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Eukaryotic initiation factor 4A is the component that interacts with ATP in protein chain initiation

[wheat germ translation *in vitro*/2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate/5′-*p*-fluorosulfonylbenzoyladenosine]

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ABSTRACT **Protein synthesis in a resolved homogenate of wheat germ requires ATP and eight factors functioning at the level of protein chain initiation. To identify the component(s) interacting with ATP, the different factors were treated with the ATP affinity analogue 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) and tested for their function in protein synthesis. The activity of eukaryotic initiation factor 4A (eIF4A) was strongly curtailed, whereas all other factors were unaffected. At a concentration of 250 μM, AMP, ADP, and ATP protected eIF4A against FSBA inactivation, whereas at a concentration 50 μM, protection was afforded only by ATP. GTP did not protect at a concentration of 250 μM. In another approach, the substrate analogue 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) was found to inhibit protein synthesis in a manner, at least in part, competitive with ATP. Supplementing a TNP-ATP inhibited reaction with eIF4A substantially reversed the inhibition. Except for a small effect by factor C1, no reversal was obtained with any other component. Finally, a preincubation of ribosomes with ATP, mRNA, and eIF4A resulted in the formation of a complex capable of TNP-ATP-resistant amino acid incorporation. These data are interpreted to indicate that the primary interaction of ATP is with eIF4A. A model is proposed reconciling this conclusion with other observations relevant to the mRNA·ribosome attachment reaction.**

The function of ATP in protein synthesis in a role other than to provide aminoacyl tRNA was initially established by its requirement in experiments with precharged aminoacyl tRNAs as the source of the incorporated amino acid (1, 2). Subsequently, formation of the 80S ribosome·mRNA complex was found to be ATP-dependent, thus localizing the action of ATP to the process of protein chain initiation (3, 4). In studies of 40S ribosome·mRNA complex formation dependent upon guanyl-5′-yl imidophosphate (GMP-PNP), we have found, in agreement with Trachsel *et al.* (4), that ATP is still stringently required, thus ruling out a function solely to regenerate GTP (5, 6) and suggesting rather a direct interaction between ATP and the factors functioning in mRNA attachment. Other insights into the function of ATP have come from ribosome binding experiments with modified mRNAs, where reducing the secondary structure of the mRNA resulted in decreased requirement both for ATP and for the cap structure of the mRNA (7–9). The data were interpreted to suggest that ATP normally interacts with a cap-recognizing factor to unwind the secondary structure of the mRNA (10). In an analysis of the crosslinking of initiation factors to reovirus mRNA oxidized at the 5′ cap, an interaction was observed with eukaryotic initiation factors 4A and 4B (eIF4A and eIF4B) in a reaction stimulated by ATP and inhibited by m^7GDP (11). The reaction was dependent upon the simultaneous presence of both factors and possibly another M_r 24,000 cap-binding protein, but the interaction between ATP and the different components was not analyzed.

In our laboratory we have investigated the mRNA interaction process in an *in vitro* system from wheat germ. We recently described the resolution of the system (12–15) and demonstrated that two factors, eIF4A and C1, were required together with ATP to facilitate the mRNA-dependent formation of 80S ribosome·Met-$tRNA_i^{Met}$ complexes (13). In direct analyses of the formation of mRNA·ribosome complexes with radioactive mRNA, we have now confirmed that these three components function specifically in the mRNA attachment reaction (unpublished data). To determine directly which of the components is interacting with ATP, we have carried out studies with two analogues of ATP; 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA), an affinity analogue whose interaction with ATP binding proteins results in irreversible inactivation (16–18), and 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP), a substrate analogue (19, 20). The results of both studies lead to the conclusion that the primary interaction of ATP is with eIF4A.

MATERIALS AND METHODS

Factor Incubation with FSBA. Two and one-half microliters of 3 mM FSBA in 10% dimethyl sulfoxide was added to 7.5 μl of each of the different factor preparations (final concentration = 0.75 mM FSBA). The mixture was incubated in ice for 45 min and an aliquot (generally 6 μl) containing the standard amount of factor (see below) was added to the protein synthesis assay. The FSBA incubation contained 75–120 mM KCl, 0.2–0.6 mM dithiothreitol, 2.5–6 mM Tris acetate (pH 7.6), and 2.5–5% glycerol, all contributed from the solution of the factor. Varying the glycerol concentration from 0.5 to 5% and the dithiothreitol concentration from 0.1 to 1 mM did not affect either the lack of inhibition of factor C1 or the extent of inhibition of eIF4A (see Table 1). Control incubations with FSBA omitted were done for each factor to monitor the effect of incubation in dimethyl sulfoxide. In no case was there any loss of factor activity. The final concentration of FSBA in the protein synthesis assay was 20–60 μM, a level that had no effect on the reaction.

