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INHIBITION OF L-GLUTAMINE:D-FRUCTOSE-6-PHOSPHATE AMINOTRANSFERASE BY METHYLGLYOXAL

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SUMMARY

1. The potent inhibitor of L-glutamine:D-fructose-6-phosphate aminotransferase (EC 2.6.1.16) present in heated ascites tumor extracts was identified as methylglyoxal. It arises from glucose 6-phosphate *via* triose phosphates. The formation of triose phosphates from glucose 6-phosphate is enzymic, but their conversion to methylglyoxal requires a brief heating.

2. The mode of methylglyoxal inhibition of aminotransferase was studied by using partially purified rat liver enzyme. The inhibition occurs readily and the concentration of methylglyoxal necessary for a 50% inhibition is about 10 μM. The inhibition is non-competitive with respect both to glutamine and fructose 6-phosphate; it is relieved by thiols, of which cysteine was found to be most effective. 11 other enzymes tested were not affected by methylglyoxal.

3. The implication of these results for the well-known cytotoxic effect of methylglyoxal was discussed.

INTRODUCTION

L-Glutamine:D-fructose-6-phosphate aminotransferase (EC 2.6.1.16) catalyzes the formation of glucosamine 6-phosphate from fructose 6-phosphate (Fru-6-*P*) and glutamine and plays an important regulatory role in the biosynthesis of UDP-*N*-acetylglucosamine[1–6]. The enzyme is extremely unstable and for its extraction from tissues, glucose 6-phosphate (Glc-6-*P*)[1–8] or Fru-6-*P* (ref. 9) have been used as protecting agents.

We reported previously that boiled extracts of ascites tumor cells potently inhibit aminotransferase enzymes partially purified from various tissues[5]. Since the presence of Glc-6-*P* was a prerequisite for the appearance of the inhibitory activity, it was suggested that the inhibitor might be a glycolytic intermediate or a related compound. The fact that rat liver extracts failed to exhibit the inhibitory activity also supported this possibility.

The present communication deals with investigations which were undertaken to disclose the nature of this inhibitor. The results presented below demonstrate that the inhibitor is methylglyoxal derived from Glc-6-*P* *via* triose phosphates.

MATERIALS AND METHODS

All the preparative experiments described below were conducted at 0–2 °C unless otherwise specified.

Preparation of tumor extract inhibitory to aminotransferase

Ehrlich ascites carcinoma cells, harvested from the peritoneal cavity of male dd mice 10 days after transplantation, were washed twice with 0.154 M KCl–1 mM EDTA–12 mM Glc-6-*P* (pH 7.5), suspended in the same volume of distilled water and homogenized in a glass–Teflon homogenizer for 1 min. After addition of the same volume of 0.308 M KCl–2 mM EDTA–24 mM Glc-6-*P* (pH 7.5), the homogenate was centrifuged at 105 000 × *g* for 1 h. The supernatant was heated for 2 min at 100 °C, chilled in an ice bath and centrifuged briefly to remove coagulated proteins. The resulting supernatant was potently inhibitory to aminotransferase.

Fractionation of heated tumor extract by Sephadex G-15 chromatography

3–5 ml of heated tumor extracts were applied to a column of Sephadex G-15 (1.5 cm × 25 cm) previously equilibrated with 50 mM sodium phosphate (pH 7.5)–1 mM EDTA and eluted with the same solution. Fractions of 1 ml were collected at a flow rate of 10–15 ml/h.

Aminotransferase preparations

Aminotransferase was prepared from rat liver as described by Miyagi and Tsuiki[6]. In most of the experiments, the $(NH_4)_2SO_4$ precipitate (the Step-2 enzyme[6]) was used because it was substantially active without thiols[5]. In a few experiments (see Fig. 5B), however, the enzyme purified further by DEAE-cellulose chromatography was employed.

Aminotransferase assay

The standard incubation mixture contained in 1 ml the following components in μmoles: glutamine, 15; Fru-6-*P*, 10; sodium phosphate (pH 7.5), 55; EDTA, 0.2; and enzyme. After incubation for 1 h at 37 °C, the reaction was stopped by heating for 2 min at 100 °C and the amount of glucosamine 6-*P* formed was determined by the method of Ghosh *et al.*[2] with free glucosamine as standard. Neither glyceraldehyde 3-*P* nor methylglyoxal interfered with the determination of glucosamine 6-*P*.

