GDP KINASE ACTIVITY ASSOCIATED WITH SALT-WASHED RIBOSOMES

Alice M. Wertheimer and M. S. Kaulenas

Department of Zoology, University of Massachusetts
Amherst, Mass. 01003

Received August 10,1977

SUMMARY: In the presence of ATP, 1M-salt-washed ribosomes and subunits of both eukaryotes and prokaryotes phosphorylate GDP to 5'GTP. A possible role for the ribosome-associated GDP kinase is presented.

INTRODUCTION: Hydrolysis of GTP is necessary for initiation of protein synthesis as well as for elongation (1). In both initiation and elongation, it has been thought that GTP is brought to the ribosome complexed with a specific factor. It has therefore been puzzling that initiation factors bind GDP much more strongly than they do GTP (2). Although a mechanism has been found for exchanging factor-bound GDP with GTP in the case of prokaryote elongation, one has not been found for initiation. The GDP kinase associated with 1M-salt-washed ribosomes and subunits of Escherichia coli and mouse liver reported here may be part of such a mechanism.

A scheme for eukaryote initiation is given which postulates that GDP rather than GTP is brought to the ribosomes, and that the ribosome phosphorylates the GDP. The scheme also incorporates (a) findings of an ATP requirement for initiation of protein synthesis in eukaryotes (3,4), (b) recycling of the stable eIF2·GDP complex, and (c) phosphorylation of a small-subunit ribosomal protein (5).

MATERIALS AND METHODS: All ribosome isolations were done at 4°C. Buffers are given in Table 1. Buffers were adjusted to pH 8.0 at room temperature.

Frozen mid-log phase E. coli (strain A-19 from General Biochemicals) were suspended in two volumes of Buf. A with DNAase (Sigma) added to a final concentration of 10 µg/ml. After sonication, the suspension was incubated at 4°C for 20 min, then centrifuged for 30 min at 15,000g.

ISSN 0006-291X

Table 1. Composition of buffers used in ribosome isolations.

Buffer	Salt (mM) Tris-HCl	NH_4Cl	KCl	MgAc	Dithiothreitol (D) β-mercaptoethanol (M)
A	50	100	-	15	5 (M)
B	50	1000	-	10	5 (M)
C	50	15	25	5	0.1 (D)
D	30	45	15	6	0.1 (D)
E	10	50	10	1	-
F	50	10	25	4	0.1 (D)
G	50	-	500	2	-
H	50	10	25	6	0.1 (D)
I	50	10	60	3	1.0 (D)
J	50	10	60	9	1.0 (D)
K	50	10	25	10	0.1 (D)

The supernatant was centrifuged at 105,000g for 90 min. The pellet was resuspended in Buf. B and incubated for 8 h at 4°C. In some experiments, Triton X-100 was added to a final concentration of either 0.5% or 1%. The supernatant from clearing at 15,000g for 15 min was centrifuged at 105,000g for 90 min. The pellets ("1M-salt-washed ribosomes") were frozen at -70°C or resuspended in Buf. C to a final concentration of 50-100 A_{260nm} units/ml and assayed directly or frozen at -20°C in aliquots. Alternatively, the ribosomes were resuspended in Buf. D and made into subunits by centrifuging into a 10%-30% sucrose gradient made up in Buf. E. Subunit fractions were collected, made 15mM in magnesium, and precipitated by addition of a half volume of cold 100% ethanol. The precipitate was collected by centrifugation at 12,500g for 10 min, resuspended in Buf. C, and assayed directly or frozen at -20°C in aliquots. Before use, ribosomes were incubated at 37°C for at least 10 min.

Crude ribosomes were extracted from starved male mice (strain CD, Charles River) essentially as in Ref. 6. The crude pellets were rinsed with distilled water, resuspended in Buf. B containing 0.25M sucrose by two strokes of a hand-held Teflon-glass homogenizer and incubated at 0°C for 2 h. Triton X-100 was added to a final concentration of 1% and the solution was cleared at 15,000g for 15 min. The supernatant was pelleted at 105,000g for 3 h through a 5-ml cushion of 500mM sucrose made up in Buf. B. The pellets ("1M-salt-washed ribosomes") were (a) frozen at -70°C until use or (b) resuspended in Buf. F to a concentration of 50-100 A_{260nm} units/ml and assayed directly or frozen at -20°C in aliquots. Alternatively, the pellets were resuspended in Buf. G, incubated with 1mM puromycin for 10 min at 4°C, then 15 min at 37°C, and centrifuged into a 10%-30% sucrose gradient made up in Buf. G (modified from Ref. 7). Subunit fractions were collected and treated as *E. coli* subunits, except that resuspension was in Buf. F.

