g x cm\textsuperscript{-3}, and Met-tRNA\textsubscript{f} bound to preparations of reticulocyte ribosomes became bound to a more rapidly sedimenting (on sucrose gradients) form of the 40 S subunit, although he did not examine buoyant density. These results imply that the native 40 S subunits of density 1.40 g x cm\textsuperscript{-3} in the cell should include complexes containing Met-tRNA\textsubscript{f}.

To provide evidence that such complexes exist in the cell, and that the binding of Met-tRNA\textsubscript{f} to 40 S ribosomal subunits in vitro is a true reflection of in vivo events, we examined Ehrlich ascites tumor cells grown in suspension culture containing \textsuperscript{35}S methionine. We found that \textsuperscript{35}S methionyl-tRNA\textsubscript{f} was present on a fraction of the native 40 S subunits of density 1.40 g x cm\textsuperscript{-3}. These data support our previous hypothesis (5) that the native 40 S subunits contain at least some of the protein factors required for initiation, and that the Met-tRNA\textsubscript{f}-40 S subunit complex is a true intermediate in the formation of the complete 80 S initiation complex.

EXPERIMENTAL PROCEDURE

Chemicals

\textsuperscript{35}S methionine was purchased from Amersham/Searle, \textsuperscript{3}H lysine from New England Nuclear Corp., poly(A,U,G) from Schwarz/Mann, and deacylated rabbit liver-soluble ribonucleic acid from General Biochemicals.

Solutions

Buffer A: 25 mM KCl, 20 mM triethanolamine-HCl (TEA-HCl), pH 7.4, (0°), 2 mM Mg(Ac)\textsubscript{2}, 1 mM dithiothreitol, 0.1 mM EDTA. Buffer B: 10 mM Tris-HCl, pH 7.1 (25°), 10 mM KCl, 1.5 mM Mg(Ac)\textsubscript{2}, 6 mM mercaptoethanol. Buffer C: 100 mM KCl, 50 mM TEA-HCl, pH 7.4, 3 mM Mg(Ac)\textsubscript{2}, 1 mM dithiothreitol.

Cells

Ehrlich ascites tumor cells were grown at 37° in spinner culture in Eagle’s Minimal Essential Medium (MEM) supplemented with 10% calf serum, as previously described (13).

Labeling of Cells and Isolation of Ribosomes

Method A—One liter of culture at approximately 1 x 10\textsuperscript{6} cells/ml was harvested by centrifugation at 1100 x g for 5 min in an

* This work was supported by United States Public Health Service Grants CA 03151 and CA 05167, and by National Science Foundation Grant GB 30540.

† Present address; Department of Biochemistry, King’s College, Strand, London WC2R 2LS, England. Supported by Damon Runyon Memorial Fund Cancer Research Fellowship DRF-615.

§ To whom reprint requests should be directed.
unrefrigerated centrifuge. The pellet was resuspended in 100 ml of a modified MEM supplemented with 10% dialyzed calf serum prewarmed to 37°C. In order to increase the specific activity of the labeled amino acid in the culture medium, the MEM was modified to contain 0.06% concentration of L-[35S]methionine or 400 μCi of L-[3H]lysine, respectively, were added to the cell cultures, so that the final specific activities of each amino acid were similar in the respective media. After 15 min of incubation at 37°C, the cells were harvested by pouring the culture medium over 50 g of crushed ice immediately prior to centrifuging. The cell pellet was washed twice with 20 ml of ice-cold Buffer A, and was finally resuspended in 3 volumes of Buffer B. The cells were allowed to swell for 10 min at 0°C, and were then lysed by 25 strokes of a hand-operated glass Dounce homogenizer. A postmitochondrial supernatant was prepared by centrifuging the lysate for 10 min at 10,000 × g. The supernatant was decanted and recentrifuged for an additional 10 min at 10,000 × g. Ribosomes were isolated from the postmitochondrial supernatant by the Mg^2+ precipitation method (4). Sixty A_{260} units of ribosomes were layered onto 28 ml 20 to 40% sucrose gradients made up in Buffer A. The gradients were centrifuged for 17 hours at 22,000 rpm and 3°C in a Spinco SW 25.1 rotor. All gradients were monitored for optical density at 280 nm, as previously described (14). Some gradients were fractionated into approximately 0.6-ml aliquots, and to each fraction, 2 drops of 0.5% bovine serum albumin were added as carrier, followed by 2 ml of ice-cold 5% trichloroacetic acid. The precipitate was collected onto Millipore filters. Each filter was washed twice with 5 ml of ice-cold 5% trichloroacetic acid, dried, and counted in a Beckman liquid scintillation counter. From other gradients, 40 S, 60 S, and 80 S ribosomal particles were collected, pooled separately, and used immediately or stored in 1-ml aliquots at −80°C.