Assay of other enzymes

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)[10], 6-phosphogluconate dehydrogenase (EC 1.1.1.44)[10] and glucosephosphate isomerase (EC 5.3.1.9)[10] of yeast, phosphofructokinase (EC 2.7.1.11)[11] and fructose 1,6-diphosphatase (EC 3.1.3.11)[12] of rat liver, fructosediphosphate aldolase (EC 4.1.2.13)[13], phosphotriose isomerase (EC 5.3.1.1)[13], pyruvate kinase (EC 2.7.1.40)[14] and lactate dehydrogenase (EC 1.1.1.27)[14] of rabbit muscle and malate dehydrogenase (EC 1.1.1.37)[15] of pig heart

were assayed as described in the references. These enzymes were purchased from Boehringer except for phosphofructokinase and fructose 1,6-diphosphatase which were prepared as described by Suzuki *et al.*[16] and by Sato and Tsuiki[17], respectively.

Analytical methods

Methylglyoxal was determined by the method of Racker[18]: 0.05 ml of samples was added to 2.95 ml of the solution containing 100 μmoles of potassium phosphate (pH 6.6), 100 mg of reduced glutathione and 1 I.U. of glyoxalase I (Boehringer) (lactoyl-glutathione lyase, EC 4.4.1.5) and the formation of thiol ester was followed spectrophotometrically at 240 nm.

Fructose 1,6-P_2, glyceraldehyde 3-P and dihydroxyacetone-P were determined enzymatically as described by Bücher and Hohorst[13]. Lactic acid was determined by the method of Barker and Summerson[19].

Chemicals

Methylglyoxal and glyoxal were purchased from Nakarai Chemicals, Kyoto. Glyceraldehyde 3-P and dihydroxyacetone-P were obtaned from Boehringer in the form of diethylacetal and dimethylketal, respectively, and freed from the substituents just prior to use. Other glycolytic intermediates were also the products of Boehringer.

RESULTS

Formation of aminotransferase inhibitor from Glc-6-P

Tumor extracts prepared without Glc-6-P did not inhibit aminotransferase after heating for 2 min at 100 °C. When these extracts (unheated) were incubated with Glc-6-P, glycolytic intermediates such as fructose 1,6-P_2, triose phosphates and lactic acid accumulated and an inhibitory activity to aminotransferase readily emerged after heating (Fig. 1). In rat liver extracts, on the other hand, no inhibitory activity emerged under the same conditions (Fig. 1). It is therefore evident that the inhibitor in question arises from Glc-6-P, most probably by its glycolytic breakdown.

TABLE I

EFFECT OF GLYCERALDEHYDE 3-PHOSPHATE AND RELATED COMPOUNDS ON RAT LIVER AMINOTRANSFERASE

Aminotransferase at the $(NH_4)_2SO_4$ step was assayed under the standard assay conditions in the presence of the compounds listed below.

Compounds	*mM*	*% Inhibition*
Fructose 1,6-P_2	0.5	18
Glyceraldehyde 3-P	0.5	71
Dihydroxyacetone-P	0.5	25
L-α-Glycerophosphate	0.5	7
Glyceraldehyde	0.1	0
Dihydroxyacetone	0.1	20
Methylglyoxal	0.1	80
Glyoxal	0.1	78
Glycolaldehyde	0.1	16

