Vol. 84, No. 4, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 30, 1978 Pages 985-992

STABILITY OF PROTEIN SYNTHESIS INITIATION COMPLEXES IN THE PRESENCE OF EDEINE

June B. Santon and Wendell M. Stanley, Jr.

Department of Molecular Biology and Biochemistry

University of California at Irvine

Irvine, California 92717

U.S.A.

Received July 24,1978

SUMMARY

The antibiotic inhibitor edeine has been used to study various aspects of the initiation of protein synthesis in a rabbit reticulocyte cell-free system. Edeine prevents assembly of the 80S initiation complex while allowing accumulation of a 44S initiation intermediate. The complete 80S initiation complex, once formed, is stable in the presence of edeine. The functioning of the initiation complex, as judged by release of methionyl-puromycin, is only partially inhibited by a concentration of edeine which fully inhibits formation of the initiation complex. The above effects of edeine on a eukaryotic system differ from the effects edeine has been found to have in a prokaryotic system.

INTRODUCTION

Initiation of protein synthesis in eukaryotes involves an initiation complex composed of the 40S and 60S ribosomal subunits, a specific region of mRNA containing an AUG triplet, Met-tRNA^{Met}[3] and initiation factors (1-7).

The initiation complex arises from the sequential formation of intermediate complexes: a ternary complex of eIf-2[4], Met-RNA$_i^{Met}$ and GTP has been

1. This work was supported in part by research grants from the American Cancer Society and the Damon Runyon-Walter Winchell Cancer Fund.

2. Present Address: Molecular Biology Department, University of Southern California, Los Angeles, California 90007.

3. Abbreviation used: Met-tRNA$_i^{Met}$ - the eukaryotic species of initiator methionyl-tRNA.

4. The nomenclature of Anderson et al. (11) for eukaryotic initiation factors has been adopted.

0006-291X/78/0844-0985$01.00/0

demonstrated (1-8). The ternary complex binds to the 40S ribosomal subunit to form an initiation intermediate (1,4,9). Messenger RNA and the 60S ribosomal subunit join the initiation intermediate to form an initiation complex. Formation of the above complexes is mediated by several initiation factors (2,4-8,10). Alternatively, mRNA may bind to the 40S subunit before binding of Met-$tRNA_i^{Met}$, but this pathway does not appear to lead to an initiation complex which is functional in a cell free system with mRNA (4).

We have investigated the effects of the antibiotic edeine in a rabbit reticulocyte cell-free protein synthesis initiation system. We report here the results of that investigation. In particular, we present information concerning the stability of the initiation complex in the presence of edeine. We find that both the initiation intermediate (formed before the point at which edeine acts in the system) and the initiation complex (formation of which is inhibited by edeine) are stable, and the initiation complex is at least partially functional in the presence of edeine.

MATERIALS AND METHODS

Preparation of initiation factors (9), ribosomes (12) and [^{35}S] Met-$tRNA_i^{Met}$ (13) have been described. The preparation of initiation factors was modified slightly as follows: after centrifugation to remove polysomes from the 0.5 M KCl wash, $(NH_4)_2SO_4$ was added to the wash, at 0°, to 70% saturation. After 30 min of stirring at 0°, the solution was centrifuged at 12,000 g for 15 min. The precipitate was resuspended in a minimum volume of 50 mM KCl, 1 mM β-mercaptoethanol, 10% glycerol and 50 mM Tris-HCl, pH 7.0 at 20°. The suspension was dialyzed against the above buffer overnight. Preparation of the factors was then continued as previously described (9). The specific activity of the Met-$tRNA_i^{Met}$ varied in different experiments between 20 and 40 Ci per mmole.

Puromycin was purchased from Sigma. Edeine was a gift from Dr. Kurylo-Borowska, Rockefeller University, New York City.

