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CERTAIN PHOSPHORYLATED SUGARS CAN PREVENT THE mRNA-INDUCED INHIBITION OF $Met\text{-}tRNA_f^{Met}$ BINDING TO INITIATION FACTOR eIF-2

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SUMMARY: The effect of mRNA on the GTP-dependent binding of initiator tRNA to partially purified reticulocyte eIF-2 has been examined in the presence of a number of sugars and phosphorylated sugars. The inhibitory effect of mRNA (0.25 µg-0.42 µg/50 µl assay) is largely prevented by the simultaneous addition of fructose 6-phosphate (0.09 mM-0.45 mM). Glucose 6-phosphate (0.45 mM) and fructose 1,6-diphosphate (0.45 mM) are 62% and 47% as effective as fructose 6-phosphate. Other sugars and phosphorylated sugars tested, including deoxyglucose 6-phosphate, give negative results.

It is generally believed that a key step in the regulation of eukaryotic peptide chain initiation is the formation of a ternary complex between GTP, initiator tRNA ($Met\text{-}tRNA_f^{Met}$) and initiator factor eIF-2 (1-5). This protein has been purified extensively from rabbit reticulocytes and has been shown to be composed of three polypeptide chains of approximate molecular weights 35,000 to 38,000 (α subunit), 48,000 to 50,000 (β subunit), and 52,000 to 55,000 (γ subunit), respectively (6-8). Numerous studies have demonstrated that the inhibition of protein synthesis in rabbit reticulocyte lysates (e.g. by the addition of the hemin-regulated inhibitor, or by addition of inhibitors activated by double-stranded RNA or oxidized glutathione) is accompanied by the increased phosphorylation of eIF-2α (1). Recently, a number of laboratories have reported that these inhibitions can be partially or totally reversed by the addition of phosphorylated sugars (9-11). The

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exact mechanism for the restorative effects of phosphorylated sugars is not known. One possible mechanism is that the phosphorylated sugars interact with eIF-2 so that it can not become the target site for the inhibitors.

In the present investigation, we have examined the interaction between phosphorylated sugars and eIF-2 by studying the inhibitory effect of mRNA on the binding of initiator tRNA to eIF-2 in the presence of a number of sugars and phosphorylated sugars. The data show that the inhibition by mRNA is effectively prevented by fructose 6-phosphate (0.09 mM-0.45 mM).

MATERIALS AND METHODS

Sugars and phosphorylated sugars were purchased from Sigma Chemical Co. L-[$^{35}_{s}$] methione was from New England Nuclear. Rabbit reticulocyte lysates were purchased from Clinical Convenience Products (Madison, Wisconsin). [^{35}s] Met-tRNA$^{Met}_{f}$ was prepared with crude rabbit liver tRNA and E.Coli tRNA-synthetase as described previously (12). The reticulocyte polysome and the 0.5 M KCl ribosomal wash were obtained as described previously (12). Initiation factor eIF-2 was prepared from the 0.5 M KCl ribosomal wash by ammonium sulphate precipitation (70%) and phosphocellulose chromatography essentially according to Walton and Gill (13) except that eIF-2 was eluted with 0.7 M KCl and concentration with solid polyethylene glycol was omitted. The eIF-2 binds 500 pmole initiator tRNA per mg protein using the conditions described.

Assay for Met-tRNA$^{Met}_{f}$.eIF-2.GTP ternary complex- eIF-2 activity was measured by its ability to form the ternary complex as quantitated by the retention of the complex on Millipore filters. The incubation mixture contained 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM dithiothreitol, 0.2 mM GTP and 1.2 pmole of [^{35}s] met-tRNA$^{Met}_{f}$ (total volume 0.05 ml). The reaction was initiated by the addition of initiator tRNA. Incubations were at 30^{o}C, 5 min. The reaction was terminated by the addition of 3 ml of ice cold buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl and 2 mM dithiothreitol), filtered under suction through Millipore filters (0.45 μM pore diameter, presoaked in buffer), washed three times with 3ml of the same buffer. Radioactivity retained on the filters was determined by liquid scintillation spectrometry using a toluene-based solution (Scintilene, Fisher).

Preparation of poly (A) rich rabbit reticulocyte mRNA- Polysomal RNA was prepared from the salt-washed ribosomes using the procedures described previously (14). The salt-washed ribosomes was suspended in 0.25 M sucrose, 1 mM

dithiothreitol and 0.1 mM EDTA, pH 7.0 to give 300 OD_{260}/ml. Two ml of 1% SDS buffer (5 mM EDTA, 10 mM Tris-HCl and 1% SDS, pH 7.5) was added to one ml of the suspension and the mixture extracted twice with 3 ml of water-saturated chloform-phenol (1:1, v/v). The acqueous phase was adjusted to 0.2 M ammonium acetate and the RNA was precipitated by 2 vol absolute ethanol (18 hr.-40°C). The precipitate was washed twice with 2 M LiCl, pH 5.5 and twice with 70% ethanol, dried in vacuo and dissolved in sterilized glass distilled water. The RNA sample (0.75 ml, 30 OD_{260}/ml) was mixed with 0.25 ml of 4 M NaCl, 1 ml of 1% SDS buffer, heated to 50°C for 2 min and applied to a small oligo (dT)-cellulose column (200 mg) equilibrated with 0.5% SDS buffer containing 0.5 M NaCl. Five ml of 0.5 M NaCl was used to wash the column. Poly (A) rich mRNA was eluted with water and concentrated by precipitation with ethanol. The precipitated RNA was dried and dissolved in glass distilled water to give 83 µg/ml (1 mg= 24 OD_{260}).