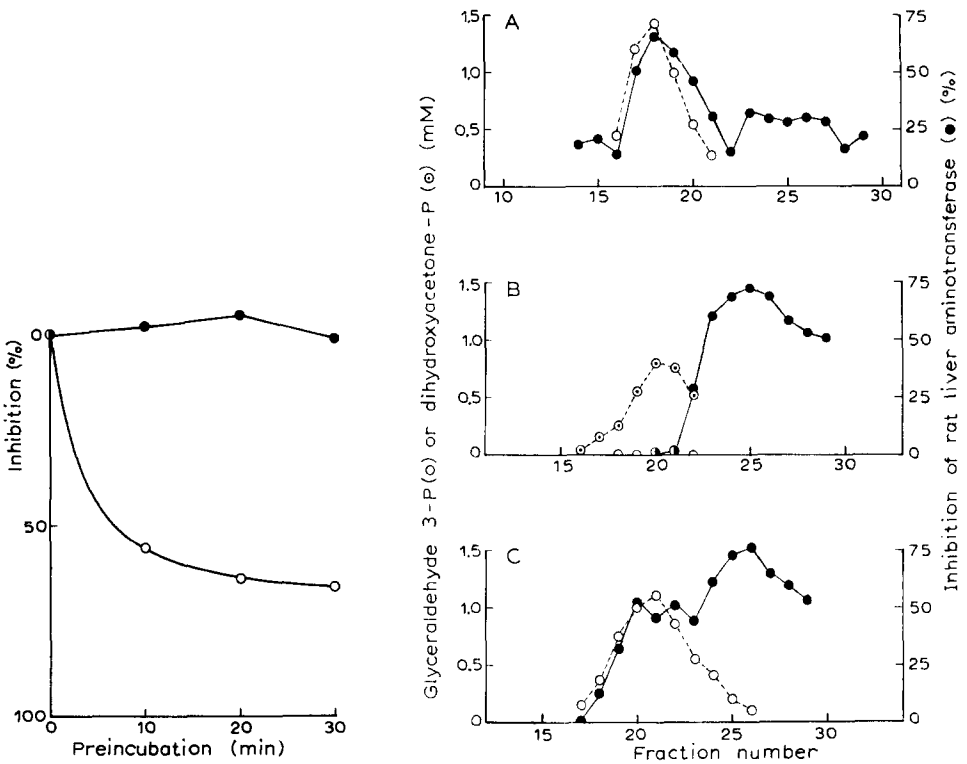

Fig. 1. Formation of aminotransferase inhibitor from Glc-6-*P*. 105 000 × *g* supernatant was prepared from mouse Ehrlich carcinoma (○—○) or rat liver (●—●) as described in text, except that Glc-6-*P* was omitted; the supernatants were then incubated with 10 mM Glc-6-*P* at 37 °C. At the times indicated, aliquots were withdrawn and their effect on aminotransferase was determined after heating for 2 min at 100 °C.

Fig. 2. Chromatography of glyceraldehyde 3-*P* and heated tumor extract on Sephadex G-15 column. The following materials were applied: A, 1.5 ml of 4.84 mM glyceraldehyde 3-*P*; B, 5 ml of a heated tumor extract prepared as described in text; and C, 3 ml of the mixture of fresh and heated (2 min at 100 °C) glyceraldehyde 3-*P* (4.84 mM) in equal amounts. 0.3 ml of each fraction was used for the assay of inhibitory activity.

Effect of glycolytic intermediates on aminotransferase activity

Of various glycolytic intermediates, only glyceraldehyde 3-*P* substantially inhibited aminotransferase (Table I), approx. 0.2 mM concentration being sufficient to bring about a 50% inhibition. Other intermediates tested were Glc-1-*P*. Glc-6-*P*, 6-phosphogluconate, 3-phosphoglycerate, 2,3-diphosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, pyruvate and lactate; none of them were inhibitory. In the experiment shown in Fig. 2A, glyceraldehyde 3-*P* was chromatographed on a column of Sephadex G-15. It is evident that the triose phosphate *per se* is inhibitory to aminotransferase.

Fractionation of heated tumor extracts by Sephadex G-15 column

When heated tumor extracts inhibitory to aminotransferase were fractionated by a Sephadex G-15 column, the triose phosphate region neither contained glyceral-

dehyde 3-*P* nor inhibited aminotransferase; an inhibitory activity could be located in the lower molecular-weight fractions (Fig. 2B). In a separated experiment, the inhibitory activity of glyceraldehyde 3-*P* was shown to be increased significantly upon heating for 2 min at 100 °C with a concomitant release of inorganic phosphate. These results suggested that glyceraldehyde 3-*P* derived from Glc-6-*P* might have been degraded upon heating to another compound that is more inhibitory to aminotransferase.

This possibility was tested by chromatographing a mixture of fresh and heated glyceraldehyde 3-*P* on a Sephadex G-15 column. As shown in Fig. 2C, two inhibitory activities emerged, one corresponding to glyceraldehyde 3-*P* and the other to the inhibitor found in heated tumor extracts. Hence the inhibitor of tumor extracts most probably arose from glyceraldehyde 3-*P*.

The same inhibitor appears to arise also from dihydroxyacetone-*P* since this triose phosphate, though scarcely inhibitory to aminotransferase, became inhibitory upon heating for 2 min at 100 °C.