Assay conditions are given in the legend to Fig. 1. Assays were run at 37°C, usually for 120 min. The assay volume was 50 µl. Controls were run under the conditions given, but the final resuspension buffer (Buf. C or Buf. F) replaced the ribosomes. Tritium-labelled nucleotides were obtained from New England Nuclear or Amersham/Searle. Unlabelled nucleotides were from Sigma or Calbiochem, and were not purified before use. Stock solutions

of sucrose (Fisher) were treated with diethylpyrocarbonate (Eastman) and filtered before use. Stock salt solutions were filtered through 0.22-μm Millipore filters.

Assay mixtures were precipitated by addition of formic acid to a final concentration of 1M. After at least 30 min in the cold, the supernatants from a 10-min spin on a bench centrifuge were spotted onto plastic-backed PEI-cellulose plates (Brinkmann Instruments) that had been washed by ascending chromatography with distilled water. The one-dimensional chromatographs were washed for 1 min in distilled water to remove salts, dried, and developed in 1.5M KH_2PO_4.

Two 2-dimensional systems were used to identify the GTP. One system was Method 2 of Ref. 8 and the other is given in Ref. 9. Fluorography was done essentially as described in Ref. 10. Kodak RP 54 film was used and exposure was for 6 days at -70°C.

Uniform zonal strips were cut from one-dimensional plates and counted in 5 ml of Liquifluor (New England Nuclear). Normalization was done by dividing the counts per minute (cpm) of each strip by the sum of the cpm of all the strips in an assay, giving the fraction of total cpm in a strip. Peaks could then easily be identified and percent of total cpm in a peak calculated. The specific activity R, in moles of product per min per mole of ribosomes was calculated using the formula

$$R = (F)(C)(P_S - P_C)/(A)(T),$$

where F is a constant factor that adjusts factors of 10 and converts A_{260nm} units into pmoles of ribosomes, C is the nmoles of substrate GTP or GDP in the assay mixture, P_S is the percent of total cpm in the product peak, P_C is the percent of total cpm in the control, A is the A_{260nm} units in the assay, and T is the duration of the assay in minutes. Error bars in Fig. 1 at the 99% confidence level were calculated as in Ref. 11 using estimated probable errors. The sum of cpm in an assay was always at least 40,000.

RESULTS: GDP kinase activity is present in 1M-salt-washed ribosomes of E. coli and mouse liver (Fig. 1). The activity is not diminished by the formation of subunits in E. coli. The formation of subunits does diminish the activity of mouse liver ribosomes, but this may be due to the ethanol precipitation step or other factors given in the Discussion. Combined reticulocyte subunits not isolated with ethanol (a kind gift of R. S. Ranu*) show an activity equal to that of our 1M-salt-washed ribosomes. In all assays, small subunits had higher activity than large. The activity of large subunits could be at least partly due to contamination by small subunits.

GTPase activity is indistinguishable from zero in eukaryote ribosomes

* Dept. of Biology, MIT.

