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AN ARTEMIA SALINA FACTOR WHICH COUNTERACTS THE mRNA-INDUCED INHIBITION OF INITIATOR Met-tRNA BINDING TO INITIATION FACTOR eIF-2

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SUMMARY: Ternary complex formation between eukaryotic initiation factor 2 (eIF-2), initiator Met-tRNA and guanosine 5'-(β, γ-imino) triphosphate [GMP-P(NH)P] is strongly inhibited by mRNA in the Artemia salina system. Developing A. salina embryos contain a factor which displays a novel activity, namely the ability to counteract the mRNA-induced inhibition of ternary complex formation. This factor is heat-labile. It is proposed that the factor may play an important role in protein biosynthesis by preventing mRNA from inhibiting an early step of peptide chain initiation.

INTRODUCTION

The eukaryotic protein synthesis initiation factor, which makes a ternary complex with GTP (or non-hydrolyzable GTP analogs) and initiator Met-tRNA ($Met\text{-}tRNA_i$)* is referred to as eIF-2 according to the nomenclature in (1). It is currently believed that formation of this ternary complex is an early and key step in eukaryotic peptide chain initiation (see refs. 2, 3 for reviews). Recent studies have shown that mRNA strongly inhibits ternary complex formation in the reticulocyte system by binding to eIF-2 (4, 5). This binding results in the dissociation of eIF-2 into its component subunits (5). The ability of mRNA to block ternary complex formation (and thereby peptide chain initiation) raises the interesting question of whether mechanisms exist in the cell to prevent this

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*Abbreviations: $Met\text{-}tRNA_i$, initiator Met-tRNA; GMP-P(NH)P, guanosine 5'-(β, γ-imino) triphosphate; MCF, messenger RNA-inhibition counteracting factor; eIF-2, eukaryotic initiation factor 2.

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inhibitory effect of mRNA. We demonstrate in this paper that developing A. salina embryos contain a heat-labile factor (MCF) which exhibits a novel activity, namely the ability to counteract the mRNA-induced inhibition of ternary complex formation. MCF might play an important role in protein biosynthesis by preventing mRNA from inhibiting an early step of peptide chain initiation.

MATERIALS AND METHODS

A. salina embryos (San Francisco Bay brine shrimp eggs distributed by Metaframe Corporation) were purchased from Aquarium Stock Company, New York City. MCF and eIF-2 were prepared by the following procedure. Development of A. salina embryos and preparation of 35-80% $(NH_4)_2SO_4$ fraction from high-salt ribosomal washes of developing embryos were carried out essentially as in (6). The $(NH_4)_2SO_4$ fraction was dialyzed against buffer (buffer A) containing 20 mM Tris-HCl, pH 7.1, 100 mM KCl, 0.2 mM dithiothreitol and 10% (v/v) glycerol for 6 hours with change of buffer after every 1 1/2 hours. The dialyzed $(NH_4)_2SO_4$ fraction was applied to a CM-Sephadex column (Pharmacia C-50) pre-equilibrated with buffer A. The column was then washed with buffer A. The material not retained by CM-Sephadex contained MCF. It was stored at -70°C in small aliquots. eIF-2 was eluted from the CM-Sephadex column by washing directly with buffer A containing 300 mM KCl (6). Fractions containing eIF-2 of highest specific activity were pooled and kept frozen at -70°C. After thawing, the KCl concentration of the pooled solution was adjusted to approximately 50 mM by diluting 6-fold with buffer (buffer B) containing 20 mM Tris-HCl, pH 7.4, 0.2 mM dithiothreitol and 10% (v/v) glycerol. The diluted solution was immediately loaded onto a DEAE-cellulose column (Whatman DE-52) pre-equilibrated with buffer B containing 50 mM KCl. The column was washed stepwise with buffer B containing 50 mM, 70 mM and 100 mM KCl, respectively. Most of the eIF-2 activity was present in fractions eluted with buffer B containing 100 mM KCl (6). Fractions containing eIF-2 of highest specific activity were pooled and stored in small aliquots at -70°C. This procedure results in about a 100-fold purification of eIF-2.

