Proceedings of the National Academy of Sciences
Vol. 67, No. 2, pp 523–528, October 1970

A Factor for the Binding of Aminoacyl Transfer RNA to Mammalian 40S Ribosomal Subunits*

David P. Leader, Ira G. Wool, and James J. Castles

DEPARTMENTS OF PHYSIOLOGY, BIOCHEMISTRY, AND MEDICINE, UNIVERSITY OF CHICAGO, CHICAGO, ILLINOIS 60637

Communicated by Dwight J. Ingle, June 22, 1970

Abstract. A factor present in rat liver supernatant catalyzes binding of Phe-tRNA to 40S ribosomal subunits from rat skeletal muscle. This factor could be distinguished from aminoacyltransferase I by a number of criteria: (1) at lower concentrations of magnesium (5 mM) the 40S binding factor was approximately seven times as effective as T-I in catalyzing binding of Phe-tRNA to 40S subunits; (2) the kinetics of the binding reaction were different when catalyzed by the 40S binding factor, in particular the initial rate was greater than in the presence of T-I—indeed, the kinetics of the T-I catalyzed reaction resembled nonenzymic binding; (3) GTP was required for maximal binding of Phe-tRNA to 40S subunits in the presence of the 40S binding factor, but not for the T-I catalyzed reaction; (4) the 40S binding factor was inactivated by *N*-ethylmaleimide whereas T-I was not; (5) finally, the 40S binding factor was more susceptible to heat inactivation. Binding of aminoacyl-tRNA to 40S ribosomal subunits may be a paradigm for the initiation of protein synthesis, and the 40S binding factor may play a role in the process.

A good deal is known of the mechanism for initiation of protein synthesis in prokaryotic cells. In the presence of messenger RNA, having at or near its 5′-end the codons AUG or GUG, an initiation complex is formed between the smaller (30 S) ribosomal subunit, initiation factors, and a specific tRNA—fmet-tRNA$_f$.[1,2] Less is known about the initiation of protein synthesis on ribosomes from eukaryotic cells. The initial tRNA is probably not fMet-tRNA$_f$[3,4]—it may be nonformylated Met-tRNA$_f$[5,6]—or possibly an acetylated aminoacyl-tRNA.[7,8] Proteins removed from ribosomes by washing with high concentrations of monovalent cations (salt-wash factors), and distinct from aminoacyltransferase I (T-I) and aminoacyltransferase II (T-II), are required for the *de novo* synthesis of hemoglobin on reticulocyte ribosomes *in vitro*.[9,10] However, the precise reactions catalyzed by the salt-wash factors have not been determined.

The likelihood that initiation in animal cells also takes place on the smaller (40 S) ribosomal subunit[11] prompted us to investigate the binding of aminoacyl-tRNA to this subunit. We report here that a factor present in rat liver supernatant—and apparently distinct from T-I—catalyzes the binding of Phe-tRNA to the 40S subunit.

Materials and Methods. The following have been described before: the source of materials and the animals used;[12] the preparation of rat skeletal muscle ribosomes;[13]

the preparation of ribosomal subunits and their reassociation.[14] Unfractionated *E. coli* B tRNA was acylated with [^{3}H]phenylalanine (5 Ci/mol) and nineteen nonradioactive amino acids,[12] and was passed through a column of Sephadex G-25 before use to remove contaminating nucleoside triphosphates.[15]

Preparation of enzyme fractions: The procedure was according to Schneir and Moldave.[16] In summary, the steps were: precipitation of the 100,000 $\times$ *g* supernatant from rat liver at pH 5.2; neutralization of the supernatant and filtration through Sephadex G-25 (the eluate is referred to as "G-25"); adsorption of the G-25 on hydroxylapatite and elution with increasing concentrations of potassium phosphate buffer; and fractionation of the 0.25 M phosphate eluate on Sephadex G-200 to give purified T-I, free from T-II.

