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REGULATION OF PROTEIN SYNTHESIS IN RABBIT RETICULOCYTE LYSATES BY 2,3-BISPHOSPHOGLYCERATE

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Summary

2,3-Bisphosphoglycerate was the most potent effector of glycolytic intermediates tested for their effects on protein synthesis in gel-filtered lysates from rabbit reticulocytes. 2,3-Bisphosphoglycerate at low levels was stimulatory but became inhibitory at high levels. Both effects were dependent on Mg^{2+} concentrations. The higher the concentration of Mg^{2+}, the higher the concentration of 2,3-bisphosphoglycerate required for maximal activation. 2,3-Bisphosphoglycerate concentrations required to exhibit an inhibitory effect increased as Mg^{2+} concentration increased. Both effects of 2,3-bisphosphoglycerate are discussed in terms of regulation of hemoglobin synthesis during maturation of erythroid cells.

Introduction

2,3-Bisphosphoglycerate is the most abundant acid-soluble organic phosphate in many mammalian reticulocytes and erythrocytes. This compound functions as an effector in a number of biological events in these cells (reviewed in Ref.1). We have found that during maturation of rabbit erythroid cells, 2,3-bisphosphoglycerate increases from the hundred-micromolar level in basophilic cells to about 5 mM in reticulocytes, and that this increase is primarily attributable to elevation of 2,3-bisphosphoglycerate synthase activity[1]. The increase in 2,3-bisphosphoglycerate levels and its synthesizing activity closely relate to the accumulation of hemoglobin with respect to maturation stages of erythroid cells. 2,3-Bisphosphoglycerate may, therefore, be involved in the regulation of hemoglobin synthesis. Studies presented here were done to examine the effects of 2,3-bisphosphoglycerate and other glycolytic intermediates on protein synthesis in gel-filtered lysates from rabbit reticulocytes.

[1] H. Narita *et al.*, manuscript in preparation

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Materials and Methods

Rabbit reticulocyte lysates were prepared according to the method of Housman *et al.*(2). The lysates were gel-filtered on a column of Sephadex G-25 previously equilibrated with 0.1 M K(OAc), 2 mM $Mg(OAc)_2$, 20 mM Hepes-KOH (pH 7.6), and 2 mM dithiothreitol. The fractions corresponding to the excluded volume were combined and stored at -80°C. The standard incubation mixture of cell-free protein synthesis assay contained the following components in 100 µl : 30 mM Hepes-KOH (pH 7.6), 80 mM K(OAc), 2 mM $Mg(OAc)_2$, 2 mM dithiothreitol, 2 mM ATP, 0.5 mM GTP, 0.1 mM spermine, 30µl/ml hemin, 3.75 µCi of L-[4,5-^{3}H]leucine (105 Ci/mmole), 50 µM each of the other non-radioactive amino acids, and 37.5 µl gel-filtered lysates. In all instances, concentrations of Hepes-KOH, K(OAc), $Mg(OAc)_2$, and dithiothreitol in reaction mixtures were corrected with those derived from the buffer solution used for gel-filtration of the lysates. Incubations were carried out at 37°C and 20 µl aliquots at intervals were applied to Whatman No.3 MM filter paper discs, previously dipped in 10% trichloroacetic acid in acetone and then dried. The discs were further processed by a slightly modified method of Giloh (Freudenberg) and Mager (3). 2,3-Bisphosphoglycerate was measured as described previously (4).

Results

Fig.1 shows the time courses of protein synthesis in gel-filtered lysates from rabbit reticulocytes in the presence and absence of 2,3-bisphosphoglycerate. When 2,3-bisphosphoglycerate was added at the physiological concentration (5 mM) in reticulocytes, protein synthesis was strongly inhibited. In contrast, the presence of 0.5 mM 2,3-bisphosphoglycerate stimulated protein synthesis by 253% and 178% after incubation for 2 min and 30 min, respectively.