Identification of the inhibitor as methylglyoxal

Several 2- and 3-carbon compounds that might be expected to arise from glyceraldehyde 3-*P* by degradation were tested for inhibition of aminotransferase. As shown in Table I, glyoxal and methylglyoxal are powerful inhibitors of aminotransferase, the concentration necessary for a 50% inhibition being 30 and 10 μM, respectively. Glycollate, glyoxylate and oxalate, also tested, were not inhibitory.

Fig. 3 shows that when fresh tumor extracts are heated at 100 °C, triose phosphates fall sharply and a glyoxal rises inversely. The latter compound is methylglyoxal rather than glyoxal since it reacted with glyoxalase I as promptly as authentic methylglyoxal. Glyoxal reacted with glyoxalase I much more slowly than methylglyoxal.

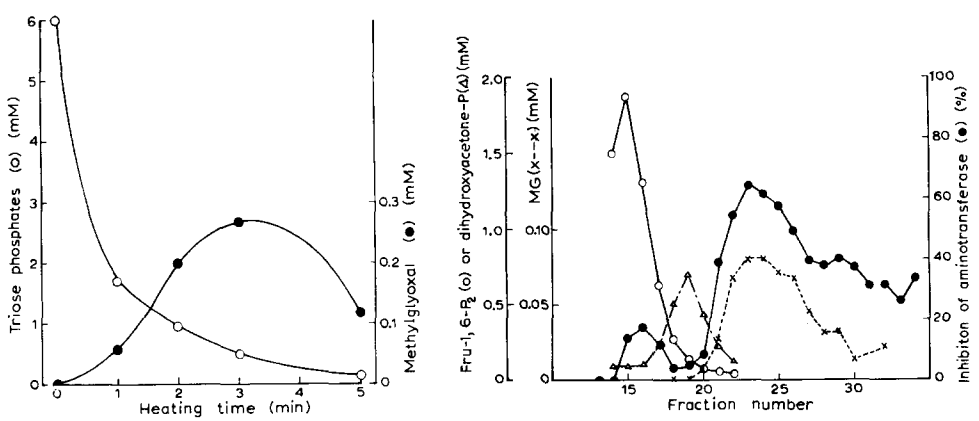

Fig. 3. Formation of methylglyoxal from triose phosphates. 105 000 × *g* supernatant prepared from Ehrlich carcinoma in the presence of Glc-6-*P* was heated at 100 °C. At the times indicated, aliquots were quickly withdrawn, chilled and assayed for triose phosphates and methylglyoxal.

Fig. 4. Identification of aminotransferase inhibitor as methylglyoxal. 3 ml of heated tumor extract prepared as described in text were chromatographed on a Sephadex G-15 column. The inhibitory activity was determined with 0.3 ml of each fraction. MG, methylglyoxal.

A heated tumor extract was fractionated by a Sephadex G-15 column and each fraction was examined for the content of methylglyoxal as well as inhibition of aminotransferase. Fig. 4 clearly shows that the inhibitor present in heated tumor extracts is methylglyoxal. Authentic methylglyoxal was eluted from the Sephadex column at the same position as the inhibitor of tumor extracts.

Inhibition of aminotransferase by methylglyoxal

The inhibition of aminotransferase by methylglyoxal occurred readily and proceeded linearly. The inhibition was of the non-competitive type, *i.e.* methylglyoxal decreased V without changing the apparent K_m for both glutamine and Fru-6-*P* (Fig. 5).

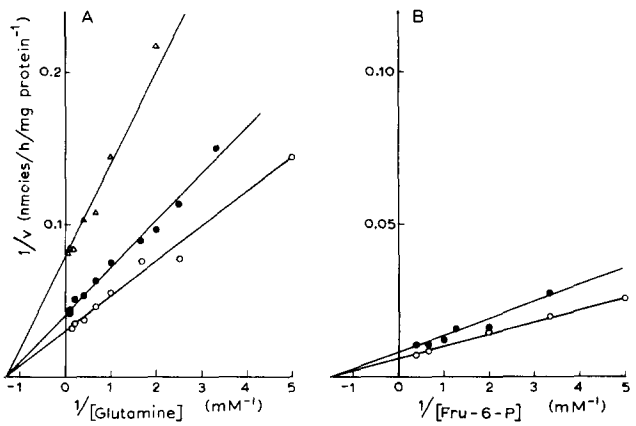

Fig. 5. Effect of methylglyoxal on the velocity of aminotransferase reaction as a function of glutamine (A) and Fru-6-*P* (B) concentration. For the experiments of B, the $(NH_4)_2SO_4$-precipitated aminotransferase was further purified by a DEAE-cellulose column so as to obtain an enzyme preparation that was free of glucose phosphate isomerase[6]. The concentration of methylglyoxal was zero (○—○), 10 (●—●) or 30 μM (△—△).