**Method B**—Cells were suspended at 1 × 10^6/ml in modified MEM, and were labeled essentially as described in Method A, except that cycloheximide (final concentration, 100 μg/ml) was added 5 min before the labeled amino acid. The cells were harvested and a postmitochondrial supernatant prepared as described above. One hundred A_{260} units of untreated postmitochondrial supernatant were layered directly onto the sucrose gradients, as described above. Particles of 40 S, 60 S, and 80 S were isolated from some gradients; other gradients were fractionated into 0.6-ml aliquots, and 0.5-ml samples were removed and placed into 2 ml of ice-cold Buffer C. The diluted fractions were quickly filtered through Millipore filters, washed twice with 5 ml of Buffer C, dried, and counted.

**CsCl Equilibrium Density Gradient Analysis of Labeled 40 S Ribosomal Subunits**

Approximately 3 A_{260} units (3.0 ml) of 40 S ribosomal subunits freshly isolated from sucrose gradients were made 4% with respect to formaldehyde (neutralized to pH 7.0 immediately before use) and analyzed by CsCl equilibrium density gradient centrifugation, as previously described (4).

**Separation of Met-tRNA_{i} and Met-tRNA_{a}**

Met-tRNA_{i} and Met-tRNA_{a} were separated as described by Samuel et al. (19) on columns of BD-cellulose (16, 17). Sodium was measured by flame photometry. RNA was purified by a cold phenol-sodium dodecyl sulfate method (18). Authentic mammalian Met-tRNA_{i} was prepared by charging rabbit liver tRNA with [35S]methionine, using an *Escherichia coli* aminoacyl-tRNA synthetase preparation (19).

**RESULTS**

Fig. 1 shows a sucrose gradient analysis of an extract of Ehrlich ascites tumor cells which had been incubated briefly (15 min) with L-[35S]methionine. The most prominent peak of cold-acid-precipitable radioactive methionine was associated with the 40 S subunits, their specific activity being considerably greater than that of the other particles. However, while this was consistent with the formation of Met-tRNA-40 S subunit complexes, much labeled methionine appeared in the region of 60 S subunits, 80 S ribosomes, and polyribosomes, and at the top of the gradient, presumably owing to the presence of methionine-containing peptides and of free methionyl-tRNA.

To eliminate protein synthesis, we repeated the experiment, labeling the cells in the presence of sufficient concentrations of cycloheximide to inhibit peptide chain elongation. As Met-tRNA_{i}-40 S subunit complexes but not free Met-tRNA_{i} bind to Millipore filters, we also filtered the gradient fractions instead of acid precipitating them. Under these conditions, [35S]methionine sedimented predominantly with the native 40 S ribosomal subunits (Fig. 2A). No label was associated with the 40 S subunits when the cells were incubated with [3H]lysine under identical conditions (Fig. 2B). Thus, cycloheximide treatment inhibited peptide chain elongation, but did not prevent the association of [35S]methionine with 40 S subunits.

The labeled methionine associated with the native 40 S ribosomal subunits was present on those subunits as aminoacyl-tRNA rather than as long chain peptides, as it was precipitated by cold acid (Table I, Line 1) but not by hot acid (Line 2) or alkali (Line 3). When RNA was purified from the native 40 S subunits and was analyzed on BD-cellulose
peptide bond formation by using the puromycin model reaction. We performed to answer the question as to whether the 80 S ribosome was active in peptide bond formation. We assayed the presence of 60 S subunits (Fig. 5A). Thus, the presence of 60 S subunits is not sufficient for any but minimal monosome formation. However, much more label was transferred when a crude preparation of initiation factors (KCl ribosomal wash) was added (Fig. 5B). To prevent ribosome (particularly 60 S subunit) precipitation by wash proteins, we deliberately added a low concentration of wash, which may account for the persistence of label in the 40 S particles (Fig. 5B). A probable requirement for messenger RNA in the formation of 80 S monosomes was demonstrated by the finding that in the absence of poly(A,U,G), less than 40% of the 35S label was transferred from the 40 S subunit to the 80 S ribosome (Table II, Experiments A and B). It is uncertain whether the minority of native 40 S subunits which formed 80 S ribosomes in the absence of poly(A,U,G) carried endogenous mRNA or received mRNA from the reticulocyte KCl wash, as it is well known that the crude reticulocyte high salt ribosomal washes contain globin messenger RNA.