Formation of the ternary complex and of the initiation complex were as previously described (9), with the following exception: the pH of the Tris-HCl used during the formation of the initiation complex was 7.4 at 20°. Incubation to form the initiation complex was for 10 min unless otherwise indicated.

Formation of the initiation complex was assayed by the use of sucrose density gradients (2). The assay for methionyl-puromycin production was performed as previously described (9).

RESULTS

In the first experiment, 80S initiation complexes were formed in the

presence and absence of edeine (Fig.1). The inhibitor, when used, was added before formation of the ternary complex and was thus present throughout the incubation. Incubation mixtures were then layered directly onto sucrose gradients and analyzed as described in the legend to Fig.1. It can be seen that edeine does not act at the level of formation of the 44S initiation intermediate. Rather, the inhibitor prevents assembly of the complete 80S initiation complex (Fig.1c). The 60S ribosomal subunit does not join the 44S initiation intermediate when edeine is present. When edeine prevents formation of the initiation complex, the initiation intermediate builds up to a high level (Fig.1c).

The stability of the initiation complex in the presence of edeine was also tested (Fig.2). The initiation complex was allowed to form, as above, for either 10 min (panel A) or 15 min (panel B) in the absence of edeine. In a third incubation (panel C), the initiation complex was allowed to form for 10 min; edeine was then added, and the incubation was continued for another 5 min. Each incubation was then layered over a sucrose gradient and analyzed as indicated in the legend to Fig.1. As shown in Fig.2, the amount of initiation complex does not decline in the presence of edeine.

When the initiation complex is incubated with puromycin, methionyl-puromycin is synthesized and released from the ribosome, in a reaction analogous to formation of the first peptide bond in protein synthesis. The effect of edeine on this reaction was measured. Edeine was added in varying concentrations to an incubation. It was added prior to formation of the ternary complex among GTP, Met-tRNA$_i^{Met}$ and eIF-2. 6.6 μM edeine, added prior to formation of the ternary complex, completely inhibits production of methionyl-puromycin. 2.5 μM edeine produces 50% inhibition (Table I). The same concentrations of edeine inhibit protein synthesis in a rabbit reticulocyte cell lysate to the same extent (data not shown).

Functioning of the 80S initiation complex in the presence of edeine was tested. In the experiment presented in Table II, edeine was added to incu-

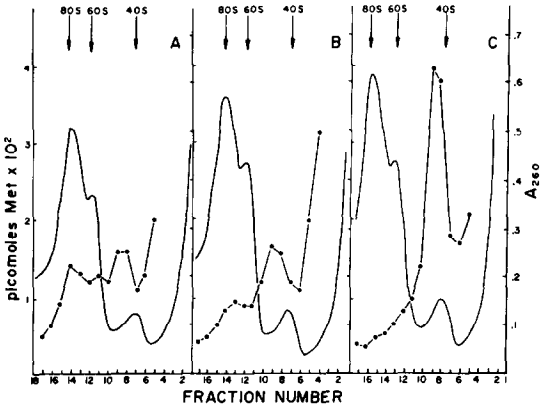

Figure 1 Initiation complexes were formed as described in Materials and Methods. After the 10 min incubation to form the initiation complex, the incubation mixtures were layered on 10 - 25% exponential sucrose gradients, made up in the following buffer: 75 mM KCl, 50 mM Tris-HCl, pH 7.4 at 20°, 5 mM $MgCl_2$ and 1 mM β-mercaptoethanol. The gradients were centrifuged at 2° in a Beckman SW41 rotor at 41,000 rpm for 4 hr. The gradients were pumped through a flow cell and the $A_{260\ nm}$ was recorded as fractions were collected. A: standard incubation to form the initiation complex. B: eIF-5 was replaced by the buffer in which it is kept. C: as in A, except 0.66 nmoles edeine (6.6 μM final concentration) was added to the incubation mixture at the beginning, before the ternary complex was formed.
———— $A_{260\ nm}$ ·———— pmoles [^{35}S]Met-tRNA$_i^{Met}$.