Preparation of the different factors, ribosomes, and [^{14}C]-aminoacyl tRNAs has been described (13). Wheat germ eIF4A is a homogeneous component with a M_r of 50,000 (13). Activity in protein synthesis was determined by tobacco mosaic virus (TMV) RNA-dependent amino acid polymerization in a 200-μl reaction mixture [as described (13, 14)], containing, in addition to the standard components (13), $K_{600}M_5$ ribosomes (60 μg of RNA), 6 μg of factor C1, 6 μg of C2(b + c) [elongation factor

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Abbreviations: FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; GMP-PNP, guanyl-5′-yl imidophosphate; eIF, eukaryotic initiation factor; EF, elongation factor; TMV, tobacco mosaic virus.

(EF) + CoeIF2], 2 μg of C2e (eIF2), 4 μg of eIF3, 2 μg of D2a, 2 μg of eIF4A, 1.2 μg of D2(c + d) (eIF4C + eIF5), and 40 μg of the phosphocellulose pass-through of fraction C2 [the 40–70% $(NH_4)_2SO_4$ precipitate of fraction C]. In one experiment (see Table 1), C2(b + c) was replaced by resolved EF1 and CoeIF2. FSBA was obtained from Sigma and TNP-ATP was from Molecular Probes (Junction City, OR).

RESULTS

Inactivation of eIF4A by Preincubation with FSBA. A number of enzymes that have ATP binding sites are inactivated by FSBA (16–18). This reagent therefore appeared to be appropriate to test the interaction of ATP with the components of the initiation system. We incubated each of the different factors with FSBA and added these back to the amino acid polymerizing system. The data of Table 1, experiment 1, show that the activity of component eIF4A was inhibited almost 80% by the FSBA incubation, whereas that of the other factors was essentially unaffected. To test the effect of the FSBA on eIF2 and CoeIF2, an experiment was done with resolved EF1 and the separated eIF2 components (experiment 2). Some inhibition of EF1 was observed, but the activity of the two initiation factors, eIF2 and CoeIF2, was unaffected.

The specificity of the FSBA inhibition as an indicator of an ATP binding site on eIF4A was tested by experiments in which different nucleotides were added to the FSBA incubation (Table 2). At the highest concentration (250 μM), both AMP and ATP were effective in protecting against the inhibition by FSBA, whereas at the lowest concentration (50 μM), 50% protection was obtained with ATP and AMP was ineffective. GTP, at 250 μM, did not protect the factor against the FSBA inactivation.

Inhibition of Translation by TNP-ATP and Its Reversal by eIF4A. In an alternative approach to localizing the site of interaction of ATP in the initiation system, we sought a substrate analogue of ATP that would, at least in part, be competitive with ATP. This would allow identification of the factor(s) reacting with ATP either by reversal of the inhibition of the analogue with excess factor or by the formation of a complex resistant to the inhibitor by prior incubation of the appropriate factor(s) with ATP. Initial experiments that varied the ATP concentration at 1.9 mM Mg^{2+} (the optimal concentration for 1 mM ATP) indicated an almost complete loss of activity at 0.5 mM ATP. The data of Fig. 1 show that this is due to the sensitivity of the protein synthesis reaction to Mg^{2+} concentration. In seeking an inhibitory analogue of ATP, it is therefore important to consider a possible effect on the free Mg^{2+} concentration. Furthermore, assays at different ATP concentrations must be monitored at the optimal Mg^{2+} concentration. Table 3 presents data with TNP-ATP, an analogue of ATP, which is a potent inhibitor of the protein synthesis reaction. The low concentrations of TNP-ATP necessary for inhibition have little effect on the free Mg^{2+} concentration, and the inhibition is, at least in

Table 1. Inhibition of eIF4A by preincubation with FSBA

Experiment	Factor	Amino acid incorporation, pmol: Factor omitted	Amino acid incorporation, pmol: Factor preincubated with FSBA
1	—	6.0	—
	C1	0.1	5.9
	eIF3	0.1	6.0
	D2a	0.3	5.4
	eIF4A	0.0	1.3
	eIF4C + eIF5	0.1	6.0
2	—	4.2	—
	EF1	0.1	3.0
	CoeIF2	1.7	3.6
	eIF2	1.3	4.2