The data in Table II also show the results of an experiment we performed to answer the question as to whether the 80 S ribosomes were active in peptide bond formation. We assayed peptide bond formation by using the puromycin model reaction. In this reaction, puromycin reacts with Met-tRNA\textsubscript{e} bound in the ribosome P site, and results in the release of methionine in the form of met-puromycin from the ribosome. We preincubated the native 35S-labeled 40 S subunit with poly-(A,U,G), 60 S subunits, and reticulocyte KCl ribosomal wash factors for 5 min at 28° to allow 80 S complex formation, and then added puromycin (1 mg/ml, final concentration) to the incubation. The 40 S and 80 S particles were separated from the gradient, and the quantity of label in each peak was estimated. The data indicate the release of the majority of Met-tRNA\textsubscript{e} from the 80 S ribosome, as the disappearance of 35S label from the 40 S subunit was not accompanied by an accumulation of label in the 80 S ribosome region of the gradient (Table II, Experiment A compared to Experiments C\textsubscript{a} and C\textsubscript{b}). From the results of these experiments, we conclude that the 80 S ribosome is capable of participating in protein synthesis.

**DISCUSSION**

The question of whether native ribosomal subunits are normal intermediates in the initiation of protein synthesis in mammalian cells or whether they result from side reactions is still unresolved. *In vitro* studies using high salt-washed total ribosomes or subunits have provided evidence for the direct

---

**Table I**

**Product analysis of L-[35S]methionine-labeled 40 S ribosomal subunits**

Two drops of 0.5% bovine serum albumin were added to duplicate 1-ml samples (3000 cpm) of L-[35S]methionine-labeled 40 S ribosomal subunits. The solutions were either (a) precipitated with ice-cold 5% trichloroacetic acid and immediately filtered (cold trichloroacetic acid treatment); (b) precipitated with 5% trichloroacetic acid and heated for 20 min at 95° before cooling to 0° and filtering (hot trichloroacetic acid treatment); or (c) mixed with 3 N NaOH to give a final concentration of 1 N, incubated for 3 hours at 37°, cooled to 0°, neutralized with an equal volume of 1 N HCl, precipitated with 5% ice-cold trichloroacetic acid (final concentration), and filtered.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cold acid-insoluble material remaining after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold trichloroacetic acid</td>
<td>2557</td>
</tr>
<tr>
<td>Hot trichloroacetic acid</td>
<td>160</td>
</tr>
<tr>
<td>1 N NaOH</td>
<td>118</td>
</tr>
</tbody>
</table>
FIG. 4. CsCl equilibrium density gradient analysis of L-[35S]methionine-labeled 40 S ribosomal subunits. A, 3 A260 units of 40 S subunits, labeled with L-[35S]methionine in the presence of cycloheximide, were collected from sucrose gradients, immediately "fixed," and analyzed as described under "Experimental Procedure." B, 4 M KCl was added dropwise to 3 A260 units of the labeled subunits to give a final concentration of 0.5 M. The solution was incubated for 15 min at 0 °C, "fixed," and analyzed as described above. The amount of 40 S subunits of density 1.40 g x cm⁻³ was measured by planimetry and the radioactivity of the peak was summed, in order to determine the specific activity of the 1.40 g x cm⁻³ subunit. The proportion of 1.40 g x cm⁻³ subunits to the total was also measured by planimetry, and using this value and the total amount of 40 S subunits determined by planimetry in Figs. 1 and 2, we estimated the specific activity of the 1.40 g x cm⁻³ subunits in these figures, assuming all the radioactivity was in this subunit.

