EFFECTS OF PHOSPHORYLATED SUGARS ON THE FORMATION AND EXPRESSION OF AN INHIBITOR OF PROTEIN SYNTHESIS ACTIVATED BY OXIDIZED GLUTATHIONE IN RABBIT RETICULOCYTE LYSATES

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1. Introduction

Initiation of protein synthesis in rabbit reticulocyte lysates is inhibited by: (i) a lack of hemin; (ii) the addition of low concentrations of natural and synthetic double-stranded RNA (dsRNA); (iii) an increase in incubation temperature to 42–45°C; and (iv) the addition of oxidized glutathione (GSSG) (review [1–3]). The inhibition of protein synthesis due to hemin deficiency has been shown to be related to the activation of an inhibitor (the hemin-controlled inhibitor) with an associated eIF-2α-specific protein kinase. The specific phosphorylation of eIF-2α has been proposed to be the major event leading to the inhibition of polypeptide synthesis [1–3]. Much progress has also been made in elucidating the mechanism(s) involved in the inhibitory activities of dsRNA. At least 3 events are pertinent to the action of dsRNA: activation of a protein kinase which selectively phosphorylates eIF2; synthesis of a unique oligonucleotide pppApApA linked by a 2′–5′ phosphodiester bond; and activation of an endonuclease by pppApApA (review [4–6]). The precise manner whereby these 3 events and the inhibition of polypeptide synthesis are related is not completely known.

The mode of action of GSSG has been studied in [7,8]. The results show that inhibition of polypeptide synthesis by GSSG is: (i) associated with the activation of a specific eIF-2α kinase; and (ii) readily reversed by the addition of G6P, F6P, dG6P and several other hexose monophosphates [7,8]. It was suggested that G6P or a derivative has a direct role in polypeptide chain initiation which is distinct from the participation of G6P in glycolysis, NADPH regeneration, or in the pentose phosphate pathway [8].

Here, the effects of selected phosphorylated sugars on the formation and expression of oxidized glutathione-induced inhibition of polypeptide synthesis [GSSG-I] are examined. The results show that whereas G6P or F6P affect both the formation and expression of GSSG-I, dG6P affects only the expression of GSSG-I. FDP is without effect.

Abbreviations: dsRNA, double-stranded RNA; eIF-2, eukaryotic initiation factor 2, designating the initiation factor which forms a ternary complex with Met-tRNA$_f^{Met}$ and GTP; pppApApA, 2′–5′-linked oligonucleotide; GSSG, oxidized glutathione; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1, 6-diphosphate; dG6P, 2-deoxyglucose 6-phosphate; GSSG-I, GSSG-activated inhibitor of protein synthesis; CP/CPK, creatine phosphate/creatine phosphokinase

2. Materials and methods

Rabbit reticulocyte lysates were obtained from Pel-Freeze Biologicals. (Lot 92578 showed a 2.8–3.2-fold stimulation by 26.7 μM hemin in 60 min, 30°C. Lot 103178 showed a 4.2-fold stimulation by 26.7 μM hemin in 60 min, 30°C.) [U-^{14}C]Leucine (270 mCi/mmol) was obtained from ICN Pharmaceuticals. [8-^{3}H]ATP (29 Ci/mmol), [U-^{14}C]G6P (241 mCi/mmol), [U-^{14}C]F6P (268 mCi/mmol) and [U-^{14}C]-FDP (327 mCi/mmol) were from Amersham/Searle. Other materials were from the sources in [9–11]. Cell free protein synthesis assays were done in 30 μl aliquots as in [9–11].

GSSG-I was activated as in [7] and partially purified by precipitation with ammonium sulfate (0–50%). The partially purified inhibitor was stored at −70°C.

