ISOLATION OF PROTEIN SYNTHESIS INITIATION FACTOR MP FROM THE HIGH-SPEED SUPERNATANT FRACTION OF WHEAT GERM

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SUMMARY

A protein which forms a ternary complex with GTP and Met-$tRNA_i$ has been partially purified from a high-speed supernatant fraction of wheat germ. This protein has properties similar to eukaryotic initiation factor MP (or IF-I) which is present in high salt washes of ribosomes from animal tissues. Ternary complex formation is optimal at low magnesium ion concentration, but diminishes as the magnesium ion concentration is increased. The factor is not specific for GTP, since a ternary complex is also formed with dGTP, CTP and UTP. At constant GTP and Met-$tRNA_i$ concentrations the amount of ternary complex formed is linear with respect to factor concentration, except at very low concentrations. Wheat germ factor is inhibited by N-ethylmaleimide and is inactivated by heating.

Work on the mechanism of initiation of protein synthesis in eukaryotic cells has led to the isolation of a number of factors which are thought to be required for the formation of initiation complexes (1-6). One of these factors which has been termed IF-MP (5) or IF-I (2, 4) forms a ternary complex with GTP and Met-$tRNA_i$*. The involvement of this factor in the initiation process was inferred from the finding that complex formation is specific for Met-$tRNA_i$. Three observations that pro-

*Abbreviations: Met-$tRNA_i$ is methionyl initiator tRNA, HEPES is N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid, EF-1 and EF-2 are eukaryotic elongation factors 1 and 2.

vide more direct evidence are 1) Met-tRNA$_i$ in a ternary complex is more efficiently incorporated into peptides than free Met-tRNA$_i$ (4, 7), 2) Met-tRNA$_i$ bound to 40S ribosomes from the ternary complex in the presence of AUG yields methionyl puromycin upon the addition of 60S ribosomes and puromycin (2, 4), and 3) addition of the factor restores protein synthesis to a reticulocyte system which has been inactivated by an inhibitor formed during incubation in the absence of hemin (8, 9).

Previous work concerned with IF-MP was done with factor isolated from 0.5 M KCl washes of ribosomes from a variety of eukaryotic sources. In the course of our work on protein synthesis in a cell-free system from wheat germ we found that the high-speed supernatant fraction contains a protein which is probably identical to IF-MP. In this communication we report properties of this factor isolated from wheat germ supernatant.

MATERIALS AND METHODS

Commercially available reagents were purchased from standard sources. Wheat germ was obtained from Dixie Portland Co., Arkansas City, Kansas; tRNA was isolated from wheat germ and purified through the DEAE-cellulose step as described by Allende (10) and was charged with [^{35}S]methionine using either Escherichia coli or wheat germ supernatant by a modification of the procedure described by Ghosh et al. (11). Specific radioactivity of the charged tRNA was 32, 000 cpm/pmole.

Wheat germ high-speed supernatant was made by centrifuging a wheat germ homogenate prepared in buffer (100 mM KCl, 20 mM HEPES pH 7.56, 5 mM $Mg(Ac)_2$, 10 mM 2-mercaptoethanol, 0.1 mM EDTA) at 15, 000 x g for 20 min and then at 105, 000 x g for 4 hr. The supernatant was treated batchwise with DEAE-cellulose, fractionated (40-65% saturation) with ammonium sulfate, and chromatographed on CM-Sephadex C-50. Further purification on DEAE-Sephadex A-50 yields a factor which will bind 120 pmole/mg protein in the standard assay described below. Experiments described in this communication were done with preparations which had been purified through the DEAE-Sephadex step.

The assay for IF-MP is based upon the GTP-dependent retention of [^{35}S]Met-tRNA$_i$ to millipore filters. The assay mixture contains in a final volume of 0.05 ml, 100 mM KCl, 20 mM HEPES, pH 7.56, 2 mM 2-mercaptoethanol, 0.2 mM GTP, 3 pmoles of [^{35}S]Met-tRNA$_i$, and

3 to 25 μg of factor depending on the stage of purification. After the samples are incubated for 10 min at 25° they are diluted by the addition of 1 ml of cold buffer containing 100 mM KCl, 20 mM HEPES, pH 7.56, and 2 mM 2-mercaptoethanol. The diluted samples are immediately filtered on nitrocellulose membranes, washed with two 2-ml portions of of dilution buffer, dried, and counted in a liquid scintillation counter.

RESULTS

The effect of magnesium ion concentration on ternary complex formation is shown in Table 1. In agreement with the results obtained with crude IF-MP from reticulocyte ribosomes (12) less complex is detected at increasing magnesium ion concentrations. Treatment of the wheat germ factor with 10 mM N-ethylmaleimide for 10 min at 25° results in complete loss of activity. Furthermore, about 50% of its acti-

Table 1

Effect of Magnesium Ion Concentration on Ternary Complex Formation

Mg^{++} (mM)	GTP (0.2 mM)	$[^{35}S]$Met-$tRNA_i$ bound (cpm)
0	+	26,000
0	-	1,200
1	+	13,300
1	-	300
3	+	4,900
3	-	320
5	+	4,000
5	-	310

The assays were performed as described in MATERIALS AND METHODS except that magnesium acetate and GTP concentrations were varied as indicated. Each tube contained 6.8 μg of IF-MP.

vity is lost when it is heated at 45° for 10 min at protein concentrations used in the assay (Table 2).