2,3-Bisphosphoglycerate forms a complex with Mg^{2+}, on the concentration of which protein synthesis is sensitively dependent. It may, therefore, be argued that the effects of 2,3-bisphosphoglycerate are caused by the change in concentrations of Mg^{2+} available for protein synthesis. Hence the effect of Mg^{2+} on protein synthesizing activity was closely investigated (Fig.2). Subsequently the effect of 2,3-bisphosphoglycerate on protein synthesis was tested by varying Mg^{2+} concentrations (Fig.3). Fig.2 shows that the Mg^{2+} concentration optimal for protein synthesis is 2.1 mM in the absence of 2,3-bisphosphoglycerate. As shown in Fig.3, stimulation of protein synthesis by 2,3-bisphosphoglycerate was observed at all Mg^{2+} concentrations tested. Optimal concentrations of 2,3-bisphosphoglycerate varied depending on Mg^{2+}

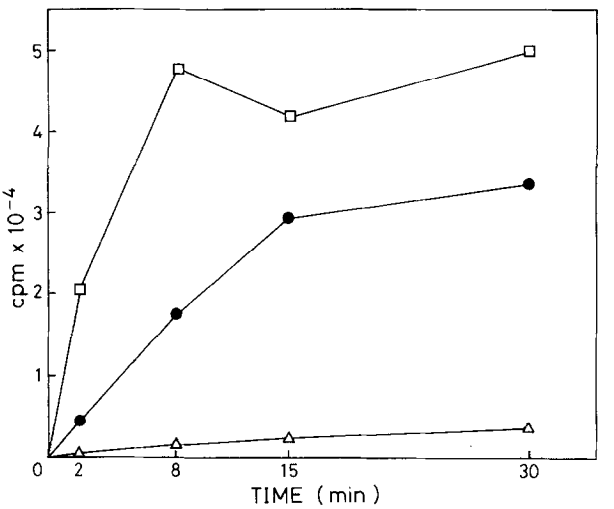

Figure 1. Effects of 2,3-bisphosphoglycerate on protein synthesis by a gel-filtered reticulocyte lysate. The lysate contained 30 μg/ml hemin and 2 mM $Mg(OAc)_2$, and was incubated at 37°C (see Materials and Methods for details of preparation of lysate and assay system of protein synthesis). The [^{3}H]leucine incorporation into protein in a 20 μl aliquot was measured with no addition (●——●), 0.5 mM 2,3-bisphosphoglycerate (□——□), and 5.0 mM 2,3-bisphosphoglycerate (△——△).

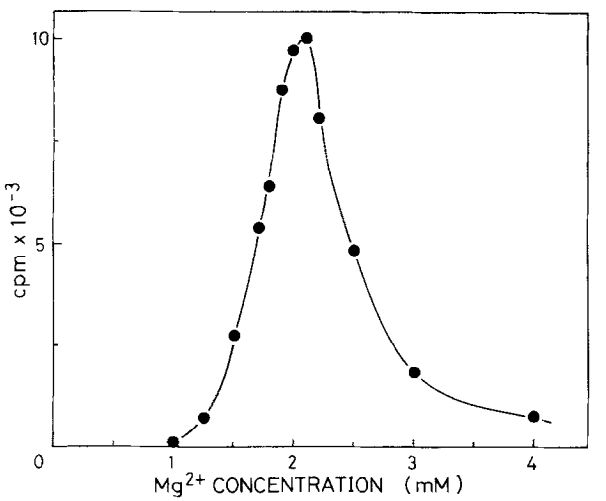

Figure 2. Mg^{2+} -response of protein synthesis in a gel-filtered lysate. Experiments were done as described in Materials and Methods except for the Mg^{2+} concentration. The final concentration of Mg^{2+} added is indicated on the abscissa. The [^{3}H]leucine incorporation at 37°C for 2 min is expressed on the ordinate.

