IDENTIFICATION AND RNA-BINDING PROPERTIES OF AN INITIATION FACTOR CAPABLE OF RELIEVING TRANSLATIONAL INHIBITION INDUCED BY HEME DEPRIVATION OR DOUBLE-STRANDED RNA

Raymond Kaempfer[§]
The Biological Laboratories
Harvard University
Cambridge, Massachusetts 02138

Received October 8,1974

ABSTRACT An initiation factor from rabbit reticulocytes can overcome the block in initiation of protein synthesis occurring in reticulocyte lysates when exogenous hemin is not present, or when double-stranded RNA is added. This factor has been identified with IF-MP, an initiation factor capable of forming ternary complexes with GTP and methionyl-$tRNA_F$. Initiation factor IF-M3 by itself is unable to overcome the block in initiation, but appears to stimulate this activity of IF-MP. IF-MP binds to single-stranded R17 RNA as well as to double-stranded RNA, while IF-M3 only binds to double-stranded RNA. The protein synthetic activity of IF-MP is sensitive to N-ethylmaleimide, but its ability to bind RNA is resistant.

Unless hemin is added, initiation of protein synthesis in rabbit reticulocyte lysates ceases after a brief delay, and polysomes disappear, while single ribosomes accumulate (1-7). The cessation of translation can be prevented not only by added hemin, but also by the addition of a partially purified initiation factor, IF-3, isolated from reticulocytes (7), or from two nonerythropoietic tissues, liver and brain (8). Apparently, endogenous initiation factor activity is lost during incubation of a reticulocyte lysate in the absence of hemin. Indeed, upon incubation of the brain factor with hemin and removal of hemin by gel filtration, the factor activity is strongly stimulated (8).

Double-stranded RNA is a powerful inhibitor of initiation of mammalian protein synthesis (9-13), but has no effect on bacterial protein synthesis (14). In reticulocyte lysates, very low concentrations of double-stranded RNA are sufficient to induce a complete inhibition (11,13). The factor from reticulocytes that relieves the block in initiation occurring during hemin deprivation, IF-3 (7), also is capable of relieving the inhibition induced by double-stranded RNA (13). This factor is able to form a tight complex with double-stranded RNA, and disap-

[§]Present address: The Hebrew University, Hadassah Medical School, Jerusalem, Israel

pearance of its activity is concomitant with the establishment of inhibition (13).

The chromatographic properties of reticulocyte IF-3, particularly its large size (7,8,13), initiallly suggested its possible identity with an initiation factor, IF-M3, isolated by Anderson and coworkers (15,16), that is required for the translation of globin messenger RNA. Indeed, according to a recent report (17), IF-M3 purified by DEAE-cellulose chromatography possesses the ability of IF-3 (7,13) to overcome the translational block caused by hemin deprivation or double-stranded RNA.

Here we report that IF-M3 obtained after greater purification does not display the activities of reticulocyte IF-3. Instead, another initiation factor, IF-MP, free of IF-M3 (18), is responsible for relieving both the block in initiation seen in the absence of hemin and that seen in the presence of double-stranded RNA. This initiation factor can form ternary complexes with GTP and Met-tRNA$_F^{Met}$ (18-23). While IF-M3 by itself is unable to overcome the block in initiation, it appears to stimulate this activity of IF-MP. Like IF-3 (7,13), IF-MP binds to single-stranded bacteriophage R17 RNA as well as to double-stranded RNA. IF-M3, by contrast, forms complexes with double-stranded RNA but fails to bind detectably to R17 RNA.

MATERIALS AND METHODS

Highly purified initiation factors IF-MP and IF-M3 were provided by Drs. W.C. Merrick and W.F. Anderson, NIH (15,16,18, and results to be published). The two preparations were free of each other as judged by the ability to form a ternary complex with GTP and Met-tRNA$_F^{Met}$ (IF-MP, ref. 18), or to stimulate globin mRNA translation (IF-M3, refs. 15,16), or by polyacrylamide gel electrophoresis in sodium dodecyl sulfate buffer (W.C. Merrick, personal communication). IF-MP (1.4 mg/ml) was stored at -80^{o}C in buffer A (0.1 M KCl, 0.02 M Tris (pH 7.8), 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol). IF-M3 (0.7 mg/ml) was stored at -80^{o}C in buffer B (0.05 M HEPES (pH 6.9), 1 mM dithiothreitol, 0.285 M KCl). ^{32}P-labeled R17 RNA (24) and double-stranded RNA from phage ϕ6 (14) were prepared as described, and were gifts from Drs. G. Jay and W.R. Abrams, respectively. Double-stranded RNA from *Penicillium chrysogenum* was prepared as described (13). Buffer C: 0.01 M Tris (pH 7.4), 0.05 M KCl, 6 mM 2-mercaptoethanol.