The various factors were incubated in a volume of 10 μl of 0.75 mM FSBA for 45 min in ice (see text), and aliquots were assayed in the standard amino acid incorporation assay. In control incubations, the same factor was deleted to demonstrate its requirement. eIF2 could not be tested in the standard amino acid incorporation assay because C2(b + c) contains sufficient eIF2 to obscure its requirement; therefore, it was tested separately in experiment 2, in which C2(b + c) was replaced by 3.2 μg of EF1 (C2b) and 1.2 μg of CoeIF2 (C2c). The concentration curve of FSBA inhibition of eIF4A activity showed a steady increase in inhibition with 87, 68, 38, 13, and 4% residual activity at FSBA concentrations of 0.1, 0.25, 0.5, 1.0, and 2.0 mM, respectively. The lack of inhibition by FSBA with the factors shown in experiment 1 was also obtained at a FSBA concentration of 2 mM.

Table 2. Protection of eIF4A from FSBA inhibition by the presence of nucleotides in the preincubation

eIF4A preincubation	Amino acid incorporation, pmol
—	8.2
0.75 mM FSBA	1.7

	Nucleotide concentration, μM: 250	100	50
FSBA			
+ ATP	7.4	5.8	4.8
+ ADP	7.0	4.3	1.9
+ AMP	6.3	2.9	1.7
+ GTP	1.9	—	—

Conditions of the assay were those given in the legend to Table 1, experiment 1, with nucleotides added to the FSBA preincubation as indicated.

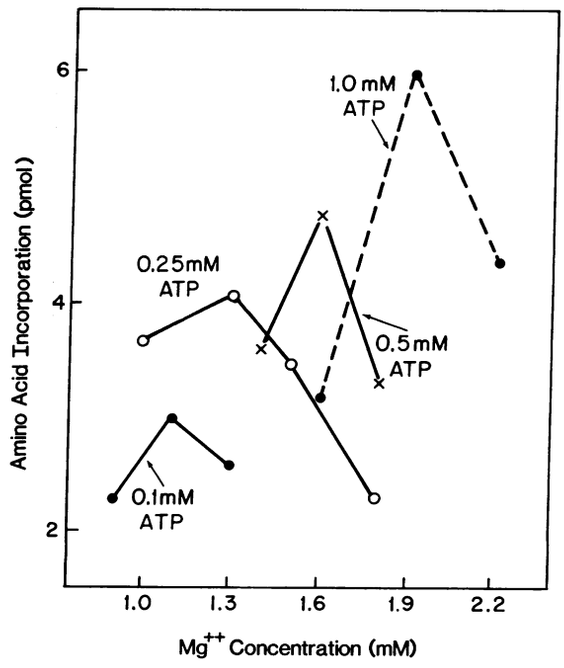

FIG. 1. Optimization of amino acid incorporation at different concentrations of ATP and Mg^{2+}. The standard amino acid incorporation incubation was carried out with ATP and Mg^{2+} varied as indicated. The following conditions resulted in a complete loss of incorporation: (*i*) omission of ATP at 0.7, 0.9, 1.3, and 1.9 mM Mg^{2+}; (*ii*) replacement of ATP by 1 mM GTP at 1.6, 1.9, and 2.2 mM Mg^{2+}.

Table 3. Inhibition of TMV RNA-dependent amino acid incorporation by TNP-ATP

TNP-ATP added, μM	Amino acid incorporation, pmol		
	At 0.25 mM ATP	At 0.50 mM ATP	At 1.0 mM ATP
—	3.0	3.9	6.1
40	0.7 (23)	1.5 (38)	2.9 (48)
80	—		0.8 (13)
120	—	—	0.1 (2)

The standard amino acid incorporation assay was used with Mg^{2+} at 1.3, 1.6, and 1.9 mM for the different concentrations of ATP. GTP was routinely added at 120 μM. In experiments with 0.50 mM ATP, the inhibition by 40 μM TNP-ATP was unaffected by varying the GTP concentration between 60 and 500 μM over a range of Mg^{2+} concentrations. The values in parentheses represent the percentage of activity relative to the incubation without TNP-ATP.

part, competitive with ATP. GTP does not reverse the TNP-ATP inhibition of protein synthesis (see legend to Table 3), and both poly(U)-dependent phenylalanine polymerization in the presence of EF1 and EF2 and eIF2-catalyzed binding of Met-$tRNA_i^{Met}$ to ribosomes are only minimally affected by 80–120 μM concentrations of this reagent (data not shown).

