A NOVEL METHOD FOR THE RAPID QUANTITATIVE DETERMINATION OF RIBOSOME DISSOCIATION FACTOR (IF-3) ACTIVITY. AN ASSAY BASED ON THE COUPLING OF THE DISSOCIATION AND PEPTIDYLTRANSFERASE REACTIONS.

Herbert A. Thompson, Isaac Sadnik and Kivie Moldave
Department of Biological Chemistry, University of California, Irvine
College of Medicine, Irvine, California 92717

Received September 22,1976

SUMMARY: An assay for factor-dependent ribosome dissociation has been developed, by coupling with the peptidyltransferase reaction between acylaminoacyl-tRNAs and puromycin. The peptidyltransferase reaction is specific for 60S subunits; it is inhibited by derived 40S subunits which combine with derived 60S subunits to form ribosomes. Native 40S subunits, and derived 40S subunits treated with an extract (IF-3) obtained from native 40S subunits, do not interact with 60S subunits and they do not affect the 60S-dependent peptidyltransferase reaction. The native 40S extract releases subunits from 80S ribosomes and the 60S subunits released, a measure of dissociation, can be determined by the peptidyltransferase reaction.

INTRODUCTION

A number of laboratories have reported the presence of a ribosome dissociation factor in the high salt "wash" of ribosomes or ribosomal subunits of various prokaryotic (1-3) and eukaryotic (4-12) cells. Recent studies in this laboratory (13) provided evidence for a ribosome dissociation activity, designated here as IF-3, in extracts prepared from native 40S subunits of rat liver. These protein factors dissociate ribosomes into subunits which can be detected by sucrose gradient sedimentation analysis. However, the determination of ribosome-dissociating activity by gradient centrifugation is limited and time consuming. In order to follow the purification of IF-3 and to examine the characteristics of the reaction that it catalyzes, it was considered desirable to develop a simple, rapid assay procedure. This communication describes a method which measures the extent to which IF-3 dissociates 80S ribosomes, or prevents the reassociation of subunits, by determining the amount of free 60S subunits resulting or remaining in the reaction. The method depends on the ability of 60S subunits specifically, in alcoholic solutions, to catalyze the peptidyltransferase reaction between an N-blocked aminoacyl-tRNA and puromycin; under the conditions employed, 80S ribosomes do not catalyze peptidyltransferase (14,15).

MATERIALS AND METHODS

Preparation of ribosomes, subunits and radioactive aminoacylated tRNAs- Derived (d40S and d60S) subunits were prepared from rat liver ribosomes and

Copyright © 1976 by Academic Press, Inc.
All rights of reproduction in any form reserved.

polysomes (16) by dissociation with 0.88 M KCl (13,17) and were resolved by zonal centrifugation (13). Free native 40S (n40S) subunits were sedimented from the post-microsomal supernatant fraction (18) and resolved by zonal centrifugation (13). The "n40S extract" containing IF-3 activity, was prepared from native 40S subunits with 0.88 M KCl (13).

Isotopically-labeled phenylalanyl-tRNA (19) and methionyl-$tRNA_m$ (20,21) were prepared as described; the radioactive aminoacyl-tRNAs were acetylated with acetic anhydride as described by Haenni and Chapeville (22) for phenylalanyl-tRNA. The specific activity of the acetyl[^{3}H]Phe-tRNA was 2300 cpm/µg of aminoacyl-tRNA and that of the tRNA-esterified phenylalanine was 4800 cpm/pmole; the specific activity of the acetyl[^{3}H]Met-$tRNA_m$ was 6700 cpm/µg of aminoacyl-tRNA and 3400 cpm/pmole of tRNA-bound methionine.

