INHIBITION BY FUSIDIC ACID OF AN EUKARYOTIC FACTOR-PROMOTED AND 40S RIBOSOMAL SUBUNIT-DEPENDENT GTP'ase ACTIVITY

Rajarshi MAZUMDER

Department of Biochemistry, New York University School of Medicine, 550 First Avenue, New York, N.Y. 10016, USA

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1. Introduction

The mechanism of polypeptide chain initiation in eukaryotic cells is being investigated in several laboratories ([1] is a recent review of the subject). However, very little information is presently available regarding the steps in which GTP is hydrolyzed and the role of GTP hydrolysis in eukaryotic chain initiation. Since polypeptide chain initiation in eukaryotes appears to involve the formation of an initiation complex on the *small* (40S) ribosomal subunit as an intermediate [1], one approach to the problem is to locate, identify and study the properties of eukaryotic factor(s) which may catalyze GTP hydrolysis dependent on *isolated* 40S ribosomal subunits. This communication documents the presence of such a factor in the embryos of the brine shrimp, *Artemia salina.*

It has been observed that DEAE-cellulose chromatography of a 0.5 M KCl wash of *A. salina* ribosomes yields a factor which promotes a 40S ribosomal subunit-dependent hydrolysis of GTP. This GTP hydrolysis is *not* due to contamination of 40S with 60S ribosomal subunits and is inhibited by fusidic acid. However, the factor appears to be distinct from elongation factor 2 (EF-2).

2. Materials and methods

The pH of all buffers was measured at 25°C. Ribosomal subunits were prepared from *A. salina* embryos essentially as described previously [2] and were stored at −20°C in a buffer containing 50% glycerol. As assayed by poly(U) translation, 40S subunits showed no detectable contamination with 60S subunits. However, 60S subunits were contaminated 10–15% with the other species. A 0.5 M KCl wash of *A. salina* ribosomes was prepared as follows. The ribosomal pellet [2], from 80 g of cysts, was suspended in and then stirred with about 48 ml of buffer containing 37 mM Tris–HCl, pH 7.8, 70 mM KCl, 9 mM Mg^{2+}, 0.1 mM EDTA and 0.1 mM dithiothreitol (DTT) for 1 hr. The suspension was centrifuged at 49 000 rev/min for 2 hr in a Ti 50 rotor and the supernatant solution was discarded. The pellet was suspended in and then stirred with about 48 ml of a buffer containing 0.5 M KCl, 50 mM Tris–HCl, pH 7.8, 0.1 mM DTT and 0.1 mM Mg^{2+} for 1 hr. The suspension was centrifuged as above in a Ti 60 rotor. The supernatant solution (0.5 M KCl ribosomal wash) was then brought to 80% saturation with $(NH_4)_2SO_4$. After stirring for about 45 min, the suspension was centrifuged and the precipitated protein was dissolved in a minimal volume of a buffer containing 50 mM KCl, 25 mM Tris–HCl, pH 7.4, 0.2 mM DTT, 0.1 mM EDTA and 5% glycerol (buffer A). The solution (1 to 1.2 ml) was then dialyzed against 100 ml of a buffer containing 100 mM KCl, 50 mM Tris–HCl, pH 7.4, 0.2 mM DTT, 0.1 mM EDTA and 5% glycerol for 4 hr with a change of buffer after each hour. The precipitate which appeared during dialysis was removed by centrifugation and discarded. An equal vol of glycerol was added to the supernatant solution which was then kept at −20°C. This solution (2 to 2.5 ml; 3–4 mg/ml) was put on a DEAE-cellulose column (0.5 × 9.0 cm) previously equilibrated with buffer A containing no glycerol (buffer B). The column was eluted successively and stepwise with buffer B, buffer B containing 0.1 M KCl and

buffer B containing 0.2 M KCl. 1.0 ml fractions were collected and the A_{280} of fractions was close to 0 when changes of buffer were made. For most of the experiments reported here, the fractions were assayed for GTP'ase activity either immediately or within 24 hr of preparation. Unless mentioned otherwise, reaction mixtures (0.1 ml) for GTP'ase assay contained: Tris–HCl buffer, pH 7.4, 35 mM; KCl, 107 mM; Mg^{2+}, 5.5 mM; DTT, 1 mM; EDTA, 0.05 mM; GTP (labeled with ^{32}P in the gamma position), 0.0025 mM. Samples, containing the above components only, were run in duplicate and routinely used as blanks. Other additions are indicated in the legends to tables. Labeled GTP was added last and reaction mixtures were incubated for 15 min at 37°C. The release of ^{32}P was measured essentially as described previously [3]. Protein concentration of DEAE-cellulose fractions and EF-2 was measured by ultraviolet absorption [4]. The amount of protein present in other fractions was determined by the method of Lowry et al. [5]. [γ-^{32}P]-GTP and DEAE-cellulose were products of ICN and Schleicher and Schuell, respectively. Brine shrimp eggs were purchased from Longlife Aquarium Products, Harrison, N. J. Highly purified EF-2 (from *A. salina* cysts) was a generous gift of Dr C. Nombela and Professor S. Ochoa, Roche Institute of Molecular Biology, Nutley, N.J. Wheat germ EF-2 and [^{14}C]phe-tRNA (for assaying poly(U) translation) were kindly provided by Mr B. Treadwell and Professor W. G. Robinson of this department.