Given the suitability of TNP-ATP as a substrate analogue of ATP, translation experiments were carried out in which the level of each of the initiation factors was doubled in an attempt to reverse the TNP-ATP inhibition (Fig. 2). Doubling the amount

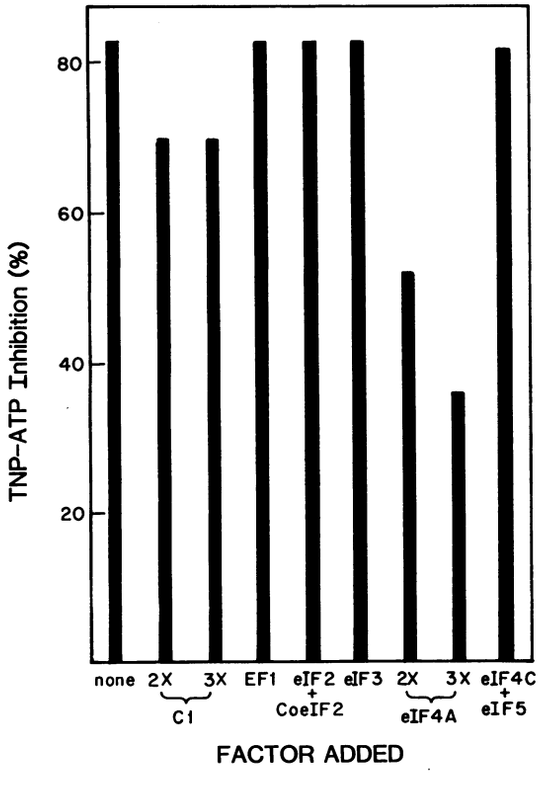

FIG. 2. Reversal of TNP-ATP inhibition by supplementation with eIF4A. The standard amino acid incorporation incubation was supplemented with an extra equivalent of each of the factors indicated. Factors C1 and eIF4A were also tested at an amount of supplement that was twice that of the initial incubation. Higher levels were not tested because of a general observation of inhibition when single factors are added in substantial excess. For each test, two incubations were done, with and without 80 μM TNP-ATP. In all cases, the noninhibited incubation resulted in 7.5–8.0 pmol incorporated and was unaffected by the extra amount of factor added.

Table 4. Preincubation of factors with ATP and the formation of complexes resistant to TNP-ATP

Components deleted from preincubation	Amino acid incorporation, pmol
None (no preincubation)	0.6
None	3.7
C1	3.3
eIF3	3.2
D2a	3.4
eIF4A	0.7
eIF4C + eIF5	3.3
TMV RNA	0.8
Ribosomes	1.4

A preincubation mixture in a volume of 180 μl contained all of the components of the standard amino acid incorporation assay, except that one component was deleted in each case as indicated. After 5 min at 20°C, the missing component was added back together with 120 μM TNP-ATP and [^{14}C]aminoacyl tRNAs to a final volume of 200 μl, and the incubation was continued for 20 min at 30°C. Similar incubations without addition of TNP-ATP resulted in the incorporation of 7.4–8.3 pmol, except when ribosomes were omitted from the preincubation, in which case the incorporation was 6.1 pmol.

of factor C1 resulted in a small but definite reversal of the inhibition, with no further effect obtained by a 3-fold level. No reversal was obtained with any of the other components, except with eIF4A. Doubling the amount of this factor reduced TNP-ATP inhibition from 83% to 52%, whereas a 3-fold level brought the inhibition down to 36%. We next attempted to form complexes resistant to TNP-ATP by preincubation in the presence of ATP. Table 4 shows that partial protection by a preincubation can indeed be obtained, resulting in an increase in incorporation in the presence of TNP-ATP from 0.6 to 3.7 pmol. Single deletion of the various components of the preincubation established that each of the factors except eIF4A and TMV RNA were not necessary, suggesting that the ATP-dependent complex could be formed in a reaction requiring only mRNA and eIF4A (and perhaps ribosomes; see legend to Table 4). Evidence for formation of such a complex is presented in Table 5. A preincubation containing mRNA, eIF4A, and ribosomes is

Table 5. Formation of TNP-ATP-resistant complexes by preincubation of eIF4A, mRNA, and ribosomes in the presence of ATP

eIF4A	TMV RNA	Amino acid incorporation, pmol	
		0 time	15 min at 2°C
+	+ (TNP-ATP added initially)	0.9	
+	+	2.4	1.0
−	+	0.7	
+	−	0.8	
+	+ (eIF3 added)	2.2	1.5
+	+ (C1 added)	2.2	1.5
+	+ (eIF3 + C1 added)	2.6	2.9