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2.1. *Thin-layer chromatography*

The metabolism of [^{3}H]ATP, [^{14}C]G6P, or [^{14}C]-F6P was determined by two separate methods:

(1) Chromatography on polyethyleneimine-cellulose (Machery-Nagel, polygram cel 300 PEI), using 1 M LiCl as the solvent (R_F-values of AMP, ADP, G6P, F6P and FDP are 0.35, 0.23, 0.1, 0.48, 0.41 and 0.02, respectively);

(2) Chromatography on cellulose plates (Eastman Kodak, no. 13254), using isobutyric acid:ammonium hydroxide:water (66:1:33) as solvent (R_F-values of AMP, ADP, ATP, G6P, F6P and FDP are 0.54, 0.42, 0.28, 0.24, 0.31 and 0.17, respectively).

3. Results and discussion

When hemin-supplemented reticulocyte lysates are incubated with GSSG and the GSSG subsequently removed with dithiothreitol, an inhibitor of protein synthesis becomes activated progressively (fig.1A,○). Addition of G6P or F6P (3 mM) at the beginning of incubation completely suppresses the inhibitor activation (fig.1A,▲,△), while identical concentrations of FDP or dG6P are without effect (fig.1A,■,□). The addition of GSSG-I to a fresh reticulocyte lysate has no effect on protein synthesis for the first 5 min. Thereafter, the rate of polypeptide synthesis is largely reduced (fig.1B,○). The addition of ATP (2 mM) and