RESULTS

Ability of IF-MP and IF-M3 to support protein synthesis in the absence of

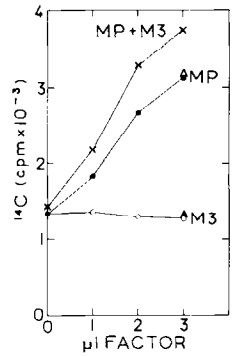

FIG. 1. Effect of IF-MP and IF-M3 on protein synthesis during hemin deprivation. Rabbit reticulocyte lysate (10 µl) was incubated at 30° in a 50-µl reaction mixture (3) containing [^{14}C]amino acids, 3 µl of buffer A and 3 µl of buffer B, except where the indicated volume of IF-MP and IF-M3 (Materials and Methods) replaced buffer A and B, respectively. Samples of the top curve received the indicated volume of each IF-MP and IF-M3. CCl_3COOH-insoluble radioactivity was determined after 75 min. In separate controls, total radioactivity incorporated in the presence of buffers A, B, or both, was the same as in their absence. Triangles: IF-MP was incubated in 0.02 M N-ethylmaleimide for 30 min at 37°, with 0.02 M 2-mercaptoethanol added before (Δ) or after (▲) incubation, and then was assayed as above.

hemin. The amount of [^{14}C]amino acids incorporated into protein by a reticulocyte lysate incubated for 75 min without added hemin is plotted in Fig. 1 as a function of the amounts of added initiation factors IF-MP or IF-M3. In the presence of increasing amounts of IF-MP, total protein synthesis increases steadily beyond the level attained in the absence of added hemin. The latter level represents synthesis supported by endogenous initiation factor activity before that activity is exhausted after 5 to 10 min (7,8). In the range tested, the extent of stimulation of protein synthesis is approximately linear with the amount of added IF-MP.

Addition of IF-M3 by itself fails to overcome the block caused by hemin deprivation (Fig. 1). However, addition of IF-MP together with IF-M3 reproducibly results in a greater stimulation of protein synthesis than that seen in the presence of IF-MP alone. This result indicates a cooperative effect between the two initiation factor preparations.

The ability of IF-MP to stimulate protein synthesis in a lysate lacking hemin is abolished completely by prior treatment of the factor with the sulfhydryl reagent, N-ethylmaleimide (Fig. 1, triangles).

Ability of IF-MP and IF-M3 to support protein synthesis in the presence of double-stranded RNA. It was shown previously that an initiation factor preparation, IF-3 (7), can overcome the block in initiation of protein synthesis observed in the presence of double-stranded RNA, and that the amount of IF-3 needed to overcome this inhibition increases as the concentration of double-stranded RNA is raised (13)

In Fig. 2, the amount of protein synthesized by a reticulocyte lysate in 75 min is plotted as a function of the concentration of added double-stranded RNA. In the absence of added hemin (open triangles), addition of double-stranded RNA causes a slight decrease in the amount of residual synthesis, in agreement with earlier observations (13). When hemin is added, protein synthesis is stimulated, but the stimulation is abolished essentially completely in the presence of 10 ng/ml double-stranded RNA (filled triangles). Addition of IF-MP allows protein synthesis to continue even in the presence of double-stranded RNA at a concentration of 50 ng/ml (filled circles). It is seen, however, that as with IF-3 (13), the total amount of protein synthesis stimulated by IF-MP decreases with increasing concentration of double-stranded RNA.

IF-M3, by contrast, is incapable of providing a detectable protection against

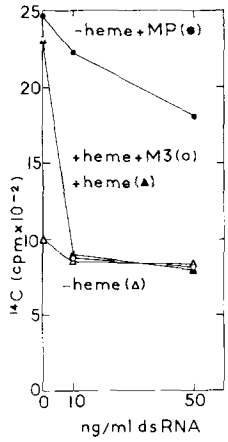

FIG. 2. Effect of IF-MP and IF-M3 on protein synthesis in the presence of double-stranded RNA. Rabbit reticulocyte lysate was incubated as in Fig. 1, but with 2 µl each of buffers A and B. *P. chrysogenum* double-stranded RNA was present at the indicated concentration. Hemin (5×10^{-5}M) was present as shown. IF-MP (2 µl) and IF-M3 (2 µl) replaced, where indicated, buffers A and B, respectively. Controls and analysis were as for Fig. 1.

inhibition by double-stranded RNA (Fig. 2, open circles). Since it was shown in Fig. 1 that IF-M3 has no effect on protein synthesis in the absence of added hemin, this assay was conducted with hemin present in the reaction mixture.

