Initiation Factor Activity Associated with Free 40 S Subunits from Rat Liver and Rabbit Reticulocytes

(Received for publication, January 31, 1974)

INGEGERD C. SUNDKVIST, WALLACE L. MCKEEHAN, MAX H. SCHREIER, AND THEOPHIL STAEBELIN*

From the Basel Institute for Immunology, CH-4068 Basel, Switzerland

SUMMARY

At least one initiation factor, required for the translation of rabbit globin messenger RNA in a partially purified cell-free system, is found to be associated with free 40 S ribosome subunits in cell extracts from rat liver and rabbit reticulocytes. The free 40 S ribosomes carrying initiation factor activity are devoid of messenger RNA activity. The initiation factor activity is dissociated from the 40 S particles by high salt concentration and then is found in the ribosome-free upper region of sucrose density gradients. The resulting salt-washed free 40 S subunits are still active in protein synthesis if supplemented with initiation factors. Our results imply that the free 40 S ribosomes in mammalian cells are intermediates in the ribosome cycle of protein synthesis and may represent at least in part true initiation complexes prior to mRNA binding.

In both bacterial and mammalian cells initiation factors for protein synthesis are usually extracted by 0.5 to 1 M KCl or NH₄Cl from crude total ribosome preparations. For Escherichia coli it has been well established that in the process of polypeptide chain initiation the initiation factors interact with the small ribosome subunits, fMet-tRNA and presumably mRNA (for review see Refs. 1-3). Furthermore, free 30 S bacterial ribosome subunits, or proteins derived from them by high salt treatment, can be used as the only source of initiation factors in bacterial systems (4-6).

The quantitative distribution of the individual initiation factors between ribosomes and the high speed supernatant cell fractions in eucaryotes may well differ from one species to another or even from one tissue to another. In immature red blood cells initiation factors required for protein synthesis in reconstituted systems have been obtained so far from crude ribosomes only (7-9). This does not exclude that at least some factor(s) may also occur free in the cell sap. The only initiation factor characterized and purified from Artemia salina embryos (10) was found exclusively in the high speed supernatant of cell extracts. The apparent functional equivalent of the Artemia factor was isolated from high speed supernatants from rat liver (11-13), rat muscle (12), and mouse Krebs ascites cells (12).

* To whom correspondence and reprint requests should be sent.

In cells active in protein synthesis one would expect some or all of the initiation factors to be associated at least temporarily with ribosomes during initiation complex formation. Whether one or more initiation factors have such a high affinity to ribosomes that, under physiological ionic conditions, their existence in the high speed supernatant is practically excluded is an open question at present. Furthermore, initiation factors obtained from the ribosome fraction in mammalian cells have been extracted only from the whole ribosome population. So far nothing is known about the distribution of initiation factors within the ribosome population.

In this study, however, we show for both rat liver and rabbit reticulocytes that initiation factor activity required for the cell-free translation of rabbit globin mRNA is found associated with free 40 S ribosome subunits at low KCl concentrations in these cell extracts. The initiation factor activity is removed from the subunits by treatment with high salt. No endogenous mRNA seems to be associated with free 40 S subunits.

METHODS AND MATERIALS

Polyosome-derived Ribosome Subunits—Polyosome derived 40 and 60 S ribosome subunits were prepared as described previously (9). Briefly, mouse liver polyosomes were obtained by centrifugation of a sodium deoxycholate-treated postmitochondrial supernatant through a double layer of 1.5 and 1 M sucrose containing 0.1 M NH₄Cl, 5 mM MgCl₂, 20 mM Tris-HCl (pH 7.6), and 1 mM dithiothreitol. The polyosomes were converted into single ribosomes by a "run-off" in an in vitro protein-synthesizing system, using a pH 5 enzyme preparation from rat liver as a source for all the components needed for polypeptide chain elongation and termination. After incubation, KCl was added to a final concentration of 0.4 M to dissociate the single ribosomes into subunits. The subunits were separated by sucrose density gradient centrifugation and the 40 and 60 S peak fractions were concentrated by centrifugation to pellet the subunits. The ribosome subunits were resuspended in 0.1 M KCl, 4 mM MgCl₂, 0.02 M Tris-HCl (pH 7.6), 1 mM dithiothreitol, and stored at -80° until used.