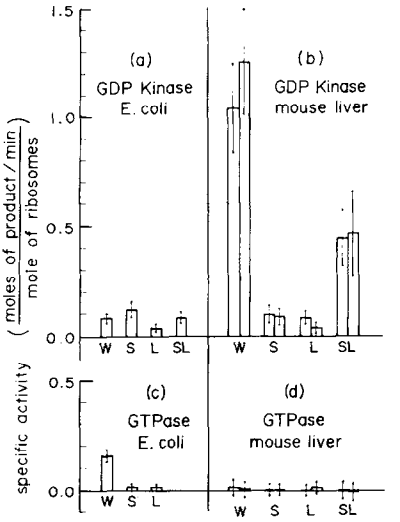

Fig. 1. GDP kinase and GTPase activities of 1M-salt-washed ribosomes and subunits of E. coli and mouse liver. W: whole ribosomes, about 1 A_{260nm} unit per assay; S: small subunits (about 0.33 A_{260nm} unit per assay); L: large subunits (about 0.67 A_{260nm} unit per assay); SL: small and large subunits combined in one assay at a ratio of 1:2 (about 1 A_{260nm} unit per assay). Assay conditions: (a) Buf. H, 4mM ATP, 0.1mM[^{3}H]GDP (specific activity 200 mCi/mmole); (b) left-hand bar: Buf. I, 2mM ATP, 0.1mM [^{3}H]GDP; right-hand bar: Buf. J, 2mM ATP, 0.1mM [^{3}H]GDP; (c) Buf. K, 0.1mM [^{3}H]GTP (specific activity 200 mCi/mmole); (d) same as (b) except [^{3}H]GDP is replaced by 0.1mM [^{3}H]GTP. Magnesium concentrations have been adjusted for presence of ATP.

and subunits, and reduced to essentially zero in E. coli subunits (Fig. 1). The rate of GDP formation by eukaryote ribosomes and subunits, and of E. coli subunits, is therefore the rate of synthesis and not a turnover rate.

The magnesium and monovalent salt conditions given in the caption to Fig. 1 are optimal. It should be noted that there are two magnesium optima for 1M-salt-washed mouse liver ribosomes.

Under the conditions of Fig. 1, CTP, GTP, and UTP can replace ATP as phosphate donors. However, the activity with UTP is half that with ATP. CTP gives a lower activity and GTP is the poorest donor (activity is less than 20% of that with ATP). Although it is not yet certain if diphosphates other than GDP can accept phosphates, visual inspection of chromatographs of mouse liver assays using ADP, CDP, and UDP as acceptors do not show the presence of any of the corresponding triphosphates.

DISCUSSION: The GDP kinase activity that has been described (see also Ref. 12) could be caused by (a) a reverse reaction of a ribosomal GTPase, (b) a factor-dependent activity observed because of residual factors bound to the ribosome, (c) a cytoplasmic contaminant that binds tightly to the ribosome throughout the isolation procedure, or (d) an authentic ribosomal activity, mediated by ribosomal proteins.

It seems improbable that the reaction is due to the ribosomal GTPase acting in reverse because the GTPase activity of the subunits and of the eukaryote ribosomes is negligible under the conditions used. The conditions are thought to approximate those found *in vivo*, and are similar to those used in *in vitro* assays of protein synthesis.

Walton and Gill (2) describe an NDP kinase associated with eIF2 that could be implicated in the ATP stimulation of initiation in eukaryotes (3,4). If the ribosome-associated GDP kinase were due to contamination by eIF2, we estimate that one out of every twenty-one 1M-salt-washed ribosomes would have to have bound eIF2.* It is probable that most ribosomes in the cell do not have bound eIF2, and that washing with 1M salt would remove most of the eIF2 from those that do. It is therefore unlikely that GDP kinase activity is due to residual eIF2. However, it seems possible that the NDP kinase observed by Walton and Gill may in fact be some of the ribosome-associated GDP kinase that partitions with eIF2 during isolation.

Although the isolation procedures were designed to eliminate contaminants, especially in the case of eukaryotes, there is no easy way to know whether or not they were successful. The decrease in activity when eukaryote ribosomes are dissociated into subunits may be due to partial removal of a contaminant, factor, or necessary ribosomal protein. The fact that no such decrease occurs with *E. coli* ribosomes shows that the activity is tightly bound. If the activity is of importance in prokaryote initiation,

*We assume a specific activity of 0.21 units/mg protein (2) and that the molecular weight is about 100,000 daltons, a typical molecular weight for NDP kinase (13).