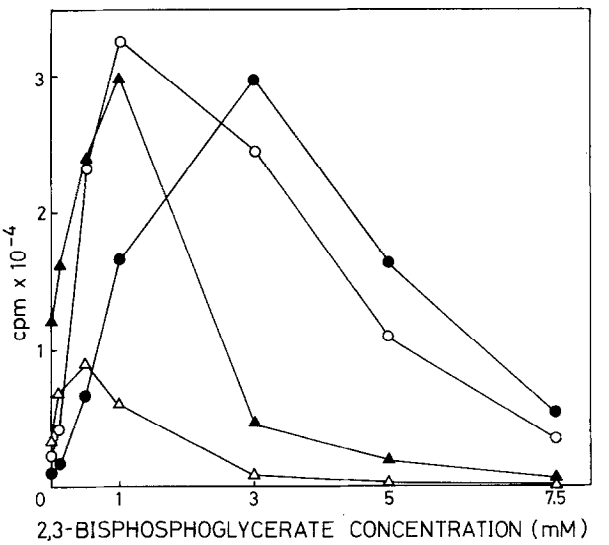

Figure 3. Effects of 2,3-bisphosphoglycerate on protein synthesis at various concentrations of Mg^{2+}. The lysate was incubated with 2,3-bisphosphoglycerate under standard conditions (see Materials and Methods) except for the Mg^{2+} concentration. The [^{3}H]leucine incorporation at 37°C for 2 min (on the ordinate) was measured at the following concentrations of Mg^{2+}, 1.5 mM (△—△), 2.0 mM (▲—▲), 3.0 mM (○—○), and 4.0 mM (●—●).

concentrations. The concentration of 2,3-bisphosphoglycerate required to give a maximal enhancement increased as the concentration of Mg^{2+} increased. The extent of stimulation also was dependent on the Mg^{2+} concentration. Maximal activation by 2,3-bisphosphoglycerate in the presence of 1.0, 2.0, 3,0, and 4.0 mM Mg^{2+} was 272, 248, 1430, and 3010%, respectively. Although addition of 2,3-bisphosphoglycerate to protein synthesizing systems containing Mg^{2+} at 1.5 mM and 2.0 mM (lower than the optimal concentration of 2.1 mM) should inhibit by reducing the concentration of Mg^{2+} available for protein synthesis, there is in fact a marked stimulation. It is evident from these results that 2,3-bisphospoglycerate exerts its stimulatory effect on protein synthesis not by varying the Mg^{2+} concentrations but by functioning as an activator for the machinary of protein synthesis. The possibility was excluded that 2,3-bisphosphoglycerate was metabolized to generate ATP or GTP and thus stimulated protein synthesis, as 2,3-bisphosphoglycerate concen-

trations were almost constant during incubation in all cases. It is likely that stimulation by 2,3-bisphosphoglycerate seen in the inhibitory range of Mg^{2+} (>2.1 mM) involves an indirect stimulatory effect due to the reduction of inhibitory Mg^{2+} as well as a direct stimulatory effect of 2,3-bisphosphoglycerate or its complex with Mg^{2+} on protein synthesis. 2,3-Bisphosphoglycerate at high concentrations became inhibitory when Mg^{2+} concentrations were low (1.5 mM and 2.0 mM). This inhibition and the decreased stimulatory effect observed with high concentrations of 2,3-bisphosphoglycerate may be caused by lowering Mg^{2+} concentrations available for protein synthesis, or the machinery for protein synthesis may have specific site(s) at which binding of 2,3-bisphosphoglycerate or a Mg•2,3-bisphosphoglycerate complex gives an inhibitory effect.