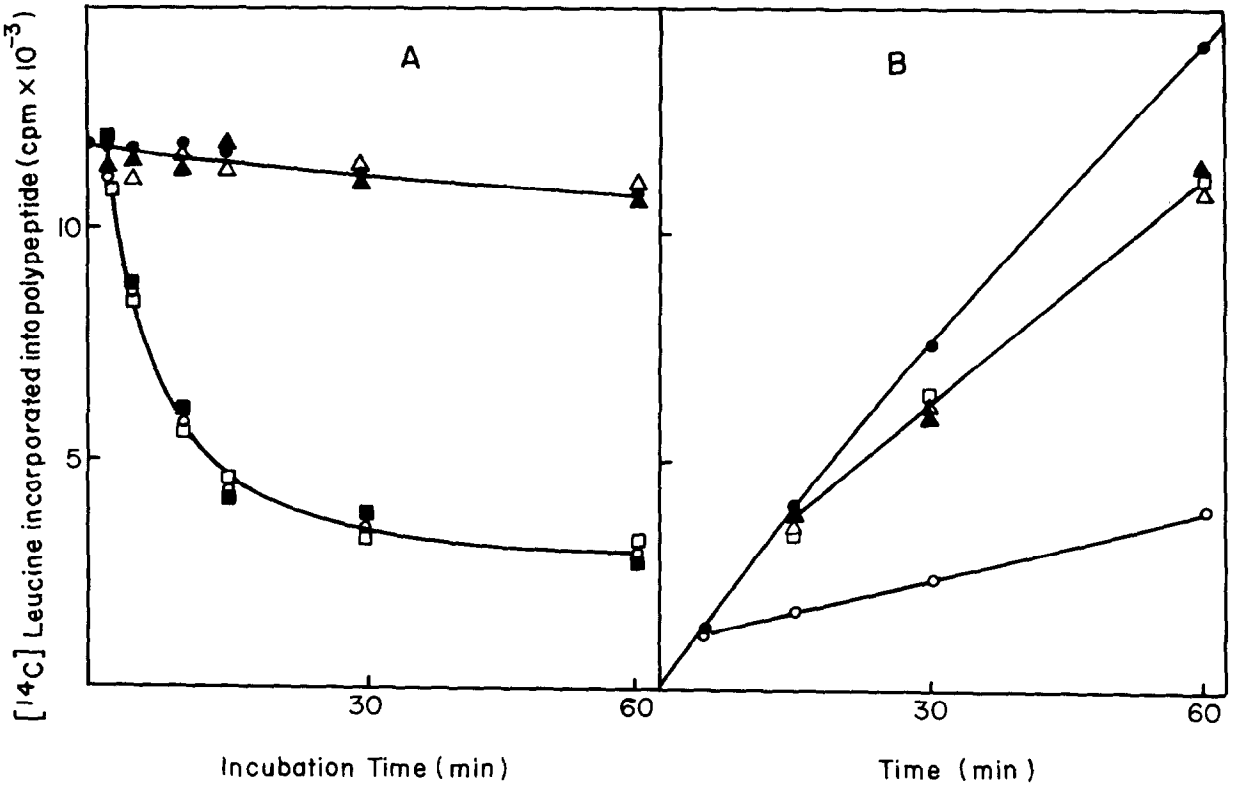

Fig.1. Effects of (A) phosphorylated sugars on the formation of GSSG-I, and (B) GSSG-I (formed in the presence of absence of phosphorylated sugars) on polypeptide synthesis in rabbit reticulocyte lysates. (A) The inhibitor was formed (as in section 2) in the absence or presence of GSSG. At the indicated times, an aliquot (1.5 μl) of the diluted preparation was assayed for inhibitory activity using a fresh protein synthesis reaction mixture (total vol. 15 μl). The incorporation of leucine into polypeptide was measured following incubations at 30°C, 60 min. (●–●) – GSSG; (○–○) + GSSG (2.5 mM); (▲–▲) + GSSG and G6P (3 mM); (△–△) + GSSG and F6P (3 mM); (■–■) + GSSG and FDP (3 mM); (□–□) + GSSG and dG6P (3 mM). (B) The incorporation of [^{14}C]leucine into polypeptides was measured by removing 5 μl aliquots at the indicated times. (●–●) Control; (□–□) control + an aliquot incubated without GSSG; (○–○) control + an aliquot incubated with GSSG (2.5 mM); (△–△) control + an aliquot incubated with GSSG and F6P (3 mM).

Table 1
Effects of CP/CPK and ATP on the activation of a translational inhibitor induced by GSSG and the effects of sugar phosphates on the activation[a]

	Polypeptide synthesis[b]				
	−GSSG	+GSSG			
		None	+G6P	+F6P	+FDP
None	12 610	3440	11 680	11 460	3020
+ATP (2 mM)	13 910	3120	11 780	10 630	2790
+CP/CPK	12 960	3040	10 420	9670	3190
+ATP and CP/CPK	12 860	2850	4010	6690	2610

[a] All sugar phosphates were added at 3 mM. Creatine phosphate (10 mM) and creatine phosphokinase (200 μg/ml) were added at the beginning of the activation step
[b] Inhibitor activation and assay of polypeptide synthesis were as in section 2

an ATP-regenerating system (provided by CP/CPK) largely abolished the ability of F6P or G6P to maintain GSSG-I in an inactive state (table 1). These results suggest that:

(i) Maintenance of inactive GSSG-I by G6P or F6P may be related to the utilization of endogenous ATP by these two phosphorylated sugars via partial glycolytic reactions;
(ii) ATP appears to be an essential component for the activation of GSSG-I.

We had shown that reticulocyte lysates do not contain detectable amounts of endogenous NAD^+ [12], hence the metabolism of glycolytic sugars is effectively blocked at the step catalyzed by glyceraldehyde-3-phosphate dehydrogenase [13]. The absence of endogenous NAD^+ in reticulocyte lysates is likely attributed to an active NAD^+ glycohydrolase present in the lysate [14].

The possibility of ATP utilization by G6P or F6P through glycolysis is tested by two additional experiments.

(1) When [^{3}H]ATP is incubated with the lysate, using conditions identical to those required for activation of GSSG-I, the hydrolysis of ATP to ADP progresses linearly for the first 20 min (fig.2,●). By 60 min, 75% of the endogenous ATP is hydrolyzed. The rate of endogenous ATP hydrolysis is not affected by the addition of dG6P (fig.2,△) but shows a 40% decrease with the addition of FDP (fig.2,△). However, when 3 mM G6P or F6P is added, >95% of the endogenous ATP is utilized within the first 2 min (fig.2,○,■).