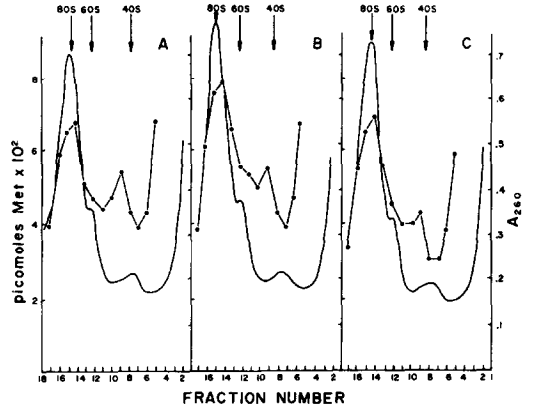

Figure 2 Conditions of centrifugation are described in the legend to Fig. 1. A: The incubation to form the initiation complex was for 10 min. B: The incubation was for 15 min. C: The incubation was for 10 min, at which time 0.66 nmoles of edeine was added, and the incubation continued for another 5 min.

TABLE I

Effect of Early Addition of Edeine on Methionyl-Puromycin Formation[a].

Concentration of Edeine (μM)	Methionyl-Puromycin Formation (fmoles)
0	62
1.3	61
2.6	31
6.6	0

[a] Edeine was added prior to the formation of the ternary complex. The ethyl acetate extraction assay for methionyl-puromycin production was performed as described in Materials and Methods. The amount of methionyl-puromycin (24 fmoles) observed in the absence of GTP and initiation factors has been substracted from these data.

TABLE II

Effect of Late Addition of Edeine on Methionyl-Puromycin Formation[b].

Sample	Methionyl-Puromycin Formation (fmoles)
Control-Initiation Complex Incubation 10 min.	117
Control-Initiation Complex Incubation 15 min.	117
+Edeine-Initiation Complex Incubation 15 min, with Edeine Added for Last 5 Min.	67

[b] The initiation complexes without edeine were formed in a 10 min or 15 min incubation, as indicated. In the sample including edeine, the initiation complex incubation was for 10 min. Edeine was then added to a final concentration of 6.6 μM and the incubation was continued for 5 min more. The samples were then made 0.2 mg puromycin per ml and the incubations were continued for an additional 30 min. Methionyl-puromycin formation was measured as described in Materials and Methods. The amount of methionyl-puromycin (43 fmoles) observed either in the absence of GTP and initiation factors, or in their presence with edeine added prior to formation of the ternary complex, has been substracted from these data.

bations similar to the incubations presented in Table I, except that here the inhibitor was added after formation of the initiation complex. In this case, the formation of methionyl-puromycin is only partially inhibited by

a concentration of edeine capable of full inhibition when added early (Tables I and II).

DISCUSSION

Initiation of protein synthesis in eukaryotes, while not yet fully understood, appears to be a considerably more complex process than in prokaryotes. We have used edeine to create a block in the assembly of the 80S initiation complex. In so doing, we have been able to examine the stability of that complex, as well as the stability of the 44S initiation intermediate.

Our data show that the 44S initiation intermediate, which accumulates in the presence of edeine, is stable (Fig.1). Additionally, the 80S initiation complex is stable for at least 5 minutes in the presence of edeine (Fig.2). This last finding is in agreement with results reported by Szer and Kurylo-Borowska (15) for a prokaryotic system. Using bacterial ribosomes, and poly U or MS_2 RNA as messengers, Szer and Kurylo-Borowska found the initiation complex to be stable in the presence of edeine.

However, the action of edeine in a eukaryotic system is not analogous to its effects in a prokaryotic system. Unlike the situation in our rabbit reticulocyte system, edeine prevents association of the initiator tRNA with a prokaryotic ribosome (15). It may be that this difference reflects a change in the ribosome during the evolution of eukaryotic systems, resulting in a changed edeine-ribosome interaction.