It has been recently reported that certain phosphorylated sugars affect protein synthesis in extracts from a variety of mammalian cells (5,6). Fructose 1,6-bisphosphate, fructose 6-phosphate and glucose 6-phosphate stimulate protein synthesis in gel-filtered rabbit reticulocyte lysates (5). In non-gel-filtered rabbit reticulocyte lysates, fructose 1,6-bisphosphate and glucose 6-phosphate inhibit protein synthesis, but the inhibitory effect becomes stimulatory in the presence of NAD^+ (6). We examined the effects of various phosphorylated sugars and NAD^+, taking into account their physiological concentrations, on protein synthesis in gel-filtered lysates from rabbit reticulocytes, to compare with the effects of 2,3-bisphosphoglycerate. Table I shows the results. Glucose 6-phosphate (0.5 mM), glucose 1,6-bisphosphate (0.5 mM), and fructose 1,6-bisphosphate (0.01 mM and 0.5 mM) showed appreciable stimulation. Fructose 6-phosphate, 3-phosphoglycerate, and NAD^+ had no effect. Of the compounds tested, 2,3-bisphosphoglycerate was the most potent activator and the only compound with inhibitory effect at physiological concentrations.

Discussion

It has been proved that both 2,3-bisphosphoglycerate synthase and phosphatase activities in erythrocytes of rabbit (7) and other mammals (8-13) are

Table I. Effects of glycolytic intermediates on protein synthesis

Compounds added	Concn. (mM)	Leucine incorporated into protein after incubation for 2 min	30 min
None	——	100%	100%
Glucose 6-P	0.1	85	100
	0.5[a]	122	124
Glucose 1,6-diP	0.1[b]	93	94
	0.5	130	138
Fructose 6-P	0.1[a]	95	107
	0.5	99	99
Fructose 1,6-diP	0.01[a]	120	102
	0.5	152	120
Glycerate 3-P	0.02[a]	103	99
	0.5	92	95
Glycerate 2,3-diP	0.5	253	178
	5.0[a]	14	15
NAD^+	0.1[b]	96	113
	0.5	108	114

Protein synthesis was measured after 2 min and 30 min incubation under standard conditions as described in Materials and Methods.
[a]Physiological concentrations in rabbit reticulocytes.
[b]Physiological concentrations in rabbit erythrocytes.

manifested by the same protein and that the synthase activity is strongly inhibited by the product, 2,3-bisphosphoglycerate (8,10,14). It is conceivable from these facts and the results presented here that hemoglobin and 2,3-bisphosphoglycerate accumulate during maturation of erythroid cells, co-operatively regulating rates of their syntheses. Hemoglobin binds with 2,3-bisphosphoglycerate. Accumulation of hemoglobin, therefore, would stimulate the synthase activity by relieving the enzyme from product inhibition, and conversely, inhibit the phosphatase activity by lowering the substrate concentration. The resulting increase of 2,3-bisphosphoglycerate stimulates hemoglobin synthesis. Our finding that hemoglobin and 2,3-bisphosphoglycerate increase concomitantly during the maturation of erythroid cells makes it likely that this regulation functions *in vivo*. Such a co-operative stimulation would operate continuously until 2,3-bisphosphoglycerate accumulates to concentrations at which the stimulatory effect of 2,3-bisphosphoglycerate on protein synthesis is overcome by its inhibitory effect. This inhibitory effect may contribute for determining the upper level of hemoglobin in matured erythrocytes. An attempt is in progress to

reveal the mechanism of the stimulatory and inhibitory effects of 2,3-bisphosphoglycerate.

The dependency of the effects of 2,3-bisphosphoglycerate on Mg^{2+} concentration suggests that Mg^{2+} participates in modulating protein synthesis. However, there is no information available on the variation of Mg^{2+} concentration in the maturation of erythroid cells.

Reticulocytes contain 5 mM 2,3-bisphosphoglycerate. It is worthwhile to mention that when reticulocyte lysates are used for experiments of protein synthesis without being subjected to gel filtration, the effects of 2,3-bisphosphoglycerate must be included in the results obtained. Results obtained in the presence of compounds which are metabolized to become substrates of 2,3-bisphosphoglycerate synthase should be interpreted carefully even when gel-filtered lysates are used.

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