Assay of binding of Phe-tRNA: The procedure of Nirenberg and Leder[17] was followed. The complete reaction mixture (0.1 ml) contained, in addition to enzyme fractions and ribosomes or ribosome subunits, 2.5 μmol Tris·HCl (pH 7.5), 8 μmol KCl, 0.05 μmol GTP, 1 μmol β-mercaptoethanol, 10 μg polyuridylic acid (poly U), 40 μg aminoacyl-tRNA (containing 25,000 cpm [^{3}H]phenylalanine) and the amount of $MgCl_2$ indicated. Variations from this protocol are noted in the text. The reaction was started by the addition of ribosomes or ribosomal subunits and incubation was for 15 min at 30°C unless stated otherwise. Bound [^{3}H]Phe-tRNA was collected on nitrocellulose filters and counted with 16% efficiency.[18]

***N*-ethylmaleimide treatment of enzyme fractions:** The enzyme fractions were incubated at 37°C for 5 min with 1 mM β-mercaptoethanol, then incubated in 0.01 M *N*-ethylmaleimide (NEM) for 10 min at 0°C. Unreacted NEM was removed by the addition of β-mercaptoethanol to a final concentration of 0.02 M.[19]

Results. Purified liver T-I stimulated the binding of Phe-tRNA to preparations of muscle ribosomes (referred to as 80S ribosomes for convenience—although they also contain polysomes) and to recombined 40S and 60S ribosomal subunits (Fig. 1). (Recombined subunits bind greater amounts of Phe-tRNA because they do not contain endogenous template, and hence bind more poly U.) We were surprised, however, to find that even with saturating amounts of T-I there was very little binding of Phe-tRNA to 40S subunits at lower concentrations of magnesium (5–7 mM)—Fig. 2—even though there was good binding to ribosomes at the same concentrations of Mg^{++} (compare binding at 5 mM $MgCl_2$ in Figs. 1 and 2).

Enzyme fractions from earlier stages of the purification of T-I did stimulate the binding of Phe-tRNA to 40S subunits at low concentrations of magnesium. Thus, with G-25, binding was substantial at 5 mM magnesium, and was maximal at 10 mM, whereas binding catalyzed by T-I, like the nonenzymic binding, was only significant at high concentrations of magnesium (Fig. 2). The [^{14}C]Phe-tRNA bound to the 40S subunit in the presence of the G-25 fraction was hydrolyzed in alkali;[18] all of the radioactivity then cochromatographed with phenylalanine—no diphenylalanine or other peptides were formed (results not shown).

These observations suggested to us that the G-25 fraction contained a factor necessary for the binding of aminoacyl-tRNA to the 40S subunit and that the factor was not T-I. In fact, all of the fractions in the purification of T-I before the Sephadex G-200 step were able to stimulate binding of Phe-tRNA to 40S subunits at low concentrations of magnesium. It seems that the binding factor is lost during the Sephadex G-200 filtration. Preliminary attempts to prevent loss of activity were unsuccessful; therefore, we used the G-25 fraction and sought to characterize the 40S binding activity of the factor.

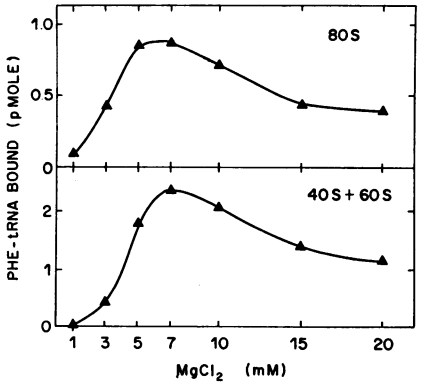

FIG. 1. Effect of magnesium concentration on binding of Phe-tRNA to ribosomes and recombined ribosomal subunits. Ribosomes (7.5 μg of ribosomal RNA) or recombined ribosomal subunits (40S, 2.14 μg of ribosomal RNA; 60S, 5.36 μg of ribosomal RNA) were incubated with T-I (25 μg) and various amounts of $MgCl_2$ in the reaction mixture described in *Materials and Methods*.