Poly (A) rich mRNA was isolated from developing A. salina embryos essentially as described by Grosfeld and Littauer (7). eIF-2 was assayed by retention of ternary complexes on millipore filters essentially as in (8). GMP-P(NH)P (rather than GTP) has been used in the present study since higher amounts of ternary complex are formed in the A. salina system with GMP-P(NH)P (8, 9). Furthermore, possible inhibition of ternary complex formation by GDP (6) is avoided when the non-hydrolyzable analog GMP-P(NH)P is used instead of GTP. CO-eIF-2 activity was assayed by measuring its stimulatory effect on ternary complex formation in the presence of saturating concentrations of albumin (9). Protein concentrations were measured by ultraviolet absorption (10). Bovine serum albumin was a product of Sigma. Unfractionated rabbit liver tRNA and unfractionated calf liver tRNA were obtained from Biogenics and Boehringer, respectively. Other materials and methods were the same as described previously (6, 8).

Table 1

Effect of Different Concentrations of A. salina mRNA on Ternary Complex Formation

mRNA addition (A_{260} unit)	$[^{35}S]$Met-tRNA$_i$ bound (cpm)	Relative Activity (%)	Inhibition by mRNA (%)
None	3923	100	-
0.0025	3299	84	16
0.005	1313	33	67
0.010	566	14	86
0.025	262	7	93

Stepwise incubations were conducted as follows.
Step I: Reaction mixtures (0.09 ml) contained 22 mM Tris-HCl buffer, pH 7.4, 1 mM dithiothreitol, 3.3% (v/v) glycerol, 110 mM KCl, 60 μg bovine serum albumin, 0.5 μg eIF-2 and indicated amounts of A. salina mRNA (when added). Incubation, 5 min. at 30°C.
Step II (final volume, 0.1 ml): GMP-P(NH)P (final concentration, 0.2 mM) followed by rabbit liver $[^{35}S]$Met-tRNA$_i$ (1.06 pmol; 22790 cpm/pmol) were added to all tubes. Incubation, 5 min. at 30°C. 2 ml of ice-cold buffer (filtration buffer) containing 20 mM Tris-HCl, pH 7.4 and 100 mM KCl was then added and the diluted reaction mixtures were passed through millipore filters. The filters were then washed three times with 2 ml of ice-cold filtration buffer. The filters were dried and counted in a Beckman Liquid Scintillation Counter.

RESULTS

The effect of different concentrations of A. salina mRNA on the activity of A. salina eIF-2 is shown in table 1. The results indicate that A. salina mRNA is a highly potent inhibitor of ternary complex formation. In this experiment, over 80% inhibition was observed at an input of 0.01 A_{260} unit[a] of mRNA.

The results given in table 2 show that the severe inhibition of ternary complex formation induced by mRNA (samples 1, 2) is almost completely reversed

[a] A_{260} unit: the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 260 nm, when measured in a 1-cm path-length cell.

Table 2

Reversal of mRNA-Induced Inhibition of Ternary Complex Formation by MCF

Sample	Additions	[^{35}S]Met-tRNA$_i$ bound (cpm)	Relative Activity (%)
1	eIF-2	2826	100
2	eIF-2 + mRNA	445	16
3	eIF-2 + MCF	6208	100
4	eIF-2 + MCF + mRNA	5762	93
5	eIF-2 + MCF + mRNA (MCF added after step I incubation)	5316	86

Conditions similar to those of table 1. A. salina mRNA (0.008 A_{260} unit) was added at step I to samples 2, 4 and 5. MCF (13.5 μg) was added to samples 3, 4 at step I and to sample 5 at step II (before GMP-P(NH)P and [^{35}S]Met-tRNA$_i$). The values given in the table are average of duplicate samples and are net values, i.e., minus eIF-2 blanks have been subtracted where applicable. These blank values (cpm) averaged 2190 (MCF alone; blank for sample 3) and 1277 (MCF plus mRNA; blank for samples 4, 5). The specific radioactivity of rabbit liver [^{35}S]Met-tRNA$_i$ was 22525 cpm/pmol.

by the addition of MCF (samples 3, 4). Interestingly, the extent of this reversal is nearly the same even when MCF is added after the first (step 1) incubation in which eIF-2 was allowed to interact with mRNA. It should be noted that the MCF preparation has CO-eIF-2 activity (9, 11, 12), i.e., it stimulates complex formation (samples 1, 3).

The effect of heating on MCF activity is shown in table 3. It may be seen that the ability of the factor to counteract the inhibitory effect of mRNA is almost completely destroyed by heating at 65° for 10 min (samples 4, 6). In contrast, the CO-eIF-2 activity present in the MCF preparation is heat-stable (samples 3, 5).