Analysis of incubations by the peptidyltransferase assay - A two-step incubation procedure was used to assay for the amount of free d60S subunits released in the first incubation (containing ribosomes and dissociation factor), capable of catalyzing the 60S-specific reaction between an acetylaminoacyl-tRNA and puromycin in the second incubation (14). The first phase contained d60S subunits and d40S or n40S subunits, with or without n40S extract, in a solution containing 30 mM morpholinopropane sulfonate (pH 7.2), 1.4 mM $MgCl_2$, 60 mM NH_4Cl and 2 mM dithiothreitol; in addition, 14 mM Tris-HCl (pH 7.3), 2.6 mM $MgCl_2$, 30 mM KCl, 0.6 mM dithiothreitol and 70 mM sucrose were contributed by the subunit and n40S extract preparations. Incubations were in a total volume of 0.05 ml, for 15 minutes at 37°. The reaction mixtures were cooled in an ice bucket for 0.5-1.0 minute before the next step, in order to avoid inactivation due to the addition of alcohol. For the second incubation, a buffered salts-alcohol solution was added to the first incubation (at 2°), to obtain the following final concentrations: 4 mM $MgCl_2$, 0.3 M KCl, 1.4 mM dithiothreitol, 0.8 mM puromycin and 33% methanol plus 40 mM Tris-HCl at pH 7.5 (in experiments with acetylphenylalanyl-tRNA) or 33% ethanol plus 40 mM Tris-HCl at pH 8.0 (in experiments with acetylmethionyl-$tRNA_m$); some incubations did not contain puromycin. Acetyl[^{3}H]phenylalanyl-tRNA containing about 19 pmoles of tritium-labeled phenylalanine or acetyl[^{3}H]-methionyl-$tRNA_m$ containing about 22 pmoles of [^{3}H]methionine was added and the reaction mixtures, in a total volume of 0.15 ml, were incubated for 10 minutes at 20°. At the end of the incubation period, the reactions were terminated, the product acetyl[^{3}H]aminoacyl-puromycin was extracted with ethyl acetate and analyzed as described previously (14,15,23,24). In most cases, the results are expressed as pmoles of labeled amino acid product extracted, after subtraction of the values obtained without puromycin; the levels of radioactivity extracted into ethyl acetate, in control experiments without puromycin, were 6-7 times lower than those with puromycin. In the purifica-

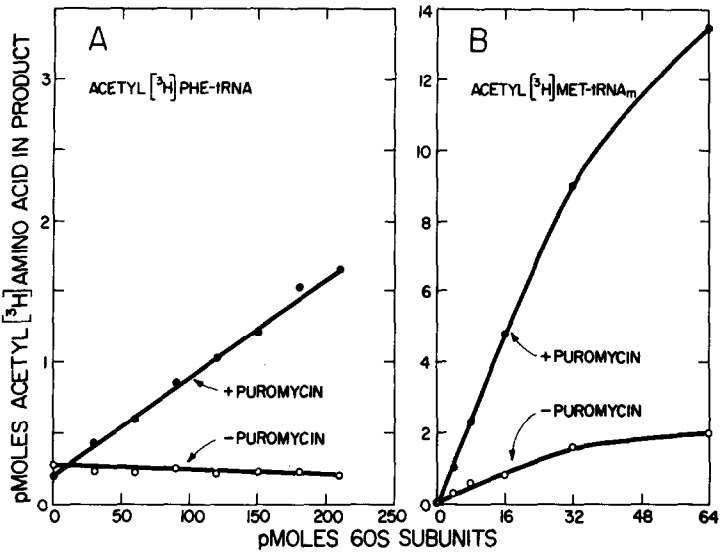

Figure 1 - The effect of d60S subunit concentration on the peptidyltransferase reaction. Varying concentrations of derived 60S subunits were incubated with acetyl[^{3}H]Phe-tRNA (A) or acetyl[^{3}H]Met-tRNA (B) in solutions containing buffered-salts, 33% alcohol, with (closed circles) and without (open circles) 0.8 mM puromycin, as described in the text. After 10 minutes at 20°, the amount of acetyl[^{3}H]aminoacyl-puromycin formed was extracted with ethyl acetate and counted:

tion procedure for IF-3, to be described elsewhere, one of the steps used was glycerol gradient centrifugation. It was noticed that high concentrations of glycerol inhibited peptidyltransferase. This difficulty was overcome by increasing the volume of the first incubation to 0.2 ml (and subsequently, the second incubation to 0.6 ml), maintaining all other components and conditions; this modification permitted analysis of larger gradient samples without significantly affecting the assay.