The effect of IF-MP concentration on binding [^{35}S] Met-tRNA$_i$ is shown in Fig. 1. At low protein concentrations little binding occurs; however, when the amount of protein in the assay reaches a critical limit, about 1.2 μg, there is a sudden increase in the binding of Met-tRNA$_i$. This observation is consistent with the occurrence of a protein-protein association effect.

Table 2

Inactivation of Wheat Germ IF-MP by Heat and N-Ethylmaleimide

Treatment	[^{35}S] Met-tRNA bound (cpm)
None	24, 000
Heat[a]	12, 200
N-Ethylmaleimide[b]	2, 800
N-Ethylmaleimide + GTP[c]	2, 500

The standard IF-MP assay as described in MATERIALS AND METHODS was used. a) An assay mixture containing 6.8 μg factor, but from which GTP and [^{35}S] Met-tRNA were omitted, was heated in a water bath at 45° for 10 min and immediately chilled in ice. The amount of ternary complex formed upon the addition of GTP and [^{35}S] Met-tRNA was determined by filtration on millipore filters as described. b) IF-MP (68 μg) in 70 μl of buffer (100 mM KCl and 20 mM HEPES, pH 7.56) was incubated with 10 mM N-ethylmaleimide for 10 min at 25°, dithiothreitol was added to a final concentration of 50 mM, and the tube was chilled in ice. The standard assay was performed using 6.8 μg of the treated IF-MP. c) Experimental conditions were the same as in b except that 0.2 mM GTP was present during the reaction with N-ethylmaleimide.

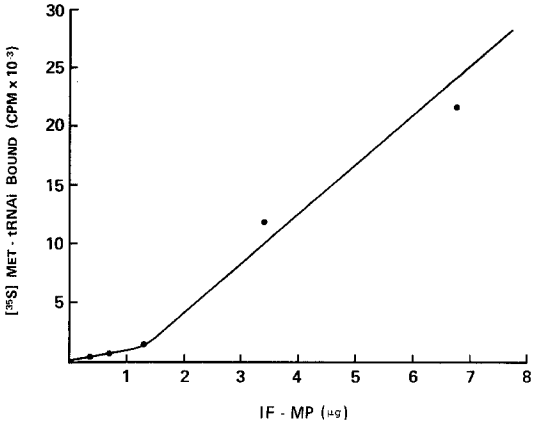

Fig. 1. Dependence of ternary complex formation upon IF-MP concentration. The binding assay was performed as described in MATERIALS AND METHODS with the amounts of IF-MP indicated.

The nucleotide specificity for ternary complex formation is shown in Table 3. $[^{35}S]$Met-tRNA$_i$ is bound to IF-MP with GTP, dGTP, UTP and CTP, but no complex is formed with ATP, dATP, dCTP, dTTP and GDP.

DISCUSSION

If initiation factor MP functions physiologically in a cyclic manner by attachment and release from ribosomes or ribosomal complexes during the initiation process, one would expect to find it in the supernatant as well as in ribosomal washes. Purification of wheat germ high-speed supernatant has revealed the presence of a factor which appears to be functionally identical to IF-MP isolated from ribosomal washes prepared from other tissues. We have not determined the presence of IF-MP in ribosomal extracts of wheat germ but believe it is undoubtedly present there.

Table 3

Nucleotide Specificity for Ternary Complex Formation

Nucleotide	[^{35}S]Met-tRNA bound (cpm)
GTP	24,222
dGTP	22,600
UTP	22,032
CTP	15,647
ATP	3,400
dATP	3,325
dCTP	4,200
dTTP	4,800
GDP	3,100
None	3,200

Incubations were as described in MATERIALS AND METHODS for the standard assay. All nucleotides were at 0.2 mM, and 6.8 μg of factor was present in each tube.

Although the data are not given we have found that our preparation is free of Met-tRNA ligase, EF-1 and EF-2. The presence of these proteins could obscure the interpretation of our results. Eukaryotic initiation factor-1 which catalyzes a GTP-independent, message-dependent binding of aminoacyl-tRNA to 40S ribosomes is present in the IF-MP preparation used in the work reported here, but the two activities differ in heat sensitivity and can be separated from each other by gradient

centrifugation. The two activities, EIF-1 and IF-MP have comparable chromatographic properties on the ion exchangers used for their purification.

The sharp break in the IF-MP concentration curve in Fig. 1 may be the result of a concentration-dependent association of IF-MP monomeric or protomeric subunits to form a protein complex which is able to bind GTP and Met-tRNA$_i$. Further purification of the factor will be needed before one can determine what this sharp break in the concentration curve means.

Since GTP is generally believed to be required for initiation of protein synthesis, we were surprised to find that wheat germ IF-MP does not have an absolute specificity for the nucleoside triphosphate in forming the ternary complex. It will be of interest to discover whether ternary complexes formed with dGTP, CTP, and UTP are able to transfer their Met-tRNA$_i$ to 40S ribosomes. An extensive study of aminoacyl-tRNA specificity has not been made, but Phe-tRNA and acPhe-tRNA (both E. coli) do not form complexes with IF-MP and GTP. The addition of E. coli Met-tRNA to the standard assay does not diminish complex formation with wheat germ [^{35}S]Met-tRNA indicating that IF-MP is specific for the eukaryotic Met-tRNA. IF-MP is probably specific for Met-tRNA$_i$ since a ternary complex is formed with wheat germ tRNA which has been charged with [^{35}S] methionine by E. coli supernatant. E. coli supernatant is known (11) to charge only the initiator species of wheat germ tRNA^{Met}.

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