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INHIBITION OF PROTEIN SYNTHESIS BY GLUCOSE 6-PHOSPHATE AND FRUCTOSE 1,6-DIPHOSPHATE IN LYSED RABBIT RETICULOCYTES AND THE REVERSAL OF INHIBITION BY NAD^+

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SUMMARY: Glucose 6-phosphate and fructose 1,6-diphosphate inhibit protein synthesis when added to lysed rabbit reticulocytes. Protein synthesis is inhibited 47% with 6 mM fructose 1,6-diphosphate and 86% with 6 mM glucose 6-phosphate. With 0.125 mM NAD^+, the inhibitory effect of glucose 6-phosphate and fructose 1,6-diphosphate becomes stimulatory. The stimulation of protein synthesis in those assays with NAD^+ and the phosphorylated sugars is 50% above those assays that contain NAD^+ alone. The inhibition of protein synthesis by glucose 6-phosphate and the reversal of this inhibition by NAD^+ occurs at a step before the synthesis of the initial dipeptide, methionyl-valine. These data illustrate the importance of NAD^+ and the activation of glycolysis in regulating protein synthesis in lysed rabbit reticulocytes.

Previous studies from this laboratory have shown that NAD^+ (up to 0.25 mM) can stimulate the initiation step of the protein synthetic process in lysed rabbit reticulocytes (1-4). NAD^+ added to lysates relieves a block at the glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase steps in glycolysis which allows for the activation of glycolysis and results in the generation of high-energy nucleoside phosphates that are depleted during protein synthesis (2). Activation of glycolysis by NAD^+ is supported by the finding that in the presence of NAD^+, there was a drastic reduction in the concentration of glucose 6-phosphate, fructose 1,6-diphosphate and other phosphorylated glycolytic intermediates; the lactate concentration was increased correspondingly (2). Glucose 6-phosphate and fructose 1,6-diphosphate have been reported by Baglioni and coworkers to stimulate protein synthesis in gel-filtered reticulocyte lysates and lysates derived from tumor cells (6,7). Studies reported here were done to examine more closely the role of phosphorylated hexoses and NAD^+ in protein synthesis

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with lysed rabbit reticulosytes. The data clearly show that glucose 6-phosphate and fructose 1,6-diphosphate inhibit protein synthesis in lysed rabbit reticulocytes. This inhibition was totally reversed by 0.125 mM NAD^+. The inhibition of protein synthesis by glucose 6-phosphate and the reversal by NAD^+ precedes the formation of the first peptide bond. The data provide new evidence for the importance of phosphorylated sugars, NAD^+ and glycolysis in the regulation of protein synthesis in rabbit reticulocyte lysates.

MATERIALS AND METHODS

Glucose 1-phosphate (dipotassium salt), glucose 6-phosphate (disodium salt), fructose 1-phosphate (di-monocyclohexylammonium salt), fructose 6-phosphate (sodium salt) and fructose 1,6-diphosphate (trisodium salt) were from Sigma Chemical Co. Pactamycin was a generous gift of Dr. S. Pestka, Roche Institute of Molecular Biology. Other chemicals were from sources described previously (1,2).

Cell-free protein synthesis assays were done in 60 µl aliquots containing 25 µl lysate, 1.45 mM $MgCl_2$, 20 mM HEPES/KOH (pH 7.4), 80 mM KCl, 15 µM hemin, 1.0 mM ATP, 0.2 mM GTP, 19 unlabeled amino acids (50 µM each) and 5 µCi of L-[4,5-^{3}H(N)]leucine (specific activity 3.85 Ci/mmole). Assays for incorporation of f[^{35}S]met-$tRNA_f^{met}$ also contained methionine (2 µM) and 5 pmole of f[^{35}S]met-$tRNA_f^{met}$ (2,850 cpm/pmole). Assays of the initial dipeptide, methionyl-valine, were as described previously (5).

RESULTS

The inhibition of protein synthesis by glucose 6-phosphate or fructose 1,6-diphosphate becomes evident at a concentration of 1 mM and increases linearly up to 3 mM glucose 6-phosphate or 6 mM fructose 1,6-diphosphate (Fig. 1, closed circles). Six mM glucose 6-phosphate and fructose 1,6-diphosphate inhibit protein synthesis by 86% and 47%, respectively. When NAD^+ (0.125 mM) was added to the lysate, there was a three-fold stimulation of leucine incorporation into polypeptides (in the absence of phosphorylated sugars)(Fig. 1, open circles). The simultaneous addition of NAD^+ with either glucose 6-phosphate or fructose 1,6-diphosphate completely reversed the inhibition of protein synthesis by glucose 6-phosphate (up to 6 mM) or fructose 1,6-diphosphate (up to 3 mM)(Fig. 1). When the phosphorylated sugars and NAD^+ were added to the lysate, there was a greater stimulation of protein synthesis compared to those assays that contain NAD^+ alone.

