Vol. 92, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1980 Pages 60-67

STIMULATION OF INITIATION FACTOR eIF-2 BY A RAT LIVER PROTEIN WITH GDPase ACTIVITY

Michael J. Clemens*, Clement O. Echetebu*†, Vivienne J. Tilleray*
and Virginia M. Pain‡

*Department of Biochemistry, St George's Hospital Medical School, Cranmer Terrace, London SE17 ORE and ‡School of Biological Sciences, University of Sussex, Falmer, Brighton, Sussex BN1 9QG, U.K.

Received November 14,1979

SUMMARY

Phosphocellulose chromatography of initiation factor eIF-2 from rat liver separates it from a protein fraction which is highly stimulatory for [eIF-2.GTP.Met-tRNA$_f$] ternary complex formation. Evidence is presented which indicates that this stimulatory fraction contains a specific GDPase activity. eIF-2 dependent formation of 40S ribosomal initiation complexes is also enhanced by the GDPase preparation. The enzyme may play a role in the recycling of eIF-2 by removing inhibitory GDP which is generated during 80S initiation complex formation.

INTRODUCTION

In many of the published procedures for the preparation of eukaryotic polypeptide chain initiation factor eIF-2 there is considerable loss of activity during the later stages of purification, particularly after phosphocellulose chromatography (1-3). We have investigated whether such losses may be due to the removal of a co-factor which is required for optimal activity of eIF-2 during initiation and our results described in this paper indicate that this is the case. A possible physiological role for the stimulatory co-factor in the recycling of eIF-2 between successive rounds of polypeptide chain initiation is considered. An abstract of this work has already been presented (4).

---

† On leave from the Department of Biochemistry, University of Nigeria, Nsukka, Nigeria.

Abbreviations: PEP, phosphoenolpyruvate; PK, pyruvate kinase; PEI-cellulose, polyethyleneimine cellulose; NDP kinase, nucleoside diphosphate kinase; CTAB, cetyl trimethyl ammonium bromide.

0006-291X/80/010060-08$01.00/0

MATERIALS AND METHODS

Materials. Cellulose phosphate (medium mesh), nucleoside di- and triphosphates, nucleoside diphosphate kinase (EC 2.7.4.6, 1080 units per mg), phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40) were from Sigma Chemicals, U.K. [^{35}S]methionine (700 Ci/mmol) and [^{3}H]nucleotides (15-20 Ci/mmol) were from the Radiochemical Centre, Amersham, U.K. PEI-cellulose plates (20x20 cm) were purchased from Camlab, Cambridge, U.K. ^{35}S labelled initiator Met-tRNA$_f$ was synthesized as described previously (5). Cellulose nitrate filters (0.45 µm pore size) were from Millipore (U.K.) Ltd.

Initiation factor eIF-2. Rat liver eIF-2 was partially purified using the procedure of Harbitz and Hauge (1). Briefly, a post-nuclear supernatant was made 0.55M in KCl, mitochondria and microsomes were removed by centrifugation, and the supernatant was then fractionated by ammonium sulphate precipitation, DEAE-cellulose chromatography and phosphocellulose chromatography. The latter step separates eIF-2 from the stimulatory fraction (see below). The eIF-2 was eluted with 0.4M KCl and was approximately 20-30% pure. Approximately 95% pure eIF-2 from rabbit reticulocytes was a generous gift from Dr W.C. Merrick.

Stimulatory fraction. Phosphocellulose column chromatograhpy of eIF-2 obtained by DEAE-cellulose purification (1) separated the initiation factor from a peak of protein which did not bind to the column in the presence of low salt buffer (20mM Tris-HCl, pH 7.6, 250mM KCl, 0.2mM EDTA, 10mM 2-mercaptoethanol, 10% v/v glycerol). This material, free of endogenous eIF-2, was used as the source of the stimulatory fraction. Details of its purification and characterization will be described elsewhere.

Assays of initiation complex formation. The formation of [eIF-2.Met-tRNA$_f$.GTP] ternary complexes was assayed by their retention on cellulose nitrate filters (1). Incubations contained the following components in a total volume of 100 µl: 25mM Tris-HCl (pH 7.6), 100mM KCl, 1.2mM Mg acetate, 1mM EDTA, 1.5mM 2-mercaptoethanol, 1.5% glycerol, up to 3.2 x 10^5 cpm of [^{35}S] Met-tRNA$_f$ and, where indicated, 0.5mM GTP and eIF-2. Other additions were as specified in the Tables and Figure. After 10 min at 30° the reaction mixtures were diluted with 1 ml of cold buffer (25 mM Tris-HCl, pH 7.6, 100mM KCl, 10mM Mg acetate) and immediately filtered through cellulose nitrate filters.