Our results agree with the conclusion of Fresno *et al* (16) that edeine inhibits transfer of the 44S initiation intermediate to an 80S initiation complex in a eukaryotic system.

In contrast to results reported by Obrig *et al* (17), we found no evidence for stimulation by edeine of methionyl-puromycin production. Obrig *et al*'s results may derive from the high magnesium concentration (30mM) present in their incubations. Such a high concentration of magnesium in protein synthesis systems frequently leads to results other than those observed at low magnesium concentrations.

The conversion of the initiation intermediate to the initiation complex may be the sole or primary site of action of edeine in inhibiting eukaryotic protein synthesis. This suggestion is consistent with the data in Table I, which shows that approximately 2.5 μM edeine is required to inhibit methionyl-puromycin synthesis by 50 percent in a rabbit reticulocyte cell-free system and 6.6 μM edeine produces complete inhibition. The same concentrations of edeine inhibit protein synthesis to the same extent in a rabbit reticulocyte cell lysate, in which the entire sequence of reactions involved in translation of a natural mRNA (globin) is observed (unpublished results).

In summary, we have found the usefulness of edeine as a tool to investigate protein synthesis to be threefold: it permits one to isolate a particular step in the assembly of the initiation complex, it also permits one to examine an intermediate complex as well as the final 80S initiation complex, and its interactions with eukaryotic and prokaryotic protein synthesis systems may well provide a useful probe in comparison of the two systems themselves.

REFERENCES

1. Dettmen, G.L., and Stanley, Jr., W.M. (1972) Biochim. Biophys. Acta 287, 124-133.

2. Cashion, L.M., and Stanley, Jr., W.M. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 436-440.

3. Dettman, G.L., and Stanley, Jr., W.M. (1973) Biochim. Biophys. Acta 299, 142-147.

4. Adams, S.L., Safer, B., Anderson, W.F., and Merrick, W.C. (1975) J. Biol. Chem. 250, 9083-9089.

5. Giesen, M., Roman, R., Seal, S.N. and Marcus, A. (1976) J. Biol. Chem. 251, 6075-6081.

6. Ilan, J. and Ilan, J. (1973) Nature New Biol. 241, 176-180.

7. Levin, D.H., and Ranu, R.S., Ernst, V., and London, I.M. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3112-3116.

8. Gupta, N.K., Woodley, C.O., Chen, Y.C. and Bose, K.K. (1973) J. Biol. Chem. 248, 4500-4511.

9. Kolb, A.J., Cooper, K.D., and Stanley, Jr., W.M. (1976) Biochim. Biophys. Acta 425, 229-233.

10. Stehelin, T., Trachsel, H., Erni, B., Boschetti, A., and Schreier, M.H. (1975) Proceedings of the Tenth FEBS Meeting, pp. 309-323.

11. Anderson, W.F., Bosch, L., Cohn, W.E., Lodish, H., Merrick, W.C., Weissbach, H., Wittmann, H.G., and Wool, I.G. (1977) FEBS Lett. 76, 1.

12. Brown, G.E., Kolb, A.J. and Stanley, Jr., W.M. (1974) in Methods in Enzumology (Moldave, K., and Grossman, L., eds.), Vol. 30, pp.368-387, Academic Press, New York.

13. Stanley, Jr., W.M. (1972) Anal. Biochem. 48, 202-216.

14. Cushion, L.M., Dettmann, G.L. and Stanley, W.M. (1974) in Methods in Enzymology (Moldave, K., and Grossman, L., eds.) Vol. 30, pp. 153-171.

15. Szer, W., and Kurylo-Borowska, Z. (1970) Biochim. Biophys. Acta 224, 477-486.

16. Fresno, M., Carrasco, L., and Vazquez, D. (1976) Eur. J. Biochem 68, 355-364.

17. Obrig, T., Irvin, J., Clup, W. and Hardesty, B. (1971) Eur. J. Biochem. 21, 31-41.