FIG. 5. Incorporation into monosomes of native 40 S subunits containing bound Met-tRNA. 1.4 A260 units (800 cpm) of native 40 S ribosomal subunits labeled with L-[35S]methionine, isolated from sucrose gradient analyses similar to Fig. 2A, were incubated for 15 min at 28 °C in 2-ml reaction mixtures that contained 50 mM TEA-HCl, pH 7.4 (20 °C), 3 mM Mg(Ac)₂, 1 mM dithiothreitol, 100 mM KCl, 6 A260 units of 0.5 M KCl-washed 60 S ribosomal subunits, and 100 µg of poly(A,U,G). The following additions were made to each incubation. A, none; B, 310 µg of 1 M KCl ribosomal wash protein. The final KCl concentration was adjusted to 100 mM, as in A. The reactions were terminated by cooling to 0 °C. Each incubation mixture was layered onto 28-ml linear 20 to 40% sucrose gradients, made up in Buffer A. After centrifugation for 17 hours at 23,000 rpm in a Spinco SW 25.1 rotor, the gradients were analyzed for absorbance at 260 nm, and radioactivity, as described under "Method A" of "Isolation of Ribosomes." The KCl ribosomal wash fraction was prepared from rabbit reticulocytes, as described previously (5) except that 1.0 M KCl was used rather than 0.5 M KCl.

TABLE II

Analysis of the native L-[35S]methionine-labeled 40 S ribosomal subunits to determine whether the subunits have bound messenger RNA and whether the 80 S ribosomes formed by incubating them with poly(A,U,G) 60 S subunits and initiation factors are active in peptide bond formation

The incubations were as described for Fig. 5B, except that 1400 cpm and 2.2 A260 units of native 40 S ribosomal subunits (prepared from Ehrlich ascites tumor cells labeled in cultures containing 750 μCi of L-[35S]methionine) were incubated with 3.4 A260 units of 0.5 M KCl-washed 60 S ribosomal subunits in a total volume of 3 ml. The incubations were layered on sucrose gradients and centrifuged as described in Fig. 5. The gradients were eluted as previously described, and the 40 S and 80 S ribosome peaks were separated from the gradient. Acetate buffer (2 M), pH 4.35, was added to each fraction to a final concentration of 0.2 M, followed by 4 M NaCl to a final concentration of 0.3 M (so that the total Na⁺ concentration was 0.5 M). To facilitate precipitation of the ribosomes, 1 mg/ml of carrier Escherichia coli RNA was added to a final concentration of 100 µg/ml. Two volumes of absolute ethanol were added to the mixtures, which were stored at −20 °C for about 4 hours. The precipitates were collected by centrifuging for 1 hour at 30,000 rpm in a Spinco angle 30 rotor. The pellets were dissolved in 100 µl of dilute NH₄OH, pH 9.5, and placed onto glass fiber filters. The centrifuge tubes were rinsed twice with the NH₄OH solution to ensure complete transfer of all the counts to the filters. The filters were thoroughly dried and counted in a Packard liquid scintillation counter. Background has been subtracted from all counts.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>40 S</th>
<th>80 S</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Complete incubation for 15 min at 28 °C (as in Fig. 5B)</td>
<td>194</td>
<td>320</td>
</tr>
<tr>
<td>B. Incubation without poly(A,U,G) for 15 min at 28 °C</td>
<td>371</td>
<td>126</td>
</tr>
<tr>
<td>C₁. Complete incubation preincubated for 5 min at 28 °C followed by a further 10 min at 28 °C with 1 mg/ml (final concentration) of puromycin</td>
<td>60</td>
<td>49</td>
</tr>
<tr>
<td>C₂. As in C₁</td>
<td>72</td>
<td>46</td>
</tr>
</tbody>
</table>