The binding of [^{35}S] Met-tRNA$_f$ to rat liver salt-washed 40S ribosomal subunits (6) was assayed in the presence of 20 mM K^+-Hepes (pH 7.6), 100mM KCl, 3mM Mg acetate, 1mM DTT, 1% glycerol, 0.2mM GTP, 0.2mM ATP and similar amounts of Met-tRNA$_f$ and eIF-2 as above. 0.45A_{260} units of 40S subunits were added per 100 µl incubation. After 15 min at 30° the incubation mixtures were fixed with 1% glutaraldehyde (buffered at pH 7.6) and layered on to 5 ml 20-50% sucrose gradients in pH 6.6 buffer as described previously (7). After centrifugation for 165 min at 234,000 x g fractions were collected and precipitated with CTAB for measurement of radioactivity (8).

Analysis of [^{3}H] nucleotides. Interconversion of guanine or adenine nucleotides was quantitated by thin layer chromatography of neutralized perchloric acid supernatants obtained after incubation of [^{3}H] nucleotides with various enzyme fractions. The incubation conditions were: 20 mM Tris-HCl, pH 7.6 , 50 mM KCl, 3 mM Mg acetate and 1 mM DTT. Nucleotides were present at 1 mM each and the protein concentrations were as specified in Table 3. After 20 min at 30° ice-cold perchloric acid was added to 0.2M and after 30 min precipitated protein was removed by centrifugation and the supernatants were neutralized with 0.2M KOH plus 0.7mM EDTA. The precipitated $KClO_4$ was removed by centrifugation. 5 µl of each supernatant was mixed with 5 µl of a mixture of unlabelled guanine and adenine nucleotides (10 µM each) as

markers and subjected to ascending thin layer chromatography on PEI-cellulose sheets. The solvent system was 3M LiCl:1M CH_3COOH (1:9 v/v), followed by 1.6M LiCl:2M CH_3COOH (1:1 v/v) (9). Nucleotide spots were identified by fluorescence under U.V. light and were cut out for estimation of radioactivity by immersion in toluene-based scintillation fluid.

RESULTS

During the early stages of isolation of eIF-2 from rat liver (by ammonium sulphate precipitation and DEAE-cellulose chromatography) the stimulatory activity described here co-purifies with the initiation factor. Subsequent fractionation by phosphocellulose chromatography separates the eIF-2 from more than 95% of the remaining protein, which is not retained on the column. The recovery of eIF-2 activity after this separation is only about 10%, however, as reported previously (1). We therefore tested the ability of the material which does not bind to phosphocellulose to restore the activity of eIF-2 for ternary complex formatiom. Table 1 shows that up to an 11-fold enhancement of Met-$tRNA_f$ binding to eIF-2 can be obtained with this fraction, although the magnitude of the effect varies considerably between experiments. The basal and stimulated binding of Met-$tRNA_f$ are both GTP-dependent and the stimulatory fraction itself does not bind Met-$tRNA_f$. The presence of an energy regenerating system in the form of PEP and pyruvate kinase also stimulates ternary complex formation, but usually not to as great an extent. Addition of the stimulatory fraction together with the PEP/PK system results in a further increase in Met-$tRNA_f$ binding (Table 1).

It has been shown previously (10) that the activity of eIF-2 *in vitro* is strongly enhanced by the enzyme nucleoside diphosphate kinase, which removes inhibitory GDP by phosphorylating it to GTP. We have tested our stimulatory fraction for NDP kinase activity and have compared it with authentic enzyme for stimulation of ternary complex formation. The results in Table 2 provide evidence that the stimulation is not due to NDP kinase activity. Under conditions where the stimulatory fraction increased Met-$tRNA_f$ binding, albeit by only 2.1-fold in this experiment, authentic NDP kinase (with or without ATP) had no effect. Only in the presence of added GDP was the NDP kinase +

Table 1. Enhancement of the binding of Met-tRNA$_f$ to eIF-2 by the stimulatory fraction and by an energy generating system.