Table 1

Effect of different concentrations of fructose 6-phosphate on reticulocyte mRNA - induced inhibition of Ternary complex formation.

mRNA added (µg)	Fructose 6-Phosphate Added (mM)	[^{35}S] Met-$tRNA_f^{Met}$ bound (pmole)[a]	Inhitition by mRNA (%)	Protection by Fructose 6-phosphate (%)
0	0.00	0.44	100	-
	0.09	0.43	98	-
	0.45	0.44	100	-
0.25 µg	0.00	0.28	36[b] (100)	0
	0.09	0.41	7 (19)	81
	0.45	0.42	5 (14)	86
0.42 µg	0.00	0.22	50[b] (100)	0
	0.09	0.40	9 (18)	82
	0.45	0.40	9 (18)	82

a. GTP-dependent binding of [^{35}S] Met-$tRNA_f^{Met}$ (1.2 pmole .4400 cpm) was assayed in a 50 µl reaction mixture containing 1.4 µg of eIF-2 (2 µl) as described in Materials and Methods. Background without eIF-2 (0.01-0.02 pmole) was not subtracted.

b. Inhibition of ternary complex formation by mRNA is taken to be 100%.

RESULTS AND DISCUSSION

In agreement with results reported by others (15-18), addition of poly (A) rich reticulocyte mRNA strongly inhibits the GTP-dependent binding of initiator tRNA to partially purified eIF-2; a 36% and a 50% inhibition is observed by 0.25 µg and 0.42 µg mRNA in a 50-µl assay (Table 1). In contrast, when reaction mixtures are supplemented with uncharged tRNA or rRNA, no inhibition is observed (data not shown). The addition of fructose 6-phosphate (0.09 mM-0.45 mM), while exerting no effect by itself, largely prevented the mRNA-dependent decrease in ternary complex formation (Table 1).

The effect of glucose, fructose and several phosphorylated sugars on the inhibition of ternary complex by mRNA is shown in Table 2. At 0.09 mM, only fructose 6-phosphate significantly prevented the inhibition (Table 2, Experiment 1). At 0.45 mM, both glucose 6-phosphate and fructose 1,6-diphosphate partially prevented the effect of mRNA and are 62% and 47% as effective as fructose 6-phosphate. All other sugars and phosphorylated sugars tested, including deoxyglucose 6-phosphate, are without effect. Under the conditions used, all sugars and phosphorylated sugars tested do not affect ternary complex formation in the absence of added mRNA (data not shown).

The ability of selected phosphorylated sugars to prevent the inhibition of ternary complex formation by mRNA can be attributed to an interaction between eIF-2 and the phosphorylated sugars. In support of this possibility, we observe that eIF-2 becomes more thermostable in the presence of fructose 6-phosphate (0.45 mM) (Table 3). We are currently studying the conformational change in eIF-2 with mRNA and phosphorylated sugars by circular dichroism.

Table 2

Effects of certain sugars and phosphorylated sugars on reticulocyte mRNA - induced inhibition of Ternary complex formation.

Addition	[^{35}S] Met-tRNA$_f^{Met}$ bound[a] (pmole)	Inhibition by mRNA (%)	Protection by Sugars or Phosphorylated sugars (%)
Experiment 1			
Minus mRNA	0.54	100	-
plus mRNA[b]	0.22	59[d](100)	0
plus mRNA and G6P (mM)			
0.09	0.25	54(92)	8
0.45	0.35	35(59)	41
plus mRNA and F6P (mM)			
0.09	0.33	39(66)	34
0.45	0.43	20(34)	66
plus mRNA and FDP (mM)			
0.09	0.26	52(88)	12
0.45	0.32	41(69)	31
plus mRNA and dG6P (mM)			
0.09	0.17	69(117)	0
0.45	0.18	67(114)	0
Experiment 2[c]			
Minus mRNA	0.54	100[d]	-
plus mRNA	0.25	54(100)	0
plus mRNA and			
glucose	0.28	48(89)	11
G1P	0.28	48(89)	11
Fructose	0.22	59(109)	0
F1P	0.18	67(124)	0
R5P	0.24	56(103)	0
G6P	0.34	37(69)	31
F6P	0.41	24(44)	56
FDP	0.34	37(69)	31
dG6P	0.25	54(100)	0

a. GTP-dependent binding of [^{35}S] Met-tRNA$_f^{Met}$ (1.2 pmole.4300 cpm) was assayed using 1.4 µg of eIF-2 as described in Materials and Methods.

b. Reticuloeyte mRNA (0.42 µg) was added

c. Concentrations of all sugars and phosphorylated sugars were 0.45 mM.

d. Inhibition by mRNA is taken to be 100%.

Table 3

Effect of fructose 6-phosphate on the heat stability of partially purified eIF-2

Treatment[a]		[35S]Met-tRNA$_f^{met}$ bound (pmole)
eIF-2 heated alone		
	0.0 min	0.52
	2.0 min	0.31
	5.0 min	0.12
eIF-2 heated with F6P (0.09 mM)		
	0.0 min	0.54
	2.0 min	0.39
	5.0 min	0.19
eIF-2 heated with F6P (0.45 mM)		
	0.0 min	0.55
	2.0 min	0.44
	5.0 min	0.27

a. eIF-2 (14 µg) was incubated with 20 mM Tris-HCl, 100 mM KCl, 1 mM dithiothreitol, 0.05 mM EDTA, 10% glycerol (total volume 0.05 ml), with or without F6P for 5 min at 4° C, and then placed in a 55° C water bath. At the indicated times, aliquots (15 µl) were removed and cooled in an ice bath for 5 min. Ternary complex formation was assayed with 1.6 µg of heated eIF-2, as described in Materials and Methods.

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