participation of 40 S subunits in initiation (7, 8), and Darnbrough et al. (1) and Baglioni (2) have demonstrated the formation of Met-tRNAₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐ¢|
variable aggregation may explain, for instance, the somewhat presence of the synthetic messenger poly(A,U,G) (Fig. 5A). In addition, associated with a fraction of the native 1.40 g x cm\(^{-2}\) particles, we have found the factor, analogous to bacterial initiation Factor IF-2 and similar to the factor described in other mammalian cells (6-11), which binds to the subunit as a Met-tRNA\(_f\)-factor-GTP complex (12). This factor is presumably responsible for the in vivo binding of Met-tRNA to the 1.40 g x cm\(^{-2}\) subunit. Radioactive methionine equilibrates rapidly between extracellular and intracellular water (20), and we have estimated from the specific activity of the subunits of density 1.40 g x cm\(^{-2}\) and of the methionine in the culture medium that about 10% of the subunits of density 1.40 g x cm\(^{-2}\) contain labeled methionine in Fig. 24, and about 7% in Fig. 4A. To avoid possible complications due to cycloheximide, we also estimated the concentration of bound [\(^{38}\)S]methionine from Fig. 1. Although the calculation is somewhat approximate, the value obtained was also 7%, indicating that cycloheximide did not produce grossly aberrant results. Thus, from the present data it appears that the native 40 S subunits of density 1.40 g x cm\(^{-2}\) are heterogeneous, and that within the cell, only a minority of them contain the bound Met-tRNA\(_{f}\) complex, under the growth conditions used.

Recent evidence has suggested that in mammalian cells, the Met-tRNA\(_f\)-IF-2-GTP complex may bind to the 40 S subunit prior to the binding of mRNA (6, 21). Our finding that addition of poly(A,U,G) greatly stimulates the formation of 80 S monosomes from native 40 S subunits and 60 S subunits (Table II) is consistent with this model, as it suggests that at least 60% of the subunits that carry bound Met-tRNA\(_f\) do not contain bound mRNA. This result must be considered tentative, as we cannot exclude other explanations for the stimulation by poly(A,U,G). However, if it is substantiated that the native 40 S subunit must form a complex with Met-tRNA\(_{f}\)-IF-2-GTP before binding to the initiation site of mRNA, the fact that only a small proportion of 1.40 g x cm\(^{-2}\) subunits contain Met-tRNA\(_{f}\) raises the possibility that the binding of Met-tRNA\(_{f}\)-IF-2-GTP to the subunit might be a limiting step in initiation. In the present experiments, the concentration of methionine may have been limiting, as it was reduced to 1/30 its usual value in order to conserve labeled methionine. Hence, the possible regulatory role of the binding of Met-tRNA\(_{f}\) must be tested under more physiological conditions.6

It is not clear why 60 S subunits fail to bind more extensively to the Met-tRNA\(_{f}\)-containing native 40 S subunits in the presence of the synthetic messenger poly(A,U,G) (Fig. 5A). Experiments involving extensive manipulation of native 40 S subunits must be interpreted cautiously. We have found that native 40 S subunits tend to aggregate extensively after centrifugal sedimentation, and to some degree, even when isolated from one sucrose gradient and rerun on another. This variable aggregation may explain, for instance, the somewhat different recoveries of total counts on the sucrose gradient analyses shown in Fig. 5, A and B. However, with this caution, the stimulatory effect of added KC1 ribosomal wash proteins (Fig. 5B) may be taken to suggest that although the native 40 S subunits of density 1.40 g x cm\(^{-2}\) contain a number of proteins that are absent from the 40 S subunits of higher density (4), they may lack, or contain in very limited amount, at least one additional factor required for formation of the complete initiation complex. The component missing from the native 40 S subunit may be an initiation factor, or a structural protein removed during preparation of the subunits (22). As the isolation procedure was mild and was carried out in low salt medium, we assume the former possibility is more likely. That the Met-tRNA\(_{f}\)-containing native 40 S subunits are able, in the presence of KC1 ribosomal wash proteins, to form initiation complexes with Met-tRNA\(_{f}\) in the peptidyl site and capable of completing a peptide bond are shown by the puromycin-dependent release of [\(^{38}\)S]methionine from the 80 S ribosome (Table II).

Acknowledgments—We are grateful for the excellent technical assistance of Ms. Anne Richards and Jeanne Thivierge, and we thank Dr. Bernard Davis for his very helpful comments on the manuscript.

REFERENCES
Binding of Met-tRNAf to native 40 S ribosomal subunits in Ehrlich ascites tumor cells.
K E Smith and E C Henshaw


Access the most updated version of this article at http://www.jbc.org/content/250/17/6880

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/17/6880.full.html#ref-list-1