Table 3

Effect of Heating on MCF Activity

Sample	Additions	[^{35}S] Met-tRNA$_i$ bound (cpm)	Relative Activity (%)
1	eIF-2	3038	100
2	eIF-2 + mRNA	764	25
3	eIF-2 + MCF	7273	100
4	eIF-2 + MCF + mRNA	6245	86
5	eIF-2 + Heated MCF	7717	100
6	eIF-2 + Heated MCF + mRNA	2310	30

Conditions similar to those of table 1. A. salina mRNA (0.01 A_{260} unit) was added at step I to samples 2, 4, 6. MCF (15 μg) was added at step I to samples 3, 4. MCF was heated at 65° for 10 min, the precipitate removed by centrifugation and 15 μg of the supernatant solution (heated MCF) was added at step I to samples 5, 6. The values given in the table are average of duplicate samples and are net values, i.e., minus eIF-2 blanks have been subtracted where applicable. These blank values (cpm) averaged 1840 (MCF alone; blank for sample 3), 921 (MCF plus mRNA; blank for sample 4), 520 (heated MCF alone; blank for sample 5) and 200 (heated MCF plus mRNA; blank for sample 6). The specific radioactivity of calf liver [^{35}S]Met-tRNA$_i$ was 28000 cpm/pmol. A different batch of MCF was used in this experiment.

The reversal of the mRNA-induced inhibition of ternary complex formation by MCF is also observed in the presence of added Mg^{2+} (data not shown).

DISCUSSION

The present study shows that, as in the case of the rabbit reticulocyte system (4, 5), ternary complex formation is strongly inhibited by mRNA in the A. salina system also (table 1). This inhibitory effect is puzzling and potentially harmful since an essential step in peptide chain initiation (i.e., ternary complex formation) is blocked by mRNA, an essential component of protein biosynthesis. The discovery of MCF in A. salina embryos (table 2) is, therefore,

significant and suggests that this factor might play an important role in protein biosynthesis by preventing mRNA from inhibiting ternary complex formation and peptide chain initiation. Curiously, there is little inhibition of complex formation even when eIF-2 is allowed to interact with mRNA in the absence of MCF and the latter factor is added subsequently at the stage of complex formation (table 2). If the assumption is made that mRNA causes dissociation of eIF-2 into subunits in the A. salina system as reported for the reticulocyte system (5), the above result suggests that the mRNA-induced dissociation of eIF-2 may also be reversed by MCF. It is presently not clear whether MCF functions by binding to mRNA or eIF-2. However, MCF appears to be distinct from CO-eIF-2 (9, 11, 12), since most of the MCF activity is destroyed by heating while CO-eIF-2 activity is stable under these conditions (table 3). Further purification of MCF is necessary for a detailed study of its mechanism of action.

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REFERENCES

1. Anderson, W.F., Bosch, L., Cohn, W.E., Lodish, H., Merrick, W.C., Weissbach, H., Wittman, H.G. and Wool, I.G. (1977) FEBS Lett. 76, 1-10.
2. Mazumder, R. and Szer, W. (1977) in Comprehensive Biochemistry (Florkin, M., Neuberger, A. and Van Deenen, L.L.M., eds) vol. 24, pp. 185-234, Elsevier, Amsterdam.
3. Weissbach, H. and Ochoa, S. (1976) Ann. Rev. Biochem. 45, 191-216.
4. Kaempfer, R., Rosen, H. and Israeli, R. (1978) Proc. Natl. Acad. Sci. USA 75, 650-654.
5. Barrieux, A. and Rosenfeld, M.G. (1978) J. Biol. Chem. 253, 6311-6314.
6. Ochiai-Yanagi, S. and Mazumder, R. (1976) Eur. J. Biochem. 68, 395-402.
7. Grosfeld, H. and Littauer, U.Z. (1976) Eur. J. Biochem. 70, 589-599.
8. Ochiai-Yanagi, S., Malathi, V.G. and Mazumder, R. (1977) FEBS Lett. 80, 148-152.
9. Malathi, V.G. and Mazumder, R. (1978) FEBS Lett. 86, 155-159.
10. Layne, E. (1957) Methods Enzymol. 3, 447-454.
11. Dasgupta, A., Majumdar, A., George, A.D. and Gupta, N.K. (1976) Biochem. Biophys. Res. Commun. 71, 1234-1241.
12. Dasgupta, A., Das, A., Roy, R., Ralston, R., Majumdar, A. and Gupta, N.K. (1978) J. Biol. Chem. 253, 6054-6059.