	Conditions				[^{35}S]Met-tRNA$_f$ bound (cpm x 10^{-3})
	eIF-2	GTP	stimulator	PEP/PK	
Exp. I	+	+	-	-	9.76
	+	+	+	-	39.71
	+	-	+	-	4.95
	+	+	-	+	18.79
	+	+	+	+	33.21
Exp. II	+	+	-	-	2.85
	+	+	+	-	30.00
	+	-	+	-	2.84
	+	+	-	+	14.44
	+	+	+	+	44.09
	-	+	+	-	0.43

Ternary complex formation was assayed as described in Materials and Methods with partially purified eIF-2 (3.3µg protein in Expt. I; 2.2µg protein in Expt. II), stimulatory fraction (26 µg protein) and 3mM phosphoenolpyruvate plus 2.1µg pyruvate kinase where indicated.

Table 2. Reversal of GDP inhibition by the stimulatory fraction and evidence that the stimulator is not nucleoside diphosphate kinase.

Conditions				[^{35}S] Met-tRNA$_f$ bound (cpm x 10^{-3})
GDP	stimulator	NDP kinase	ATP	
-	-	-	-	61.9
-	+	-	-	131.6
-	-	+	-	41.9
-	-	+	+	40.9
+	-	-	-	12.0
+	+	-	-	96.2
+	-	+	-	12.8
+	-	+	+	19.4

Ternary complex formation was assayed as described in Materials and Methods with stimulatory fraction (26 µg protein), NDP kinase (4µg), GDP (0.5mM) and ATP (0.5mM) where indicated. Partially purified eIF-2 (2.2µg protein) and GTP (0.5mM) were present throughout.

Table 3. Hydrolysis of GDP by the stimulatory fraction; comparison with the effects of nucleoside diphosphate kinase.

Substrates	Protein fraction	Radioactivity in nucleotides (% of total)					
		GTP	ATP	GDP	ADP	GMP	AMP
[^{3}H]GDP+ATP	NDP kinase	43.7	6.8	41.9	0.6	6.4	0.6
[^{3}H]GDP+ATP	stimulator	3.7	1.6	0.9	2.0	90.9	1.8
[^{3}H]ADP+GTP	NDP kinase	2.9	36.5	2.0	41.5	1.3	15.8
[^{3}H]ADP+GTP	stimulator	2.3	7.0	1.1	82.9	2.0	4.8

50µl incubations contained 1µCi of either [^{3}H]GDP or [^{3}H]ADP together with 50 nmoles of each of the unlabelled nucleotide pairs indicated. 2µg of NDP kinase or 15µg of stimulatory fraction was added and the other conditions were as specified in Materials and Methods. After incuabation, precipitation and thin-layer chromatography of the neutralized supernatants on PEI-cellulose (Materials and Methods), the radioactivity associated with the 6 nucleotide spots shown was assessed. The results are expressed as % of the total radioactivity recovered (ca. 3 x 10^4 cpm in each case). Control incubations (without any added protein fraction) showed greater than 90% of the radioactivity to be still associated with the original [^{3}H]nucleotide.

ATP combination stimulatory, and then by only 62%. In contrast, the stimulator from rat liver overcame much of the inhibitory effect of GDP, with an 8-fold enhancement of ternary complex formation (Table 2).

Table 3 illustrates the lack of NDP kinase activity in the stimulatory fraction, as assayed by direct analysis of [^{3}H]nucleotides by thin layer chromatography. This Table also presents data which provide a basis for the ability of this material to reverse the inhibitory effects of GDP on eIF-2. A potent GDPase activity converts greater than 90% of added GDP to GMP. This is in contrast with the effect of NDP kinase where only about half the GDP is phosphorylated to GTP, in agreement with the position at equilibrium of the reaction catalysed by this enzyme. [^{3}H]ADP cannot replace GDP as a substrate for the GDPase, but is phosphorylated by NDP kinase to give [^{3}H]ATP (Table 3). Further experiments of this type indicated the inability of GTP or ATP to undergo any chemical transformation by the eIF-2 stimulator (unpublished data of C.O.E.).