Binding of IF-MP to single-stranded and double-stranded RNA. Reticulocyte IF-3 retains single-stranded R17 phage RNA on nitrocellulose filters (7), and binds tightly to double-stranded RNA (13). As seen in Fig. 3, IF-MP likewise causes both labeled R17 RNA and double-stranded RNA from phage ϕ6 to be retained on filters. Binding of IF-MP to R17 RNA is not noticeably stimulated by the addition of IF-M3 (Fig. 3, open circles). The ability of IF-MP to form complexes with R17 RNA or double-stranded RNA is completely resistant to treatment with N-ethylmaleimide (Fig. 3, triangles), in contrast to the sensitivity of its protein synthetic activity noted in Fig. 1. This differential sensitivity to N-ethylmaleimide is also displayed by reticulocyte IF-3 (unpublished results).

Selective binding of IF-M3 to double-stranded RNA. As seen in Fig. 4, IF-M3, in contrast to IF-MP, fails to form complexes with R17 RNA. It does, however, form complexes with ϕ6 double-stranded RNA. This selectivity for double-stranded RNA further distinguishes IF-M3 from IF-MP.

DISCUSSION

Purified IF-MP is able to relieve the block in initiation of protein synthesis induced by heme deprivation or by double-stranded RNA, activities originally found in a partially purified reticulocyte initiation factor preparation, IF-3 (7,13). The present findings make it possible to bring together a number of observations. IF-MP can form a ternary complex with GTP and Met-tRNA$_F^{Met}$ (18), a property already reported for very likely identical factor preparations (19-23). This factor is known to be required for the binding of Met-tRNA$_F^{Met}$ to 40S ribosomal subunits during initiation (21-23), and 40S·Met-tRNA$_F^{Met}$ complexes disappear during heme deprivation (25) or in the presence of double-stranded RNA (26). The Met-tRNA$_F^{Met}$-binding factor is found both in the supernatant and on ribosomes (22), as is the IF-3 activity from brain (8) and reticulocytes (unpublished results). The sensitivity of the protein synthetic activity of IF-MP to N-ethylmaleimide

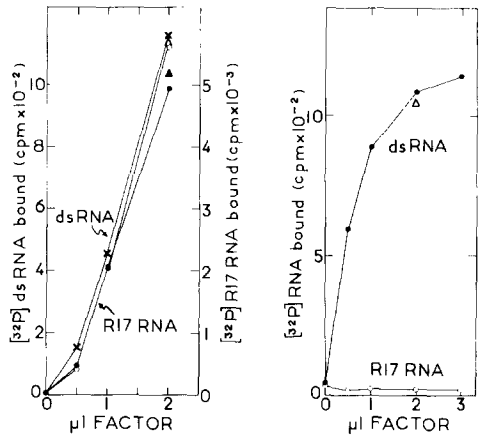

FIG. 3 (left panel). *Binding of IF-MP to R17 RNA and double-stranded RNA.* ^{32}P-labeled R17 RNA (0.04 A_{260}; 10,150 cpm) (●,o) or ϕ6 double-stranded RNA (0.015 A_{260}; 1,600 cpm) (×) were incubated for 10 min in buffer C with the indicated volumes of IF-MP (●,×) or each IF-MP and IF-M3 (o), in a total volume of 75 µl. Samples were diluted with 1 ml of buffer C and passed through Millipore HA 0.45 µm filters, washing extensively with buffer C. Filters were dried and analyzed for radioactivity. Triangles: IF-MP was treated with N-ethylmaleimide as for Fig. 2, with 2-mercaptoethanol added after treatment, and then was included in a binding reaction mixture containing R17 RNA (▲) or ϕ6 double-stranded RNA (Δ).

FIG. 4 (right panel). *Binding of IF-M3 to R17 RNA and double-stranded RNA.* Incubation of the indicated volumes of IF-M3 with R17 RNA or ϕ6 double-stranded RNA, N-ethylmaleimide treatment (Δ), filtration, and analysis were as described in the legend of Fig. 3.

(Fig. 1), furthermore, is matched by the Met-$tRNA_F^{Met}$-binding factor (22), and stands in contrast to the resistance of two other activities displayed by IF-MP, complex formation with R17 RNA or with double-stranded RNA (Fig. 3; refs. 7,13).