RESULTS AND DISCUSSION

The peptidyltransferase reaction was proportional to the amount of 60S subunits present, over a wide concentration range, when assayed with acetylphenylalanyl-tRNA (Figure 1, A) or with acetylmethionyl-tRNA$_m$ (Figure 1, B). As shown previously (14), and in Figure 2, the addition of derived 40S subunits to an incubation containing d60S subunits inhibited the reaction between the acetylaminoacyl-tRNA and puromycin (open circles), due to the formation of 80S ribosomes (13). The extent of inhibition was dependent on the concentration of d40S subunits in the incubation with 60S particles, prior to the assay for peptidyltransferase in the presence of alcohol. However,

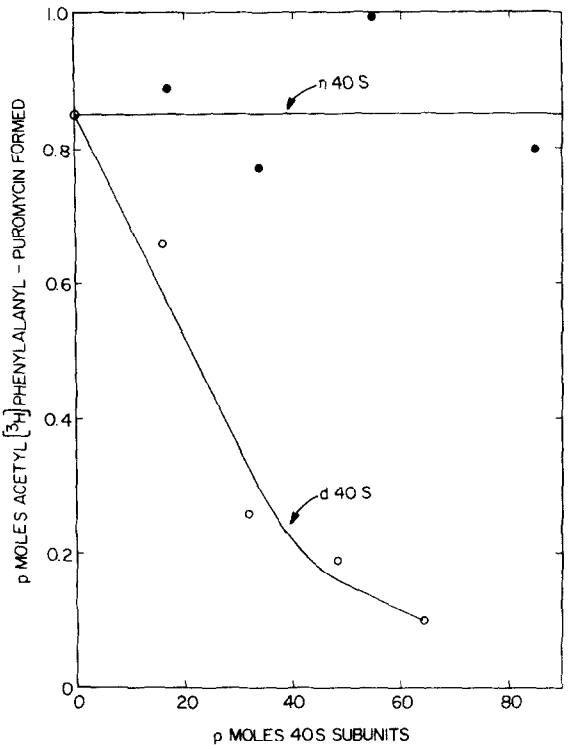

Figure 2 - The effect of native 40S subunit and derived 40S subunit concentrations on the peptidyltransferase reaction catalyzed by d60S subunits. Approximately 54 pmoles of d60S subunits were preincubated for 2 minutes at 37° as such, or with varying concentrations of n40S subunits (closed circles) or d40S subunits (open circles), as noted. The incubation mixtures were then assayed for peptidyltransferase with buffered salts-methanol, acetyl-[^{3}H]Phe-tRNA and puromycin, as described in the text.

TABLE I

The Effect of d40S Subunits and IF-3 on the Peptidyltransferase Reaction Catalyzed by d60S Subunits

First incubation components	Second incubation additions	pmoles of acetyl[^{3}H]Met-puromycin formed
None	d60S subunits	2.39
d40S subunits	d60S subunits	0.38
d40S subunits + IF-3	d60S subunits	1.89

The first incubation, for 10 minutes at 37°, contained 24 pmoles of d40S subunits and 47 µg of n40S extract protein (IF-3) where noted. Derived 60S subunits (24 pmoles) were then added to all of the incubations, which were allowed to continue for 5 minutes at 37°. At the end of the second phase, buffered salts-methanol, acetyl[^{3}H]Met-tRNA and puromycin were added; after 10 minutes at 20°, the reactions were analyzed for acetylmethionyl-puromycin.