We have previously reported that thiols can activate partially purified aminotransferase[5]. With the $(NH_4)_2SO_4$-precipitated enzyme, activation by dithiothreitol and cysteine was approx. 1.7- and 1.2-fold, respectively. Fig. 6 shows that cysteine completely prevents methylglyoxal inhibition of aminotransferase. Methylglyoxal inhibition may therefore involve thiol groups of aminotransferase. In this respect, dithiothreitol (4 mM) was not as effective as cysteine: it raised the concentration of methylglyoxal necessary for a 50% inhibition from 10 to 70 μM.

Effect of methylglyoxal on enzymes other than aminotransferase

Methylglyoxal at concentrations as high as 0.5 mM failed to affect the activities of the following enzymes, some of which are known to possess thiol groups critical to catalytic activity: glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glucosephosphate isomerase, phosphofructokinase, fructose 1,6-diphosphatase, fructosediphosphate aldolase, phosphotriose isomerase, L-α-glycerophosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase and malate dehydrogenase.

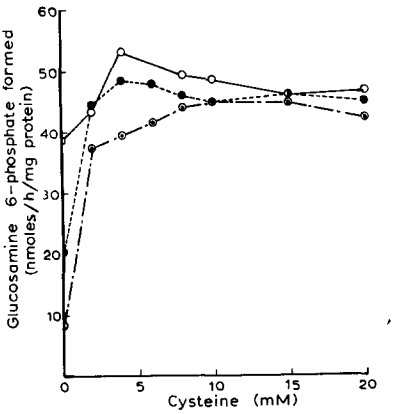

Fig. 6. Effect of cysteine on the methylglyoxal inhibition of aminotransferase. The enzyme was assayed in the presence of varying concentrations of cysteine. The concentration of methylglyoxal was zero (○—○), 10 (●—●) or 50 μM (⊙—⊙).

Aminotransferase enzymes partially purified from mouse liver, mouse Ehrlich carcinoma and rat Yoshida sarcoma were all inhibited by methylglyoxal as severely as the rat liver enzyme.

DISCUSSION

The inhibitor of aminotransferase present in heated tumor extracts was found to be methylglyoxal. The inhibition appears to be reasonably specific since none of the 11 other enzymes tested were inhibited by methylglyoxal. It has been reported earlier that hexokinase (EC 2.7.1.11), triose phosphate dehydrogenase (EC 1.2.1.9)[20], and succinate dehydrogenase (EC 1.3.99.1)[21] are inhibited by methylglyoxal. But much higher concentrations appear to be required.

Methylglyoxal has been considered by Együd[22] and Együd and Szent-Györgyi[23,24] to be closely related to retine, an endogenous growth-retarding substance found by them. They reported that 1 mM methylglyoxal stops the division of a variety of cells. Protein synthesis is affected severely and rapidly enough to be considered as a cause of inhibition of cell division[24,25]. These cytotoxic effects were prevented most effectively by cysteine.

Aminotransferase is the first and rate-limiting enzyme in the pathway specific for the formation of UDP-*N*-acetylglucosamine and CMP-*N*-acetylneuraminic acid[1–6]. Rapidly growing tissues evidently require these nucleotide sugars as building blocks of membrane glycoproteins and glycolipids and interestingly enough, fetal liver[26] and hepatomas[5] were found to possess much larger amounts of aminotransferase than noncancerous adult liver. The extremely sensitive nature of aminotransferase to inhibition by methylglyoxal may therefore be an important cause for the cytotoxicity of methylglyoxal.

Methylglyoxal found in heated tumor extracts was derived from Glc-6-*P* *via* triose phosphates. Although the formation of triose phosphates was enzymic, its conversion to methylglyoxal required a brief heating. The mechanism of conversion, however, may be analogous to that reported by Riddle and Lorenz[27] who showed

that polyanions such as phosphate converts trioses and triose phosphates to methylglyoxal under physiological conditions of pH and temperature. The possibility of enzyme-mediated formation of methylglyoxal in liver and other tissues is an unsettled problem and is under current investigation.

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