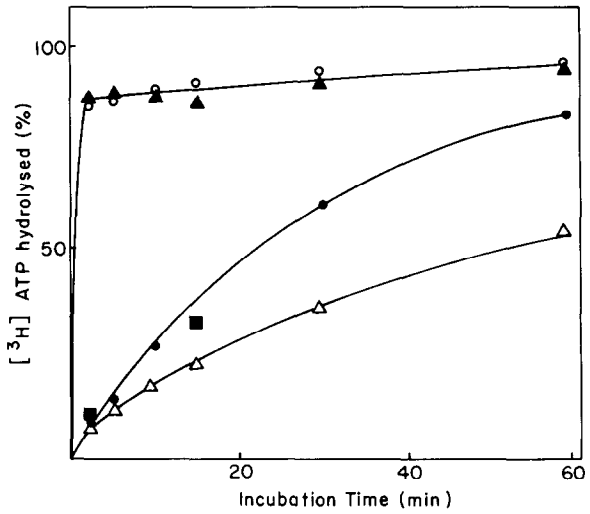

Fig.2. Utilization of endogenous ATP during the activation of an inhibitor of protein synthesis by addition of GSSG and sugar phosphates. The reaction mixture for the activation of GSSG-I (as in section 2) (100 μl) was supplemented with 10 μCi [^{3}H]ATP (29 Ci/nmol). The reaction was started at 37°C. At specified times, 15 μl aliquots were removed and mixed with 15 μl ice-cold glass-distilled water, and immediately put into a boiling water bath, 5 min. The reaction mixture was centrifuged in a Brinkmann centrifuge, 2 min. The deproteinized supernatant (5 μl) was spotted onto either PEI-cellulose or cellulose plates with markers and developed in the solvent systems (as in section 2). Sections (1 cm) of the plates were cut out, put into mini-vials and counted for radioactivity. The radioactivity in the ATP region was determined as % ATP hydrolyzed. The values obtained by both methods were averaged (values obtained agree to within 10%) and plotted as a function of time of incubation. (●–●) Control (+ or − 2.5 mM GSSG); (○–○) + GSSG and G6P (3 mM); (▲–▲) + GSSG and F6P (3 mM); (△–△) + GSSG and FDP (3 mM); (■–■) + GSSG and dG6P (determined only for 2 min and 15 min incubations).

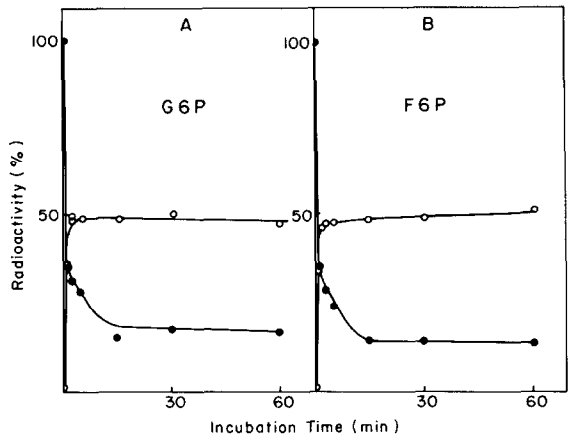

Fig.3. The metabolism of G6P or F6P during the activation of GSSG-I; reaction mixture for the activation of GSSG-I (as in section 2) (100 μl) was supplemented with 3 mM G6P and 0.5 μCi [U-^{14}C]G6P (A), or with 3 mM F6P and 0.5 μCi [U-^{14}C]F6P (B). The reactions started, terminated and chromatographed as in fig.2. (A) (●–●) radioactivity in G6P region; (○–○) radioactivity in FDP region. (B) (●–●) radioactivity in F6P region; (○–○) radioactivity in FDP region.