Rat Liver pH 5 Enzymes—Rat liver pH 5 enzymes were prepared according to Fahey and Staehelin (14).

In Vitro Protein Synthesis—The system is basically that described by Schreier and Staehelin (9). Incubation mixtures, 0.1 ml, contained free 40 S ribosome subunits present in pH 5 enzyme preparations of either rat liver or rabbit reticulocyte high speed supernatants, or polyosome-derived 40 S subunits. In either case polysome-derived 60 S subunits were added. A standard assay contained 0.36 A₄₅₀ units of 60 S subunits and 0.14 A₄₅₀ units of 40 S subunits, or, as indicated for each experiment, 50 μ of rat liver pH 5 enzymes, 2 μg of rabbit globin mRNA, and the following reactants per ml: 20 μoles of creatine phosphate, 3.75 units of creatine kinase, 1 μole of ATP, 0.4 μoles of GTP, 80 μoles of KCl, 4.5 μoles of MgCl₂, 30 μoles of Tris-HCl (pH 7.6), 1
μmole of dithiothreitol, 30 nmoles of each L-amino acid and 0.75 
μCl of [14C]leucine (final specific activity, 22 mCi per mmole). 
Incubation was for 40 min at 30°. The samples were processed 
for hot trichloroacetic acid (10%)-insoluble radioactivity mea-
surements as described previously (9). One thousand counts per 
min correspond to 26 pmoles of leucine. If exogenous initiation 
Factors were used for control experiments, they were prepared as 
an "A-fraction" from rabbit reticulocyte ribosomes as described 
previously (9).

Density Gradient Centrifugation—Density gradient centrifuga-
tion of pH 5 enzymes for analysis or preparative use of free 40 S 
subunits was done in sucrose or glycerol gradients.

Analytical centrifugations were done using 4-ml convol-
exponential 10 to 32% (w/v) sucrose gradients (15) containing 0.3 
m KCl, 3 mM MgCl₂, 20 mM Tris-HCl (pH 7.6). Centrifugation 
was at 50,000 rpm for 70 min at 4° in a Beckman SW 56 Ti rotor.
Preparative centrifugations were done using 3.5- or 4.5-ml convex 
exponential 20 to 70% (w/v) glycerol gradients. Low salt gra-
dients contained 0.03 M KCl, 2 mM MgCl₂, 20 mM Tris-HCl (pH 
7.6) and 1 mM dithiothreitol. High salt gradients contained 0.5 
mKCl, 3 mM MgCl₂, 20 mM Tris-HCl (pH 7.6) and 1 mM dithio-
the late. Centrifugation was at 50,000 rpm at 4° for 2.5 hours 
without using the brake in a Beckman SW 50.1 rotor. The ab-
sorption was monitored continuously as previously described (9) 
and the preparative gradients were collected into 0.25-ml frac-
tions.

RESULTS

Correlation between Initiation Factor Activity and Presence of 
Free 40 S Subunits in pH 5 Enzyme Preparations—A highly 
efficient mammalian system for the in vitro translation of exog-
ogenous rabbit globin mRNA has been reported previously (9).
This partly purified cell-free system was shown to be dependent 
on the addition of initiation factors and messenger RNA. How-
ever, we noticed that the system also was able to promote at low, 
but significant, efficiency de novo synthesis of rabbit globin in 
the absence of added initiation factors. This synthesis was 
found to be inversely related to the length of centrifugation time 
time used to prepare the high speed supernatant for the pH 5 
enzyme preparation. These rat liver pH 5 enzymes contain all the 
components required for amino acid activation, polypeptide 
elongation, and termination in the cell-free system. Sucrose 
gradient analysis of different pH 5 enzyme preparations revealed a 
s triking correlation between the amount of 40 S subunits (Fig. 
1) and their ability to promote de novo protein synthesis in the 
presence of polysome-derived 40 and 60 S subunits and rabbit 
globin mRNA (Table I). It should be noted that the synthetic 
activities of the pH 5 enzyme preparations A, B, and C without 
added initiation factors were about 22, 7, and 1%, respectively, 
compared to similar incubation mixtures supplemented with 
saturating amounts of partially purified rabbit reticulocyte initia-
tion factors (results not shown).