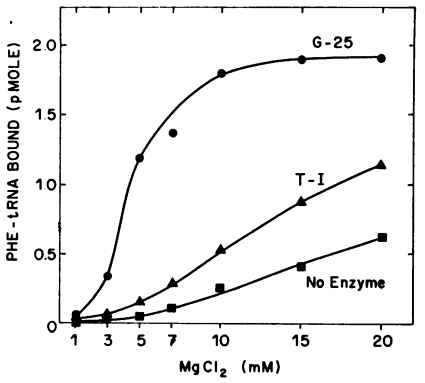

FIG. 2. Effect of magnesium concentration on binding of Phe-tRNA to 40S ribosomal subunits. 40S subunits (2.14 μg of ribosomal RNA) were incubated with various amounts of $MgCl_2$ and G-25 (250 μg) or T-I (25 μg) in the reaction mixture described in *Materials and Methods*.

Binding of Phe-tRNA to 40S subunits was dependent on poly U (Table 1). The binding catalyzed by G-25 appeared to be slightly stimulated by β-mercaptoethanol (greater stimulation has been observed in other experiments). However, it is very striking that binding to 40S subunits dependent on G-25 was stimulated by GTP whereas the binding dependent on T-I, like the nonenzymic binding, was not. The T-I catalyzed binding of aminoacyl-tRNA to 80S ribosomes is GTP dependent.[20–22]

The kinetics of the binding reaction catalyzed by G-25 were distinct from those with T-I (Fig. 3); the initial rate was much greater with G-25. The time course of binding with T-I was similar to that for nonenzymic binding and suggested a less specific association with the 40S subunit. (The decline in the binding catalyzed by G-25 after 15 min is probably due to deacylation of [^{3}H]Phe-tRNA.[23])

TABLE 1. *Characteristics of binding of Phe-tRNA to 40S ribosomal subunits.*

	Phe-tRNA bound (pmol)			
Incubation condition	G-25 (5 mM $MgCl_2$)	G-25 (10 mM $MgCl_2$)	T-I (10 mM $MgCl_2$)	No enzyme (10 mM $MgCl_2$)
Complete	0.99	1.51	0.44	0.26
− Poly U	0.06	0.05	0.03	0.03
− GTP	0.45	0.66	0.50	0.28
− β-mercaptoethanol	0.82	1.31	0.47	0.24

The assay was performed with 40S subunits (2.14 μg of ribosomal RNA) and G-25 (250 μg) or T-I (25 μg) in the reaction mixture described in *Materials and Methods* and at the $MgCl_2$ concentration shown. For this experiment, the subunits were dialyzed against buffer from which β-mercaptoethanol had been omitted.

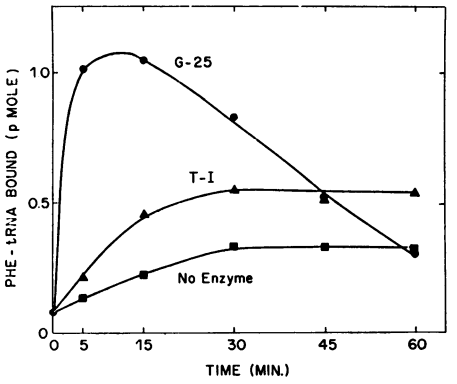

FIG. 3. Effect of incubation time on binding of Phe-tRNA to 40S ribosomal subunits. 40S subunits (2.14 μg of ribosomal RNA) were incubated at 10 mM $MgCl_2$ with G-25 (150 μg) or T-I (25 μg) in the reaction mixture described in *Materials and Methods*.