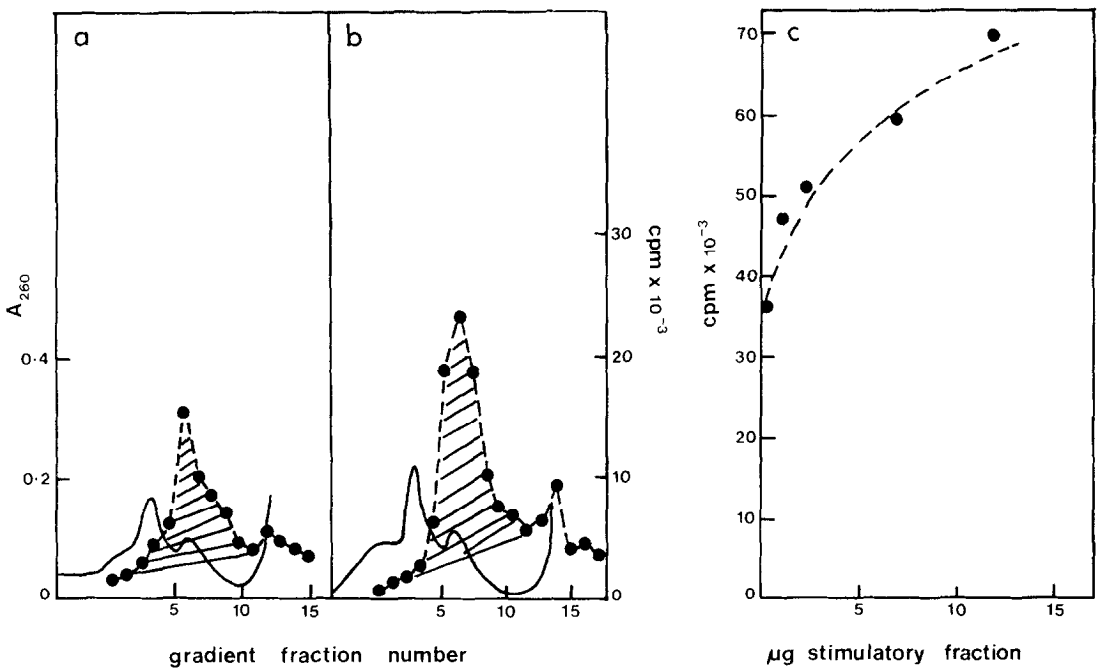

Figure 1. Enhancement of Met-tRNA$_f$ binding to 40S ribosomal subunits by the stimulatory fraction.

Salt-washed 40S subunits were incubated with 4.4µg of highly purified reticulocyte eIF-2, [^{35}S]Met-tRNA$_f$, GTP and various amounts of stimulatory fraction under conditions described in Materials and Methods. The initiation complexes were fixed with glutaraldehyde and isolated by sucrose gradient centrifugation. Sedimentation was from right to left. (——), A_{260}, (● ------ ●), radioactivity. The faster-sedimenting peak is 40S subunit dimers. (a) Control incubation; (b) With 11.6µg stimulatory fraction. The radioactivity associated with the 40S monomer peak was quantitated as shown, after subtracting an estimated base-line. (c) Concentration dependence of the stimulation of Met-tRNA$_f$ binding by the stimulatory fraction.

We have investigated whether the subsequent stage in initiation complex formation is also affected. With purified, salt-washed 40S ribosomal subunits and very pure eIF-2 there is a 2-3-fold increase in Met-tRNA$_f$ binding to the subunits in the presence of the stimulator (Figure 1). This is unlikely to be due to increased stability of the 40S initiation complexes during centrifugation, since glutaraldehyde fixation was carried out prior to their isolation on sucrose gradients.

DISCUSSION

Two important questions are raised by the results described here, which relate to: (i) whether the stimulatory effects on eIF-2 are due to the GDPase

activity; (ii) what physiological relevance this GDPase has to the control of eIF-2 activity *in vivo*. The first of these points can only be settled with certainty by use of GDPase purified to homogeneity. Such purification is currently under way in this laboratory and, thus far, there is no evidence for any separation of the eIF-2-stimulating and GDPase activities (V.J.T., unpublished data). Futhermore, other characteristics of the material, such as its heat lability (results not shown) and the fact that it reverses the inhibitory effect of GDP on eIF-2 (Table 2), are consistent with the proposal that its activity is due to its ability to hydrolyse the nucleotide.