Free 40 S Subunits Present in pH 5 Enzymes Participate in 
Globin Synthesis—The results described above do not prove that 
the free 40 S subunits present in rat liver pH 5 enzymes have 
initiation factor activity or can participate in protein synthesis. 
Therefore, we used the pH 5 enzyme Preparations A, B, and C 
as the exclusive source of both initiation factors and 40 S ribo-
some subunits in the cell-free system. Increasing amounts of 
polyasome-derived 60 S subunits were added to incubation mix-
tures containing globin mRNA, pH 5 enzymes (A, B, or C) and 
the other components required for protein synthesis (Fig. 2). 
The results show that the rat liver 40 S subunits present in the 
ph 5 enzyme preparations actually participate in globin syn-
thesis together with the added mouse liver 60 S subunits. The 
activity of pH 5 enzyme Preparation A in the absence of added 
60 S subunits is due to the small amount of 60 S subunits present 
in this pH 5 enzyme preparation (Fig. 1A). From Curve A and 
B in Fig. 2 we calculate that maximum activity was reached at 
a 60:40 S A₅₅₀ ratio of about 2.8 which corresponds to a molar 
ratio of slightly more than 1. In the linear range of Curve A 
of Fig. 2, where 60 S subunits are limiting, about 3.5 to 4 pmoles 
of globin were synthesized per pmole of 60 S subunits.

Initiation Factor Activity Is Bound to Free 40 S Subunits at 
Low Ionic Strength—In order to localize the initiation factor 
activity we centrifuged rat liver pH 5 enzymes rich in free 40 S 
subunits into glycerol density gradients containing either low 
Salt (0.03 M KCl and 0.002 M MgCl₂) or high salt (0.5 M KCl and 
0.003 M MgCl₂) concentrations. Aliquots of each gradient frac-

Fig. 1. Analytical sucrose density gradients of different pH 5 
enzyme preparations obtained from high speed supernatants of 
rat liver. The high speed supernatants had been centrifuged for 
2 hours (A), 3 hours (B), and 4 hours (C), in the IEC A170 angle 
rotor at 40,000 rpm. The upper three fourths of the content of 
the centrifuge tubes had been removed and processed for pH 5 
enzyme preparations. Fifty microliters of pH 5 enzyme prepara-
tions were analysed on each gradient.

Table I

| pH 5 enzyme preparation | Centrifugation p.m.s. a at 40,000 rpm | Free 40 S subunit content of pH 5 enzymes per assay | Polysome-
derived 40 and 60 S subunits per assay | Protein synthesis without exogenous initiation factors: [14C]leucine incorporated |
|-------------------------|------------------------------------|-----------------------------------------------|------------------------------------|-----------------------------------------------|
| A                       | 2                                  | ~2.2                                          | 9                                  | 95                                            |
| B                       | 3                                  | ~0.6                                          | 9                                  | 31                                            |
| C                       | 4                                  | ~0.15                                         | 9                                  | 4.5                                           |

* p.m.s., postmitochondrial supernatant.
Protein synthesis with pH 5 enzymes as the only source of 40 S subunits and initiation factors. Twenty-five microliters of pH 5 enzyme Preparations A, B, and C (Fig. 1) in 0.1-ml incubation mixtures containing 2 μg of globin mRNA and the other components for protein synthesis except ribosomes and initiation factors were supplemented with purified polysome-derived 60 S subunits as indicated on the abscissa. Incubation and sample processing for radioactivity determination was as described under "Methods and Materials;" 1000 cpm = 26 pmoles of leucine incorporated.