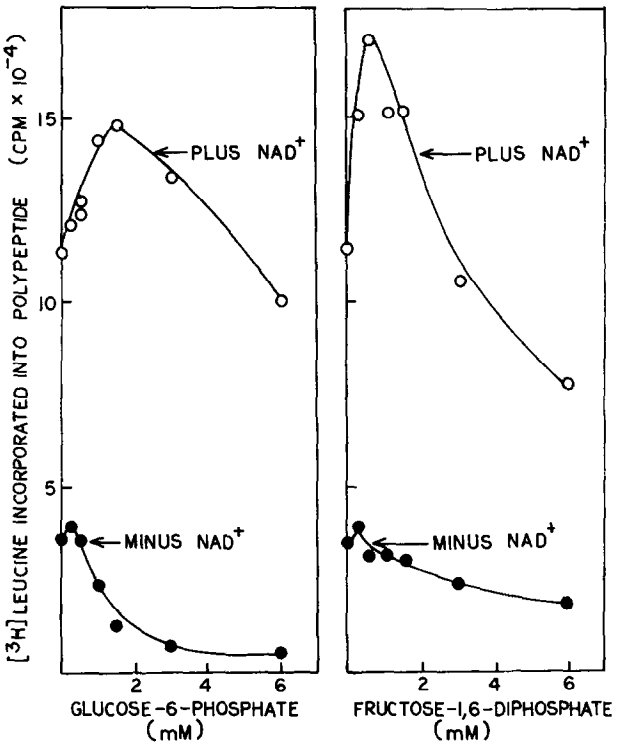

Figure 1. Inhibition of protein synthesis in rabbit reticulocyte lysates by glucose 6-phosphate or fructose 1,6-diphosphate and the reversal of inhibition with NAD^+ (0.125 mM). The lysate contained 15 μM hemin and was incubated for 45 min at 30°C as described under Materials and Methods. The [^{3}H]leucine incorporation was measured in 15 μl aliquots. •-•, minus NAD^+; o-o, plus NAD^+.

The concentration at which this additional stimulation of polypeptide occurs was 1.5 mM for glucose 6-phosphate and 0.5 mM for fructose 1,6-diphosphate. An examination of the kinetics of leucine incorporation into polypeptide showed that the inhibitory effect of glucose 6-phosphate only occurs after 5-10 min (Fig. 2). The addition of NAD^+ allows a higher rate of leucine incorporation into polypeptide for the first 15 min after which the rate of protein synthesis becomes slower. The addition of both NAD^+ and glucose 6-phosphate eliminates the inhibitory effect of glucose 6-phosphate; protein synthesis continues linearly for 45 min (Fig. 2).

Measurement of the initial dipeptide, methionyl-valine, in the presence of pactamycin reveals that glucose 6-phosphate also inhibits the initial

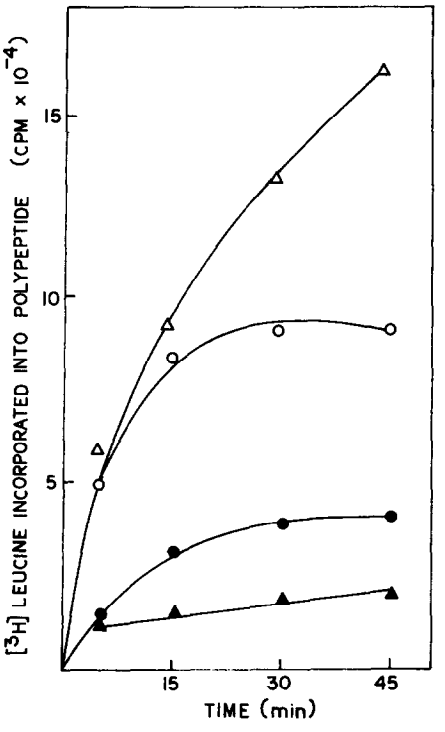

Figure 2. Kinetics of protein synthesis and the effects of glucose 6-phosphate and NAD^+. Experiments were done as described in Materials and Methods. Temperature of incubation was 30°C. Concentration of hemin was 15 μM. ●-●, minus NAD^+, minus glucose 6-phosphate; ▲-▲, minus NAD^+, plus glucose 6-phosphate (3 mM); o-o, plus NAD^+ (0.125 mM), minus glucose 6-phosphate; Δ-Δ, plus NAD^+, plus glucose 6-phosphate.

dipeptide accumulation (Table 1). When NAD^+ is added, the inhibition of dipeptide synthesis is reversed (Table 1). The incorporation of formyl-methionine into polypeptide is also inhibited by glucose 6-phosphate and reversed by NAD^+ (Table 1). Similar results were obtained with fructose 1,6-diphosphate (data not shown).