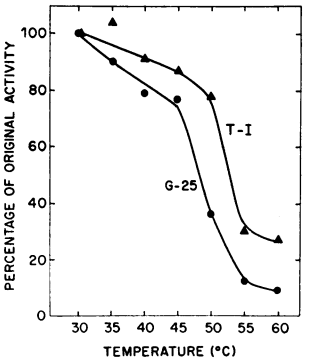

FIG. 4. Heat inactivation of different enzyme fractions. Enzyme fractions were heated for 5 min at the temperature shown and then incubated with 40S subunits (2.14 μg of ribosomal RNA) in the reaction mixture described in *Materials and Methods*. For the binding assay G-25 (250 μg) was incubated at 5 mM $MgCl_2$, T-I (25 μg) at 10 mM $MgCl_2$.

As we suspected that the 40S binding factor was inactivated in the last stage of the preparation of T-I, we compared the heat inactivation of G-25 and T-I (Fig. 4). Indeed, G-25 was inactivated at a lower temperature than T-I. The (necessarily) different protein concentrations of the two fractions (we used 250 μg of G-25 and 25 μg of T-I) makes a precise interpretation of this result difficult. However, heat inactivation of enzymes normally decreases with increased protein concentration; since G-25 was present in a higher concentration and was inactivated at a lower temperature, the results suggest that a factor, more labile than T-I, is present in the G-25 fraction.

Treatment of T-I with NEM did not affect the ability of the enzyme to direct binding of Phe-tRNA to 40S subunits (Table 2); NEM does not alter T-I

TABLE 2. *Effect of N-ethylmaleimide treatment of enzyme fractions on their ability to catalyze binding of Phe-tRNA to 40S ribosomal subunits.*

	Phe-tRNA bound (pmol)		
Enzyme fraction	Untreated enzyme	NEM-treated enzyme	No enzyme
G-25 (5 mM $MgCl_2$)	1.21	0.04	0.13
T-I (10 mM $MgCl_2$)	0.51	0.48	0.25

The assay was performed with 40S ribosomal subunits (2.14 μg of ribosomal RNA) and G-25 (250 μg) or T-I (25 μg) in the reaction mixture described in *Materials and Methods* and at the $MgCl_2$ concentration shown.

catalyzed binding to 80S ribosomes either.[19] However, G-25 catalyzed binding was abolished by NEM. (T-I was assayed at 10 mM magnesium, whereas G-25 was assayed at 5 mM because it contains T-I.) Aminoacyltransferase II which is present in G-25, is also inactivated by NEM.[19] However, preparations of purified T-II did not catalyze the binding of Phe-tRNA to 40S subunits (results not shown).

Discussion. A factor present in rat liver supernatant catalyzes binding of Phe-tRNA to 40S ribosomal subunits. We cannot be certain of the nature or function of the factor, which is still quite crude, until it has been separated from T-I and purified. However, the activity of the 40S binding factor can be distinguished from that of T-I by a number of characteristics: (1) at lower concentrations of magnesium (5 mM) the binding factor is approximately seven times as effective as T-I in catalyzing binding of Phe-tRNA to 40S subunits; (2) the kinetics of the binding reaction are different when catalyzed by the 40S binding factor, in particular the initial rate is greater than in the presence of T-I—indeed, the kinetics of the T-I catalyzed reaction resemble nonenzymic binding; (3) GTP is required for maximal binding of Phe-tRNA to 40S subunits in the presence of the factor but not for the T-I catalyzed reaction; (4) the 40S binding factor is inactivated by NEM, whereas T-I is not; (5) finally, the 40S factor is more susceptible to heat inactivation.

Rao and Moldave have reported that T-I binds to liver 40S subunits in the presence of Phe-tRNA—this result implied that T-I catalyzed binding of Phe-tRNA had occurred.[24] Our results are not inconsistent with theirs, since in their experiments GTP was not required and the magnesium concentration was 10 mM.[24] We found that T-I did in fact catalyze binding of Phe-tRNA to 40S subunits in the conditions they used, but in the presence of GTP the reaction catalyzed by the 40S binding factor was far more efficient and, what is more, binding was substantial in 5 mM magnesium.