3. Results

GTP hydrolysis which requires the presence of *both* factor (either DEAE-cellulose fraction or EF-2) and ribosomal subunits (either 40S or 60S or 40S plus 60S) was calculated as follows: the sum of the activities observed with subunit(s) *alone* and factor *alone* was subtracted from the activity observed upon combination of the two components. For example, in table 1 (exp. 2), GTP'ase activity (6.95 pmol) due to *both* 40S subunit and 0.2 M KCl eluate was calculated by subtracting the *sum* of activities observed with 40S subunit *alone* (1.55 pmol) and the DEAE fraction *alone* (2.97 pmol) from the activity (11.47 pmol) observed upon their combination. When the fractions resulting from the stepwise elution of DEAE-cellulose columns were assayed in this manner, it was observed that the bulk of the factor(s) which catalyzed GTP hydrolysis dependent on isolated 40S subunits, was eluted with 0.2 M KCl (table 1, expts. 1,2). It is important to note that although ribosome-dependent GTP'ase activity is also observed using fraction eluted with 0.1 M KCl, these fractions catalyze little or no GTP hydrolysis unless 60S subunits are also present in addition to 40S subunits (last column of table 1, expts. 1,2). In contrast, ribosome-dependent GTP hydrolysis using 0.2 M KCl eluate occurs predominantly with 40S subunit (last column of table 1, expts, 1–3) and the stimulation by 60S subunit is less than the additive effect (table 1, expt. 3). The above results, along with the fact that these 40S subunits are essentially free of 60S subunits as judged by poly(U) translation and EF-2-catalyzed GTP'ase (see table 3), indicate that the observed GTP hydrolysis, dependent on isolated 40S subunits and the factor(s) present in 0.2 M KCl eluate, is *not* due to contamination with 60S subunits. This factor appears to be quite labile; there is an apparent loss of 40–60% of the activity upon storage of DEAE-cellulose fractions at 4°C for about 20 hr.

Fusidic acid (2 mM) strongly inhibits the factor and 40S subunit-dependent GTP hydrolysis (table 2). The value for this GTP hydrolysis in the presence of fusidic acid is calculated in the same way as described in a preceding paragraph. The inhibition obtained with 0.6 mM fusidic acid is around 50% (data not shown). Since the reticulocyte elongation factor, EF-2, is also inhibited by fusidic acid [6–8], it is important to establish that the *Artemia* factor catalyzing 40S subunit-dependent GTP hydrolysis has properties which are distinct from those of authentic *Artemia* EF-2. The ribosomal subunit requirements for EF-2-dependent GTP'ase, was therefore, tested in a homologous system using highly purified EF-2 derived from *A. salina* embryos. The results are summarized in table 3. It may be seen that, in this case, there is little or no hydrolysis of GTP with isolated 40S subunits (expts. 1,2). In contrast, substantial hydrolysis is observed with 60S subunits which is stimulated by 40S subunits. This stimulation is greater than the additive effect (expt. 2). Similar results have been obtained by other investigators using EF-2 from reticulocytes [8,9]. These results strongly suggest that the factor, described in this paper, is distinct from EF-2. The other

Table 1
GTP hydrolysis dependent on DEAE-cellulose fractions and ribosomal subunits

Expt. No.	Incubation	Net release of ^{32}P*	Factor and ribosome-dependent GTP'ase**	
		(pmol)	(pmol)	40S / 40S + 60S
1	40S	0.86	–	–
	40 S + 60S	1.67	–	–
	0.1 M KCl eluate	0.98	–	–
	+ 40S	3.86	2.02	–
	+ 40S + 60S	17.92	15.27	0.13
	0.2 M KCl eluate	2.75	–	–
	+ 40S	9.23	5.62	–
	+ 40S + 60S	12.80	8.38	0.67
2	40S	1.55	–	–
	40 S + 60S	3.54	–	–
	0.1 M KCl eluate	0.85	–	–
	+ 40S	2.27	0	–
	+ 40S + 60S	5.79	1.40	v. low
	0.2 M KCl eluate	2.97	–	–
	+ 40 S	11.47	6.95	–
	+ 40S + 60S	15.40	8.89	0.78
3	40S	1.89	–	–
	60S	2.09	–	–
	40S + 60S	3.39	–	–
	0.2 M KCl eluate	2.93	–	–
	+ 40S	6.40	1.58	–
	+ 60S	6.03	1.01	–
	+ 40S + 60S	8.70	2.38	0.66

* Average of duplicate runs; blank values of about 3 pmol have been subtracted.