IF-M3 lacks the protein synthetic activities of IF-MP, yet, as seen in Fig. 1, it is capable of stimulating synthesis supported by IF-MP. That IF-M3 is distinct from IF-MP is evidenced by the finding that IF-M3 is unable to bind to R17 RNA, although it binds to double-stranded RNA (Fig. 4). The mechanism of stimulation by IF-M3 remains obscure, but if IF-M3 is analogous to IF-E3 (22), as is likely, both IF-MP and IF-M3 may be required for the binding of Met-$tRNA_F^{Met}$ to the 40S ribosomal subunit. Indeed, there are preliminary indications that IF-MP and IF-M3 may interact physically. Reticulocyte (7,13) and brain IF-3 (8) share with IF-M3 (15) and IF-E3 (22) their large molecular size. Brain IF-3 activity is stimulated strongly by incubation with hemin, a stimulation involving the con-

version of inactive, lower molecular weight components to active, high molecular weight IF-3 (8). This indicates that hemin mediates the assembly of an active factor complex (8). A complex between activities corresponding to IF-MP and IF-M3 has been observed in the early stages of purification (23), and such a complex may well account for the recovery of IF-MP activity with partially purified IF-M3 (17). While other explanations are possible, one hypothesis is that cyclic association and dissociation of IF-MP and IF-M3 must occur during each round of initiation, but is prevented by heme deprivation or by double-stranded RNA.

I thank Drs. William C. Merrick and W. French Anderson for offering the initiation factor preparations, Susan Curran for excellent assistance, and Bernard van Engel for art work.

REFERENCES

1. Waxman, H. & Rabinovitz, M. (1966) Biochim. Biophys. Acta 129, 369-379.
2. Zucker, W. & Schulman, H. (1968) Proc. Nat. Acad. Sci. USA 59, 582-589.
3. Adamson, S., Herbert, E. & Godschaux, W. (1968) Arch. Biochem. Biophys. 125, 671-683.
4. Adamson, S., Herbert, E. & Kemp, S. (1969) J. Mol. Biol. 42, 247-258.
5. Rabinovitz, M., Freedman, M., Fisher, J. & Maxwell, C. (1969) Cold Spring Harbor Symp. Quant. Biol. 34, 567-578.
6. Hunt, T., Vanderhoff, G. & London, I. (1972) J. Mol. Biol. 66, 471-481.
7. Kaempfer, R. & Kaufman, J. (1972) Proc. Nat. Acad. Sci. USA 69, 3317-3321.
8. Raffel, C., Stein, S. & Kaempfer, R. (1974) Proc. Nat. Acad. Sci. USA, in press.
9. Hunt, T. & Ehrenfeld, E. (1971) Nature New Biol. 230, 91-94.
10. Ehrenfeld, E. & Hunt, T. (1971) Proc. Nat. Acad. Sci. USA 68, 1075-1078.
11. Hunter, A., Hunt, T., Jackson, R. & Robertson, H. (1972) in Synthese, Struktur und Funktion des Haemoglobins, eds. Martin, H. & Nowicki, L. (Lehmanns, Munich), 133-145.
12. Robertson, H. & Mathews, M. (1973) Proc. Nat. Acad. Sci. USA 70, 225-229.
13. Kaempfer, R. & Kaufman, J. (1973) Proc. Nat. Acad. Sci. USA 70, 1222-1226.
14. Jay, G., Abrams, W. & Kaempfer, R. (1974) Biochem. Biophys. Res. Commun., in press.
15. Shafritz, D., Pritchard, P., Gilbert, J., Merrick, W. & Anderson, W. (1972) Proc. Nat. Acad. Sci. USA 69, 983-987.
16. Prichard, P. & Anderson, W. (1974) Methods Enzymol. XXX, 136-141.
17. Beuzard, V. & London, I. (1974) Proc. Nat. Acad. Sci. USA 71, 2863-2866.
18. Elson, N., Adams, S., Merrick, W. & Anderson, W. (1974) J. Biol. Chem., in press.
19. Chen, Y., Woodley, C., Bose, K. & Gupta, N. (1972) Biochem. Biophys. Res. Commun. 48, 1-9.
20. Dettman, G. & Stanley, W. (1972) Biochim. Biophys. Acta 287, 124-133.
21. Levin, D., Kyner, D. & Acs, G. (1973) Proc. Nat. Acad. Sci. USA 70, 41-45.
22. Schreier, M. & Staehelin, T. (1973) Nature New Biol. 242, 35-38.
23. Cashion, L. & Stanley, W. (1974) Proc. Nat. Acad. Sci. USA 71, 436-440.
24. Jay, G. & Kaempfer, R. (1974) J. Mol. Biol. 82, 193-212.
25. Legon, S., Jackson, R. & Hunt, T. (1973) Nature New Biol. 241, 150-152.
26. Darnbrough, C., Hunt, T. & Jackson, R. (1972) Biochem. Biophys. Res. Commun. 48, 1556-1564.