The effect of glucose and several phosphorylated hexoses on protein synthesis is shown in Table 2. Fructose 6-phosphate and glucose 6-phosphate inhibit protein synthesis by 80-85%. Fructose 1,6-diphosphate is only half as effective as fructose 6-phosphate in inhibiting protein synthesis. Glucose, glucose 1-phosphate and fructose 1-phosphate are without effect. The addition of NAD^+ completely reverses the inhibitory effect of glucose

TABLE 1

Effect of glucose 6-phosphate and NAD^+ on initial dipeptide synthesis and formyl-methionine incorporation into polypeptide

	Control	Glucose 6-phosphate (3 mM) Added
	cpm	cpm
Dipeptide synthesis[a]		
Minus NAD^+	2,110	1,460
Plus NAD^+	3,100	2,570
Formyl-methionine incorporation[b]		
Minus NAD^+	1,100	320
Plus NAD^+	2,150	1,650

[a]Dipeptide synthesis was measured after 10 min incubation, 30°C, as described under Materials and Methods.

[b]Formyl-methionine incorporation into polypeptide was measured by incubating lysate with 5 pmole of f[^{35}S]met-$tRNA_f^{met}$ (2,850 cpm/pmole) and other components for protein synthesis (total volume 60 µl), 15 min, 30°C, as described previously (4). Aliquots of 25 µl were removed to determine the incorporation of formyl-methionine.

6-phosphate, fructose 6-phosphate, and fructose 1,6-diphosphate. Finally, there is a significant stimulation of protein synthesis in those assays that contain NAD^+ and either glucose 6-phosphate, fructose 6-phosphate, or fructose 1,6-diphosphate compared to those assays that contain NAD^+ alone. Glucose 1-phosphate and NAD^+ also give a much greater stimulation of protein synthesis than NAD^+ alone (Table 2).

DISCUSSION

The experiments described show that the addition of glucose 6-phosphate and fructose 1,6-diphosphate to rabbit reticulocyte lysates inhibits leucine incorporation into polypeptide (Fig. 1). In addition, the formation of

TABLE 2

Effects of glucose, hexose phosphates, and NAD^+ on polypeptide synthesis[a]

Addition	Polypeptide synthesis	
	Minus NAD^+	Plus NAD^+
	cpm	cpm
Control	37,240	87,390
Glucose	35,380	94,040
Glucose 6-phosphate	7,550	122,590
Glucose 1-phosphate	37,490	123,550
Fructose 1-phosphate	33,970	81,840
Fructose 6-phosphate	5,600	129,430
Fructose 1,6-diphosphate	21,680	108,600

[a] NAD^+ concentration was 0.125 mM. The concentrations of hexoses and hexose phosphates were 3 mM. Incubations were at 30°C, 45 min. The [^{3}H]leucine incorporation was measured in 15 µl aliquots.

the initial dipeptide, methionyl-valine, is also decreased by the addition of these two phosphorylated sugars. These findings are in contrast to the stimulatory effects of fructose 1,6-diphosphate on cell-free protein synthesizing systems derived from Ascites cells, L cells, HeLa cells, and myeloma cells, and the stimulatory effects of glucose 6-phosphate on gel-filtered rabbit reticulocyte lysates (7). However, when NAD^+ is added to rabbit reticulocyte lysates, the effect of these phosphorylated sugars changes from inhibitory to stimulatory (Fig. 1). The addition of phosphorylated sugars and NAD^+ stimulates protein synthesis to a level greater than with NAD^+ alone. These results strongly support the notion that NAD^+ and the activation of glycolysis is central to the regulation of protein synthesis in lysed rabbit reticulocytes. The effect of NAD^+ on the activation of glycolysis is important in at least two ways. First, glycolysis generates high energy nucleoside triphosphates. Second, glycolysis permits

the efficient removal of the inhibitory phosphorylated hexoses. These two effects of glycolysis result in a higher rate of initiation of protein synthesis.

It is not immediately apparent why the results described here differ from those described by Hickey et al. (6). It is possible that in mammalian tumor (Ascites) cells or cells adapted to continuous growth in tissue culture (L cells or HeLa cells), NAD^+ is present in concentrations high enough to overcome the inhibition by these phosphorylated sugars. It is known that cells transformed by oncogenic viruses have a greater glycolytic flux than corresponding normal cells (8). Therefore, it is possible that the level of NAD^+ in lysates derived from tumor cells may be different than in lysates derived from normal cells. Alternatively, NAD^+ metabolizing enzymes may be less active in L cell or HeLa cell lysates. Previously we have found that the NAD^+ concentration of the rabbit reticulocyte lysate is less than 1 μM (1). Therefore, NAD^+ must be supplied exogenously to stimulate the initiation step of protein synthesis. Wu et al. have shown that lysed rabbit reticulocytes contain NAD^+ glycohydrolase, ADP-ribose phosphatase, and other enzymes that metabolize NAD^+ to ATP and ribose 5-phosphate (9). A higher NAD^+ concentration and less active NAD^+ metabolizing enzymes in tumor cell lysates could explain the stimulation of protein synthesis by fructose 1,6-diphosphate because the presence of NAD^+ would allow fructose 1,6-diphosphate to be metabolized through glycolysis and subsequently used for generation of high-energy nucleoside phosphates.

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