The possible functional relationship of the GDPase to eIF-2 *in vivo* is difficult to determine from the present results. The enzyme co-purifies with the initiation factor through ammonium sulphate precipitation and DEAE-cellulose chromatography and could therefore be physically associated with it. There have been several recent reports of proteins with stimulatory activity towards eIF-2 (11-14) and it will be of considerable interest to discover which of these, if any, corresponds to the activity described here. Based on similar behaviour on DEAE-cellulose and phosphocellulose chromatography, the GDPase seems most like the factors ESP of de Haro et al. (12) and SF of Ranu and London (13). A recent paper (15) has also warned of the possibility of non-specific, apparent stimulation of ternary complex formation by proteins which reduce the adsorption of eIF-2 to plastic reaction vessels. We have confirmed that the latter phenomenon does occur but it is not prevented by our GDPase preparation and does not appear to be the basis for the observed stimulation (V.J.T., unpublished data).

It is established that GTP is hydrolysed to GDP and phosphate on joining of 60S ribosomal subunits with 40S pre-initiation complexes (16-18). The eIF-2 is also released at this stage (17, 19) and may remain associated with the GDP, for which it has a strong affinity (10). The role of the GDPase thus might be to permit recycling of eIF-2 by hydrolysing the inhibitory nucleotide, allowing the binding of another molecule of GTP and of Met-tRNA$_f$.

Our preliminary results suggest that the reticulocyte haem controlled repressor (20) can modify the response of the initiation factor to the GDPase (V.J.T. & M.J.C., unpublished observations). The recycling mechanism and the effect of the haem controlled repressor on it are therefore currently under investigation.

Acknowledgements

This work was supported by grants from the Medical Research Council, the Cancer Research Campaign and the Anna Fuller Fund. C.O.E. is grateful to the Inter-University Council for a maintenance grant.

REFERENCES

1. Harbitz, I. and Hauge, J. (1976). Arch.Biochem.Biophys. 176, 766-778.
2. Cashion, L.M. and Stanley, W.M. Jr. (1973). Biochim.Biophys.Acta 324, 410-419.
3. Spremulli, L.L., Walthall, B.J., Lax, S.R. and Ravel, J.M. (1977). Arch. Biochem.Biophys. 178, 565-575.
4. Pain, V.M., Echetebu, C.O., Tilleray, V.J. and Clemens, M.J. (1979). Proc. XIth International Congress of Biochemistry, Toronto. Abstract number 02-8-H49.
5. Clemens, M.J. and Williams, B.R.G. (1978). Cell 13, 565-572.
6. Schreier, M.H. and Staehelin, T. (1973). J.Mol.Biol. 73, 329-349.
7. Pain, V.M., Lewis, J.A., Huvos, P., Henshaw, E.C. and Clemens, M.J. (1979). J.Biol.Chem. (in press).
8. Legon, S., Jackson, R.J. and Hunt, T. (1973). Nature New Biol. 241, 150-152.
9. Neuhard, J. (1966). Biochim.Biophys.Acta 129, 104-115.
10. Walton, G.M. and Gill, G.N. (1975). Biochim.Biophys.Acta 390, 231-245.
11. Dasgupta, A., Das, A., Roy, R., Ralston, R., Majumdar, A. and Gupta, N. (1978). J.Biol.Chem. 253, 6054-6059.
12. De Haro, C. and Ochoa, S. (1978). Proc.Nat.Acad.Sci. U.S.A. 75, 2713-2716.
13. Ranu, R.S. and London. I.M. (1979). Proc.Nat.Acad.Sci. U.S.A. 76, 1079-1083.
14. Das, A., Ralston, R.O., Grace, M., Roy, R., Ghosh-Dastidar, P., Das, H.K., Yaghmai, B., Palmieri, S. and Gupta, N.K. (1979). Proc.Nat.Acad. Sci. U.S.A. (in press).
15. Benne, R., Amesz, H., Hershey, J.W.B. and Voorma, H.O. (1979). J.Biol Chem. 254, 3201-3205.
16. Safer, B. and Anderson, W.F. (1978). Crit.Rev.Biochem. 5, 261-290.
17. Peterson, D.T., Merrick, W.C. and Safer, B. (1979). J.Biol.Chem. 254, 2909-2916.
18. Merrick, W.C. (1979). J.Biol.Chem. 254, 3708-3711.
19. Trachsel, H. and Staehelin, T. (1978). Proc.Nat.Acad.Sci. U.S.A. 75, 204-208.
20. Trachsel, H., Ranu, R.S. and London, I.M. (1978). Proc.Nat.Acad.Sci. U.S.A. 75, 3654-3658.