Distribution of initiation factor activity of rat liver pH 5 enzymes centrifuged either in low salt or high salt glycerol density gradients. Two 1.5-ml samples of liver pH 5 enzymes (Fig. 1A) were adjusted to the ionic conditions of preparative glycerol gradients containing either low salt (0.03 M KCl, 0.002 M MgCl₂) or high salt conditions (0.5 M KCl, and 0.003 M MgCl₂). The samples were layered onto the 3.5-ml convex gradients (see "Methods and Materials") and centrifuged as described under "Methods and Materials." After centrifugation the optical density was recorded and 0.25-ml fractions were collected. Ten microliters of each fraction was assayed for initiation factor activity with a complete globin-synthesizing system containing polysome-derived subunits and an absolutely ribosome-free pH 5 enzyme preparation. The results from both gradients are plotted into the same figure. Initiation factor activity coincides with the position of the 40 S subunits in the gradient containing 0.03 M KCl. However, in the gradient containing 0.5 M KCl all the initiation factor activity is found on the top of the gradient. The sample had been adjusted to 0.5 M KCl before layering on the high salt gradient. Since all the assays for globin synthesis now contained 40 S-free pH 5 enzymes, we conclude from these experiments that at least one initiation factor occurs at low ionic strength exclusively on the 40 S subunits, while others might be free or subunit-bound, or both. As expected, 0.5 M KCl completely dissociates factor activity from the subunits.

Protein Synthesis Promoted by Free 40 S Subunits is mRNA-Dependent—In Fig. 4 we show the mRNA dependence of protein synthesis by free 40 S ribosome subunits in rat liver pH 5 enzymes supplemented with an equimolar amount of polysome-derived 60 S subunits. Each assay contained about 2 pmoles each of free 40 S subunits and polysome-derived 60 S subunits. Globin messenger RNA was titrated into the system and at saturation about 2.5 pmoles of globin were synthesized per pmole of ribosomes. The strong dependence on, and very high response to exogenous mRNA indicate that the majority of the free 40 S subunits isolated with pH 5 enzymes is at a functional stage of the ribosome cycle prior to mRNA binding.

Experiments with pH 5 Enzyme Preparations Obtained from Rabbit Reticulocytes—In order to substantiate and generalize our findings with free 40 S subunits from rat liver, we did similar experiments with pH 5 enzyme preparations obtained from high speed supernatants of rabbit reticulocytes. Whereas from rat liver only the upper three-fourths of the high speed supernatant
Two 0.5-ml samples of a reticulocyte pH 5 enzyme preparation obtained from a 2-hour centrifuged high speed supernatant were adjusted to the ionic conditions of 4.5-ml preparative glycerol gradients containing either low salt (0.03 M KCl, 0.002 M MgCl₂) or high salt (0.5 M KCl, 0.003 M MgCl₂). The gradients were centrifuged and collected as previously described (Fig. 3). Twenty microliters of each fraction were assayed for initiation factor activity with a complete globin-synthesizing system containing an absolutely ribosome-free pH 5 enzyme preparation. The low salt gradient also was assayed with an incubation mixture without globin messenger. A, low salt gradient. Initiation factor activity in the presence (O- - -O) and absence (△△△) of messenger. B, high salt gradient. Initiation factor activity in the presence of messenger (O- - -O); 1000 cpm = 26 pmoles of leucine incorporated.

Each fraction of the low salt gradient also was assayed for messenger activity using the same system as above but with the omission of globin mRNA. The result demonstrates that in reticulocytes, too, no endogenous mRNA is associated with the free 40 S subunits (Fig. 5A, open triangles).