The preincubation mixture in 170 μl contained ribosomes, TMV RNA, eIF4A, eIF3, and C1, as indicated, in addition to other nonfactor components normally present in the complete amino acid incorporation assay. After 5 min at 20°C, the missing factors were added together with 80 μM TNP-ATP and [^{14}C]aminoacyl tRNAs to a volume of 200 μl, and the incubation was continued for 20 min at 30°C. An assay without TNP-ATP that was not preincubated incorporated 6.2 pmol. In a second set, the indicated preincubated mixtures were kept for 15 min in ice (after the 20°C preincubation) prior to the assay of incorporation.

capable of significant amino acid polymerization in the presence of TNP-ATP, and further addition of eIF3 and factor C1 has little effect on the extent of complex formation. However, the primary complex is unstable and is lost upon incubation for 15 min in ice. Formation of a secondary complex containing both factor C1 and eIF3 provides increased stability.

eIF4A Is Not a Cap-Binding Protein. The earlier suggestion that ATP interacts with a cap-binding protein (see Introduction) made it of interest to ascertain whether eIF4A was a cap-binding protein. In an experiment done in collaboration with N. Sonenberg (Department of Biochemistry, McGill University), incubation of eIF4A with cap-oxidized methyl-labeled reovirus RNA (21) did not result in crosslinking between the protein and the cap end of the mRNA. In a more sensitive test, 5 mg of fraction D2(a + b), from which pure eIF4A is obtained in a yield of 10% (13), was passed through a column of m^7GDP-agarose and the bound protein (0.26 mg) was eluted with m^7GDP. In an assay of eIF4A activity, in which 0.0 pmol of amino acid was incorporated in the absence of eIF4A and 6.7 and 9.8 pmol were incorporated in the presence of 1 and 2 μg of the factor, respectively, addition of 1 μg of the m^7GDP eluate resulted in 0.0 pmol incorporated. In addition, full recovery of the eIF4A activity was obtained in the protein fraction passing through the m^7GDP agarose column. eIF4A is clearly not a cap-binding protein.

DISCUSSION

mRNAs with reduced secondary structure form ribosome complexes with a lesser requirement for both ATP and the cap structure of the mRNA (7–9). Crosslinking of the cap structure of such mRNAs to several cap-binding proteins is also less dependent on ATP (22). Both of these results would suggest an ATP interaction with a cap-binding entity. Nevertheless, the results presented here provide strong evidence that the component of the wheat germ initiation system that directly interacts with ATP is eIF4A. Because eIF4A alone is not a cap-binding protein, we suggest that the interaction between ATP and the cap-binding entity is a secondary consequence of the eIF4A reaction. A reasonable mechanism consistent with this consideration would involve the separate attachment of a cap-binding entity (and perhaps an additional mRNA-binding protein) to a region near the 5′ end of the mRNA and an ATP-dependent attachment of eIF4A to a region downstream from these factors. The eIF4A attachment would result in a change in the orientation of the mRNA such that the cap-binding entity could interact directly with the cap of the mRNA. An unwinding function would then be carried out by the coupled system, perhaps with the further hydrolysis of ATP (8). The 40S ribosome would attach to the mRNA either initially as part of a complex with the mRNA-binding factors or subsequently after a part of the mRNA was converted to a single-stranded form. Such a mechanism is consistent with the ability to form an ATP-dependent complex in a reaction containing only eIF4A and mRNA (Tables 4 and 5) and with the stabilization of the complex by component C1 (Table 5). The two-step mechanism would further allow: (*i*) that the ATP interaction is solely with eIF4A, although its function would be related to that of the cap-binding entity, and (*ii*) that the determining step as to whether a particular mRNA was or was not translated could be its affinity for a mRNA-interacting protein other than eIF4A. In the wheat germ system under conditions of mRNA competition (23), translation of competitively inhibited mRNAs is restored specifically by addition of increasing amounts of factor C1 (13). This factor would be providing the "mRNA-interacting" components that function separately from the eIF4A–ATP reaction. (*iii*) mRNAs with decreased secondary structure could to a considerable degree bypass the ATP–eIF4A reaction and interact directly with the ribosome. Thus, although both the ATP–eIF4A reaction and the cap structure of the mRNA normally function to increase the affinity of a mRNA for the ribosome attaching system, a single-stranded RNA would have a greater affinity in its own right and render both ATP and the cap structure unnecessary. A test of aspects of this scheme should be possible by the analysis of the factor requirement for the ribosome binding of mRNAs having little secondary structure. Direct analyses of intermediate complexes may also be possible by short-range crosslinking (24).

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