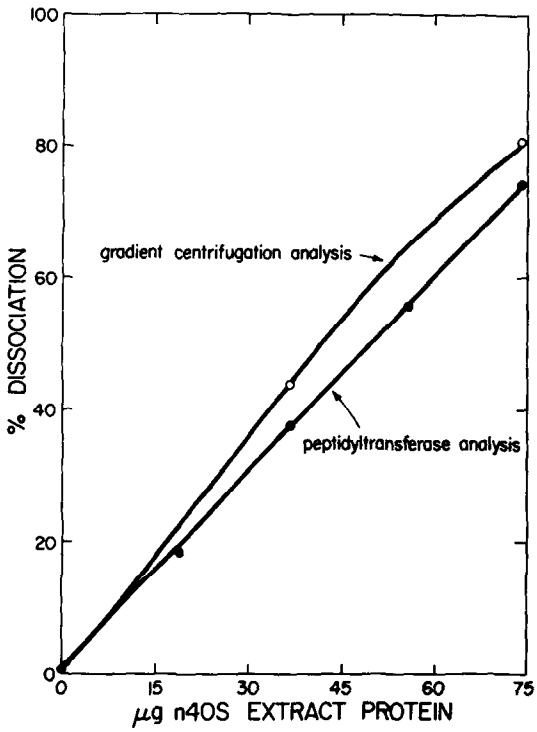

Figure 3 - The effect of varying concentrations of n40S extract on the dissociation of 80S ribosomes. Approximately 8 pmoles of d40S subunits and 8 pmoles of d60S subunits were preincubated as such for 10 minutes at 37° to form ribosomes. The ribosomes were incubated with the amounts of n40S extract indicated, for 15 minutes at 37°. Duplicate reaction mixtures were then analyzed by centrifugation through linear (10-30%) sucrose gradients (13) or by the peptidyltransferase method with acetyl[^{3}H]Met-tRNA and puromycin as described above. In the gradient centrifugation analyses, the percent dissociation (open circles) was estimated from the areas under the peaks obtained in the continuous spectrophotometric patterns. In the peptidyltransferase analyses (closed circles), the values obtained from incubations containing d60S subunits (without d40S subunits), and from incubations containing d60S plus d40S subunits (without n40S extract), were chosen to represent 100% and 0% dissociation, respectively.

when n40S subunits which do not readily associate with 60S particles to form 80S ribosomes were added, peptidyltransferase was not inhibited (closed circles). The decrease in transpeptidation by d40S subunits was not due to an inactivation of the peptidyltransferase catalytic center, since d40S subunits did not inhibit when acylaminoacyl-oligonucleotide fragment was used as a substrate, instead of acylaminoacyl-tRNA (25).

The effect of n40S extract, containing IF-3, using this "coupled" assay system, is shown in Table I. In this and subsequent experiments, peptidyltransferase was assayed with acetylmethionyl-tRNA$_m$ (instead of acetylphenyl-

alanyl-tRNA) because, as shown in Figure 1, the reaction was considerably more sensitive with this substrate. Derived 60S subunits alone catalyzed the formation of significant amounts of acetylmethionyl-puromycin (line 1); the addition of d40S subunits during the first phase of the incubation (line 2) inhibited the 60S-dependent activity about 85%, but when d40S subunits and IF-3 were used in the first incubation, peptidyltransferase was inhibited only 20% indicating that the 40S subunits did not associate with 60S particles in the presence of dissociation factor.

When d40S subunits were present in the 1st incubation with 60S subunits (in the absence of IF-3), peptidyltransferase activity was decreased 4- to 5-fold as compared to reactions in which the first incubation contained 60S subunits only; the decrease in activity, as described above, was due to the reaction of d40S with d60S subunits to form 80S ribosomes. However, when IF-3 was incubated with preformed 80S ribosomes, the subsequent peptidyl-transferase reaction was markedly stimulated. The dissociation of ribosomes was dependent on the concentration of n40S extract protein used (Figure 3, closed circles). When duplicate reaction mixtures were centrifuged through sucrose gradients (13), the extent of dissociation calculated from the concentration of subunits obtained from the optical density profile (Figure 3, open circles), agreed well with that obtained with the peptidyltransferase assay. Although not shown here, d40S subunits and n40S extract, individually or together, did not carry out transpeptidation between acetylaminoacyl-tRNA and puromycin.

Additional evidence for a ribosome dissociation activity in n40S subunits, in addition to that obtained previously by sucrose gradient analysis (13), is provided by the following observation with the peptidyltransferase-coupled assay: whereas d40S subunits inhibit markedly the 60S-dependent reaction, the effect of n40S particles on the initial velocity of the transpeptidation reaction is negligible; the addition of n40S extract to incubations containing d40S and d60S subunits prevents the marked inhibition (by d40S subunits) of the peptidyltransferase reaction, indicating that subunit reassociation is prevented. The peptidyltransferase reaction is readily catalyzed by 60S particles in the presence of IF-3; this observation suggests that the dissociation factor does not interfere with the functions of the peptidyltransferase "active center" or the "P" and "A" sites on 60S particles that interact with the appropriate substrates.