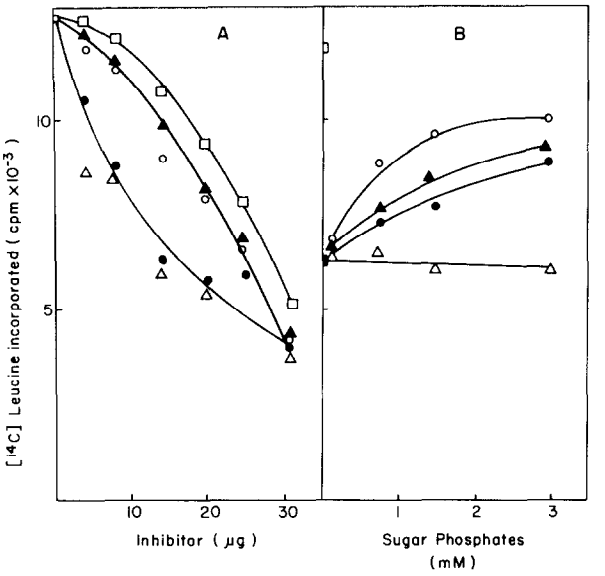

Fig.4. Effects on phosphorylated sugars on the expression of a partially purified inhibitor of protein synthesis activated by GSSG. (A) Effects of phosphorylated sugars on varying concentrations of GSSG-I. Phosphorylated sugars (3 mM were added to protein synthesis assay mixtures, (total vol. 15 μl) to which varying concentrations of GSSG-I have been added. Incubations were at 30°C, 60 min, at the end of which 5 μl aliquots were removed to determine the incorporation of leucine into polypeptides. (●–●) control; (▲–▲) control + F6P; (○–○) control + G6P; (△–△) control + FDP; (□–□) + dG6P. (B) Effects of varying concentrations of phosphorylated sugars on the inhibition of protein synthesis by GSSG-I (15 μg/15 μl assay). (□) Control; (■) control + GSSG-I; (○–○) dG6P + GSSG-I; (▲–▲) G6P + GSSG-I; (●–●) F6P + GSSG-I.

(2) When the lysates are incubated with [U-^{14}C]G6P (3 mM) or [U-^{14}C]F6P using the same conditions as those for GSSG-I activation, ~60% of G6P or F6P is metabolized in the first min (fig.3) and 90% of the metabolized product appears as FDP by thin-layer chromatography, using two separate solvent systems (see section 2).

These data strongly support the notion that the effects of G6P or F6P in suppressing activation of GSSG-I is likely the ability of these two phosphorylated sugars to utilize ATP via parital glycolytic reactions, and lend further support to the proposal that ATP is essential for GSSG-I activation. The exact role(s) of ATP in the activation process, however, still remains to be established.

The effects of selected phosphorylated sugars on the expression of the inhibitory activity by partially purified GSSG-I were also studied. An increase in protein synthesis inhibition was observed with the addition of increasing amounts of GSSG-I (fig.4A,●). At non-saturating concentrations of GSSG-I (<15 μg/15 μl assay), dG6P effectively prevents the expression of the inhibitory activity (fig.4A,□), G6P and F6P are 70–80% as effective (fig.4A,▲,○), whereas FDP is completely without effect (fig.4A,△). At saturating concentrations of GSSG-I (30 μg/15 μl assay) however, G6P or F6P cannot reverse the inhibitory effect of GSSG-I; some reversal (10–15%) of inhibition was consistently observed with dG6P (fig.4A). When inhibition of protein synthesis was assayed using a non-saturating concentration of GSSG-I, dG6P is effective in preventing GSSG-I inhibition of protein synthesis (fig.4B,○), G6P or F6P is much less effective than dG6P (35–50% as effective) at low concentrations (1 mM) but approaches the effect of dG6P at higher concentrations (3 mM) (fig.4B,▲,●). This is presumably a result of the rapid metabolism of G6P or F6P via partially glycolytic reactions, whereas dG6P apparently is quite stable.

Although there has been much progress in the identification and purification of protein factors and other components required for the assembly of the initiation complex, the details of the control of pro-

tein synthesis at the translational level still remains relatively unknown [1–3]. The phosphorylated sugars have a vital role(s) in regulating the initiation step of the protein synthetic process [7,8,15–17]. These results show that phosphorylated sugars can participate in reactions involved in both the formation and expression of an inhibitor of protein synthesis induced by GSSG. Studies to define the role(s) of ATP and GSSG in the activation of GSSG-I, and the role(s) of phosphorylated sugars in preventing the expression of GSSG-I are in progress.

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