The binding of Phe-tRNA to 40S ribosomal subunits in the assay we have used may be a paradigm for the formation of the initiation complex *in vivo;* the assay should then be useful in identification and in determination of the mode of action of initiation factors for eukaryotic ribosomes. The 40S binding factor whose characteristics we have described may be one such initiation factor.

* The expenses of the research were met by grants from the National Institutes of Health (AM-04842 and AM-13807) and the John A. Hartford Foundation. D. P. L. is the recipient of a Fulbright-Hays Travel Grant. J. J. C. is the recipient of a U.S. Public Health Service Research Career Development Award.

Abbreviations: NEM, *N*-ethylmaleimide; Poly U, polyuridylic acid; G-25, the eluate from a Sephadex G-25 column (a partially purified preparation of aminoacyltransferase I).

[1] Stanley, W. M., M. Salas, A. J. Wahba, and S. Ochoa, *Proc. Nat. Acad. Sci. USA*, **56**, 290 (1966).
[2] Nomura, M., and C. V. Lowry, *Proc. Nat. Acad. Sci. USA*, **58**, 946 (1967).
[3] Smith, A. E., and K. A. Marcker, *J. Mol. Biol.*, **38**, 241 (1968).
[4] Marcker, K. A., and A. E. Smith, *Bull. Soc. Chim. Biol.*, **51**, 1453 (1969).
[5] Smith, A. E., and K. A. Marcker, *Nature*, **226**, 607 (1970).
[6] Brown, J. C., and A. E. Smith, *Nature*, **226**, 610 (1970).
[7] Laycock, D. G., and J. A. Hunt, *Nature*, **221**, 1118 (1969).
[8] Liew, C. C., G. W. Haslett, and V. G. Allfrey, *Nature*, **226**, 414 (1970).
[9] Miller, R. L., and R. Schweet, *Arch. Biochem. Biophys.*, **125**, 632 (1968).
[10] Prichard, P. M., J. M. Gilbert, D. A. Shafritz, and W. F. Anderson, *Nature*, **226**, 511 (1970).
[11] Kaempfer, R., *Nature*, **222**, 950 (1969).
[12] Wool, I. G., and P. Cavicchi, *Biochemistry*, **6**, 1231 (1967).
[13] Martin, T. E., F. S. Rolleston, R. B. Low, and I. G. Wool, *J. Mol. Biol.*, **43**, 135 (1969).
[14] Martin, T. E., and I. G. Wool, *Proc. Nat. Acad. Sci. USA*, **60**, 569 (1968).

[15] Ravel, J. M., R. D. Mosteller, and B. Hardesty, *Proc. Nat. Acad. Sci. USA*, **56**, 701 (1967).
[16] Schneir, M., and K. Moldave, *Biochim. Biophys. Acta*, **166**, 58 (1968).
[17] Nirenberg, M., and P. Leder, *Science*, **145**, 1399 (1964).
[18] Castles, J. J., and I. G. Wool, *Biochemistry*, **9**, 1909 (1970).
[19] McKeehan, W. L., and B. Hardesty, *J. Biol. Chem.*, **244**, 4330 (1969).
[20] Ibuki, F., and K. Moldave, *J. Biol. Chem.*, **243**, 791 (1968).
[21] Lin, S-Y, W. L. McKeehan, W. Culp, and B. Hardesty, *J. Biol. Chem.*, **244**, 4340 (1969).
[22] We have found that T-I catalyzed binding of aminoacyl-tRNA to recombined subunits is also GTP dependent (unpublished work).
[23] Pestka, S., *J. Biol. Chem.*, **242**, 4939 (1967).
[24] Rao, P., and K. Moldave, *J. Mol. Biol.*, **46**, 447 (1969).