Acknowledgements. This work was supported in part by research grants from the American Cancer Society (NP-88) and the U.S. Public Health Service (AM-15156). The authors thank Mrs. Eva Mack, Mr. Wayne Sabo, Mr. Peter Hui and Mr. Peter Moldave for their technical assistance, and Flor Herrera for some of the preparations used in this study.

REFERENCES

1. Subramanian, A.R., Ron, E.Z., and Davis, B.D. (1968) Proc. Nat. Acad. Sci. U.S. 61, 761-767.
2. Subramanian, A.R., Davis, B.D., and Beller, R.J. (1969) Cold Spring Harbor Symp. Quant. Biol. 34, 223-230.
3. Sabol, S., and Ochoa, S. (1977) Nature (London) 234, 233-239.
4. Petre, J. (1970) Eur. J. Biochem. 14, 399-405.
5. Lubsen, N.H., and Davis, B.D. (1972) Proc. Nat. Acad. Sci. U.S. 69, 353-357.
6. Merrick, W.C., Lubsen, N.H., and Anderson, W.F. (1973) Proc. Nat. Acad. Sci. U.S. 70, 2220-2223.
7. Mizuno, S., and Rabinowitz, M. (1973) Proc. Nat. Acad. Sci. U.S. 70, 787-791.
8. Favelukes, G., Sorgentini, D., Bard, E., and de Barrajo, C.M. (1973) in Gene Expression and its Regulation (Kenney, F.T., Hamkalo, B.A., Favelukes, B., and August, J.T., eds.) Basic Life Sciences 1, 393-409, Plenum Press, New York and London.
9. Ayuso-Parilla, M., Henshaw, E.C., and Hirsch, C.A. (1973) J. Biol. Chem. 248, 4386-4393.
10. Lubsen, N.H., and Davis, B.D. (1974) Biochim. Biophys. Acta 335, 196-200.
11. Lawford, G.R., Kaiser, J., and Hey, W.D. (1971) Canad. J. Biochem. 49, 1301-1306.
12. Sundkvist, I.C., and Staehelin, T. (1975) J. Mol. Biol. 99, 401-418.
13. Sadnik, I., Herrera, F., McCuiston, J., Thompson, H.A., and Moldave, K. (1975) Biochemistry 14, 5328-5335.
14. Thompson, H.A., and Moldave, K. (1974) Biochemistry 13, 1348-1353.
15. Edens, B., Thompson, H.A., and Moldave, K. (1975) Biochemistry 14, 54-61.
16. Skogerson, L., and Moldave, K. (1967) Biochem. Biophys. Res. Commun. 27, 568-572.
17. Martin, T.E., and Wool, I.G. (1968) Proc. Nat. Acad. Sci. U.S. 60, 569-574.
18. Moldave, K., Galasinski, W., and Rao, P. (1971) in Methods in Enzymology, (Moldave, K. and Grossman, L., eds.), Vol. XX C, p. 337-348, Academic Press, New York.
19. Siler, J., and Moldave, K. (1969) Biochim. Biophys. Acta 195, 123-129.
20. Smith, A.E., and Marcker, K.A. (1970) Nature (London) 226, 607-610.
21. Schroer, R.A., and Moldave, K. (1973) Arch. Biochem. Biophys. 154, 422-430.
22. Haenni, A.-L., and Chapeville, F. (1966) Biochim. Biophys. Acta 114, 135-148.
23. Leder, P., and Bursztyn, H. (1966) Biochem. Biophys. Res. Commun. 25,
24. Maden, B.E.H., and Monro, R.E. (1968) Eur. J. Biochem. 6, 309-316.
25. Edens, B., Thompson, H.A., and Moldave, K. (1974) in Lipmann Symposium: Energy, Biosynthesis and Regulation in Molecular Biology, Richter, D., ed., Walter de Gruyter Verlag, West Berlin, 179-191.