** See Results for a description of this calculation.

Conditions of GTP'ase assay (see Methods). Other additions (where indicated) were: 40S, 0.5 A_{260} unit; 60S, 1.0 A_{260} unit; 0.1 M KCl eluate, 3.4 μg (expt. 1) and 5 μg (expt. 2); 0.2 M KCl eluate, 7.8 μg (expt. 1) and 9.5 μg (expts. 2, 3). DEAE-cellulose fractions used in expt. 1 and expts. 2, 3 were prepared from different batches of 0.5 M KCl ribosomal wash. 0.2 M KCl eluate used in expt. 3 had been stored for about 48 hr at 4°C. The specific radioactivity of [γ-^{32}P]GTP used was 2720, 1830 and 3280 cpm per pmol, respectively in expts. 1, 2 and 3.

Table 2
Effect of fusidic acid on GTP hydrolysis

Incubation	Net release of ^{32}P*	Factor and ribosome-dependent GTP'ase**	
	(pmol)	(pmol)	Inhibition by fusidic acid (%)
40S	0.92	–	–
40S + fusidic acid	0.89	–	–
0.2 M KCl eluate	2.79	–	–
+ fusidic acid	1.70	–	–
+ 40S	7.25	3.54	–
+ 40S + fusidic acid	2.11	0	100

* Average of duplicate runs; a blank value of 3 pmol has been subtracted.
** See Results for a description of this calculation.

Conditions same as those of expt. 1 (table 1), except that 2 mM fusidic acid was present (where indicated) and 0.2 M KCl eluate used had been stored overnight at 4°C. The specific radioactivity of [γ-^{32}P]GTP used was 2590 cpm per pmol.

Table 3
GTP hydrolysis dependent on purified *A. salina* EF-2 and ribosomal subunits

Expt. No.	Incubation	Net release of ^{32}P*	EF-2 and ribosome-dependent GTP'ase**	
		(pmol)	(pmol)	$\frac{40S}{40S + 60S}$
1	40S	0.99	–	–
	40S + 60S	2.00	–	–
	EF-2	0.28	–	–
	+ 40S	0.97	0	–
	+ 40S + 60S	3.82	1.54	v. low
2	40S	1.17	–	–
	60S	4.65	–	–
	40S + 60S	4.84	–	–
	EF-2	1.32	–	–
	+ 40S	2.66	0.17	–
	+ 60S	9.20	3.23	–
	+ 40S + 60S	12.72	6.56	0.03

* Average of duplicate runs; blank values of 3 pmol (expt. 1) and 4.7 pmol (expt. 2) have been subtracted.
** See Results for a description of this calculation.

Conditions similar to those of table 1 with EF-2, 1.5 μg (expt. 1) and 1.0 μg (expt. 2). Glycerol, 1.5% (expt. 1) and 1% (expt. 2) was present in all samples. Two different preparations of ribosomal subunits and purified *A. salina* EF-2 were used in expts. 1 and 2. The specific radioactivity of [γ-^{32}P]GTP used was 2535 cpm/pmol (expt. 1) and 3245 cpm/pmol (expt. 2).

conclusion which emerges from the present study is that the inhibitory effect of fusidic acid in eukaryotic systems is probably *not* restricted to EF-2-dependent functions only.

4. Discussion

It has been reported [8] that *partially purified* preparations of the reticulocyte initiation factor M_{2A} catalyzed a 40S subunit-dependent hydrolysis of both GTP and ATP. However, in contrast to the results reported here, this hydrolysis of GTP was *not* inhibited by fusidic acid [8]. Furthermore, it is not known with certainty whether the 40S subunit-dependent GTP'ase and ATP'ase activities as well as the M_{2A} activity are all mediated by the *same* protein. Several laboratories have described the partial purification of another initiation factor from eukaryotic cells which makes a ternary complex with GTP and initiator Met-$tRNA_f$ [1]. This factor has been named IF-MP by one group of investigators [10]. Experiments are now in progress to see whether the *A. salina* factor, described in this paper, has functional properties similar to those of IF-MP.

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