In the following experiment we tested the activity of the 0.5 M KCl-washed 40 S subunits present in the glycerol gradient shown in Fig. 5B, when supplemented with the washed off initiation factors (gradient Fractions 16 and 17), polysome-derived 60 S subunits, and globin mRNA. The result is shown in Fig. 6. The washed 40 S subunits recovered in the glycerol gradient were indeed active in protein synthesis in the presence of initiation factors recovered from the top of the same gradient. The relatively high incorporation in the absence of 40 S subunits was due to contamination of polysome-derived 40 S subunits in the polysome-derived 60 S subunit preparation used.

**DISCUSSION**

In this study we show for two mammalian cell types of different species the free 40 S ribosome subunit as the carrier of initiation factor activity. Since 40 S-free pH 5 enzymes cannot promote protein synthesis in our system, they must lack at least one component essential for chain initiation. Thus, one or more initiation factor(s) are exclusively bound to the 40 S subunit at the low ionic strength used here.

By *in vitro* reconstitution from polysome-derived 40 S subunits and purified initiation factors we have demonstrated a class of "40 S" subunits carrying the large initiation factor IF-E₄ (16). IF-E₄ could indeed be purified from isolated free 40 S subunits of mouse Krebs II ascites cells and rabbit reticulocytes. This factor seems to be bound exclusively to ribosomes under our conditions of cell disruption and ribosome isolation, whereas other initiation factors are also detectable in the ribosome-free supernatant. The results presented here give strong support to the interpretation of Ayuso-Parilla et al. (17) that at least

1 The abbreviation used is: IF-E₄, initiation factor E₄.
2 I. C. Sundkvist and T. Staehelin, manuscript in preparation.
3 Unpublished results.
some or possibly all of the extra protein they found associated with free 40 S subunits obtained from Ehrlich ascites cells might be due to initiation factors. Very few, if any, free 40 S subunits have been shown to carry mRNA (18-21) but a significant amount seem to have bound initiator Met-tRNAf (18). The 40 S subunits obtained from Ehrlich ascites cells might be due to initiation factors. Very few, if any, free 40 S subunits have been shown to carry mRNA (18-21) but a significant amount seem to have bound initiator Met-tRNAf (18).

To what extent free subunits serve as carrier of initiation factors not directly engaged in initiation cannot be determined for the moment. This problem may be a question of semantics depending on the definition of individual steps of initiation complex formation. As it has been shown, however, that the initiation of protein synthesis occurs on ribosomes drawn from the pool of free or so-called native subunits in the mammalian cell (22, 23), we would suggest that free 40 S subunits represent at least in part intermediates in a stage prior to messenger binding in the process of chain initiation carrying specifically the factors required for their formation. Further evidence for this conclusion will be published elsewhere.2

Another problem of importance is the stability of initiation factor-ribosome complexes. The conditions used for the isolation procedure may determine whether a given initiation factor will be mainly bound to ribosomes and therefore called a “ribosome factor” or dissociated into the ribosome free supernatant and be called a “supernatant factor” or “cytosol factor.” In order to illustrate this problem, which may be a serious source of confusion between different investigators, we extracted protein from a liver microsome fraction with different concentrations of KCl and tested the protein fractions for initiation factor activity in our cell-free system. The results are shown in Fig. 7. Even at 0.1 M KCl significant amounts of the most tightly bound initiation factor(s) were extracted. The extraction was complete at 0.5 M KCl. To what extent the hydrostatic pressure during centrifugation helped dissociate initiation factors from ribosomes at the lower KCl concentrations is not known. However, this experiment at least suggests that even the initiation factor with the highest affinity to ribosomes might be partly free under the ionic conditions of protein synthesis. Although different organisms, tissues, or developmental stages may show differences in the distribution of homologous initiation factors between ribosomes and supernatant under the same fractionation procedure, any classification of initiation factors as either “ribosome factor” or “supernatant factor” should be strictly operational and will be dependent on the experimental conditions.