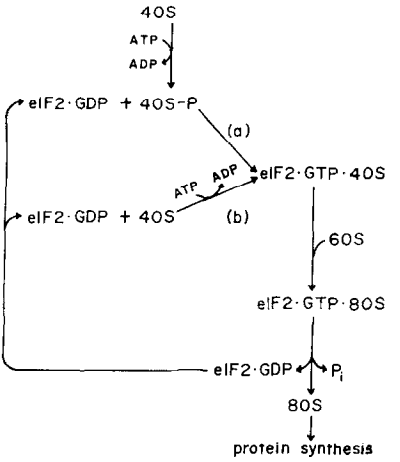

Fig. 2. A partial scheme of eukaryotic initiation. The involvement and sequence of events concerning tRNA, mRNA, and factors other than eIF2 are not represented. Reaction (a) involves a stable phosphorylated subunit as an intermediate and requires ATP. The subunit would thus be similar to known NDP kinases (including succinyl thiokinase) which all have stable phosphorylated intermediates (13). The small subunit may be phosphorylated while still attached to the large subunit. Reaction (b) does not involve ribosomal phosphorylation, but does require ATP. The chief feature is that the ribosome phosphorylates and dephosphorylates eIF2-bound GDP and the stable eIF2·GDP complex is thus recycled.

Reaction 2 of Fig. 2 would be the likely route. As the same substrates are used in guanosine tetraphosphate formation, the activity may somehow be related to that reaction. The failure of others to notice this activity in E. coli may be due to (a) use of suboptimal magnesium concentration (concentrations higher than 8mM are inhibitory), (b) presence of GTPase antagonizing the activity, or (c) more intensive washing.

If the reaction is indeed a true ribosomal one, it would have interesting implications for understanding how the ribosome works. Figure 2 gives a proposed scheme for eukaryote initiation that incorporates the events listed in the Introduction. The major feature is that eIF2-bound GDP and the eIF2·GDP complex are recycled in a simple way.

Even if the activity described is only a tightly bound contaminant, its presence should be taken into account when assaying nucleotide cycling

in protein synthesis, binding of nucleotides to components of protein synthesis, phosphorylation of factors and ribosomal proteins, and the role of the phosphorylation in control of initiation.

ACKNOWLEDGEMENTS: This research was supported in part by a grant to MSK from the Research Council, Univ. of Massachusetts. AMW would like to thank G. Brown, R. Duhamel, G. Fredericks and R. Yenofsky for valuable criticism, and O. Martini and D. Richter for their hospitality and a chance to do an interesting preliminary experiment.

REFERENCES

1. For example, Haselkorn, R., and Rothman-Denes, L. B. (1973) Annu. Rev. Biochem. 42, 397-438.
2. Walton, G. M., and Gill, G. N. (1975) Biochim. Biophys. Acta 390, 231-245.
3. Marcus, A. (1970) J. Biol. Chem. 245, 955-961.
4. Schreier, M. H., and Staehelin, T. (1973) in Regulation of Transcription and Translation in Eukaryotes, edited by E. Bantz, P. Karlson, and H. Kersten, pp. 335-349, Springer-Verlag, New York.
5. Wool, I. G., and Stöffler, G. (1974) in Ribosomes, edited by M. Nomura, A. Tissiéres, and P. Lengyel, pp. 417-460, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. Schreier, M. H., and Staehelin, T. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 462-465.
7. Sherton, C. C., and Wool, I. G. (1974) J. Biol. Chem. 249, 2258-2267.
8. Gallant, J., Shell, L., and Bittner, R. (1976) Cell 7, 75-84.
9. Cashel, M., Lazzarini, R. A., and Kalbacher, B. (1969) J. Chromatogr. 40, 103-109; Cashel, M., and Kalbacher, B. (1970) J. Biol. Chem. 245, 2309-2318.
10. Randerath, K. (1970) Anal. Biochem. 34, 188-205.
11. Margenau, H., and Murphy, G. M. (1956) The Mathematics of Chemistry and Physics, 2nd ed., Ch. 13, D. Van Nostrand Company, Inc., Princeton, N.J.
12. Wertheimer, A. M., (1977) Fed. Proc. 36, 871 (Abstr. No. 3164) (a preliminary report of some of the present material).
13. Parks, Jr., R. E., and Agarwal, R. P. (1973) in The Enzymes, Vol. VIII, Part A, edited by P. D. Boyer, pp. 307-333, Academic Press, New York.