REFERENCES

1. Davis, B. D. (1971) Nature 231, 153
2. Grünberg-Manago, M., Godeboy-Colburn, T. H., Wolfe, A. D., Draken, P., Pantafo, D., Springer, M., Grapp, M., Dondon, J., and Kay, A. (1973) in 24th Mosbach Colloquium (Bauft, E. F., Karlson, P., and Kersten, H., eds) p. 213, Springer-Verlag, Heidelberg
3. Noll, H., Noll, M., Hafke, B., and van Dieijen, G. (1973) in 24th Mosbach Colloquium (Bauft, E. F., Karlson, P., and Kersten, H., eds) p. 237, Springer-Verlag, Heidelberg
4. Parenti-Rosina, R., Eisenstadt, A., and Eisenstadt, J. M. (1969) Nature 221, 353
5. Eisenstadt, J. M., and Hauwerman, G. (1967) Proc. Nat. Acad. Sci. U. S. A. 66, 1560
6. Miller, M. J., Zaloff, M., and Ochoa, S. (1969) Fed. Eur. Biochem. Soc. Lett. 3, 50
7. Miller, R. L., and Schweet, R. (1968) Arch. Biochem. Biophys. 125, 632
8. Prichard, P. M., Gilbert, J. M., Shafritz, D. A., and Anderson, W. F. (1970) Nature 222, 511
9. Schreiber, M. H., and Starehalin, T. (1975) J. Mol. Biol. 77, 329
10. Zaloff, M., and Ochoa, S. (1971) Proc. Nat. Acad. Sci. U. S. A. 66, 3039
11. Gaisier, E., Rao, P., and Moldave, K. (1971) Biochem. Biophys. Acta 264, 331
12. Falvey, D. P., Krin-Bekhaan, H., Wool, I. G., and Fox, A. (1972) Biochem. Biophys. Res. Commun. 46, 215
13. Leader, D. P., and Wool, I. G. (1973) Biochim. Biophys. Acta 262, 300
14. Falvey, A. K., and Starehalin, T. (1973) J. Mol. Biol. 73, 1
15. Noll, H. (1966) in Techniques in Protein Biosynthesis (Sargent, J., and Campbell, P., eds) Vol. 2, pp. 101-179, Academic Press, London
16. Schreiber, M. H., and Starehalin, T. (1978) Nature New Biol. 242, 35
17. Ayuso-Parilla, M., Henshaw, E. C., and Hirsch, C. A. (1973) J. Biol. Chem. 248, 4386
18. Darnbrough, C., Legon, S., Hunt, T., and Jackson, R. J. (1973) J. Mol. Biol. 76, 379
19. Terada, M., Mefora, S., Banks, J., Dow, L. W., Bank, A., and Marks, P. A. (1972) Biochem. Biophys. Res. Commun. 47, 766
20. Jacobs-Lorena, M., and Baglioni, C. (1972) Proc. Nat. Acad. Sci. U. S. A. 69, 1425
21. Olsen, G. D., Ganzigg, P., and Katt, D. (1972) Biochim. Biophys. Acta 272, 297
22. Howard, G. A., Adamson, S. D., and Herbert, E. (1970) J. Biol. Chem. 245, 6037
23. Henshaw, E. C., Guiney, D. G., and Hirsch, C. A. (1973) J. Biol. Chem. 248, 4367
24. Schreiber, M. H., and Starehalin, T. (1973) Proc. Nat. Acad. Sci. U. S. A. 70, 463
Initiation Factor Activity Associated with Free 40 S Subunits from Rat Liver and Rabbit Reticulocytes
Ingegerd C. Sundkvist, Wallace L. McKeehan, Max H. Schreier and Theophil Staehelin

J. Biol. Chem. 1974, 249:6512-6516.

Access the most updated version of this article at http://www.jbc.org/content/249/20/6512

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/249/20/6512.full.html#ref-list-1