THE REGULATION OF *ESCHERICHIA COLI* METHYLGLYOXAL SYNTHASE; A NEW CONTROL SITE IN GLYCOLYSIS?

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1. Introduction

A novel sequence of reactions for the conversion of dihydroxyacetone phosphate (DHAP) to pyruvate was described recently [1]. These reactions in which methylglyoxal and D-lactate are intermediates provide a possible alternative to the glycolytic conversion of DHAP to pyruvate but their physiological significance is uncertain. In this paper we show that the activity of the first enzyme of this pathway, methylglyoxal synthase, is regulated by Pi and DHAP in a way which suggests that it may play a role in the control of glycolysis.

2. Material and methods

The various bacteria used in this work are indicated in table 2. Cultures were grown aerobically in either minimal medium or nutrient broth as described previously [2]. The cells were resuspended in 50 mM iminazole buffer pH 7.0, disrupted by ultrasonic oscillations then centrifuged at 30,000 *g* for 15 min at 4° and the supernatant used for the assay of methylglyoxal synthase. Soluble protein was measured colorimetrically [3] using crystalline bovine serum albumin as the standard.

Three assays were used for the estimation of methylglyoxal synthase. Assay 1 was as described previously [1] but with iminazole buffer pH 7.0 instead of sodium maleate buffer pH 6.6. For assay 2, the reaction mixture contained, in 1 ml: iminazole buffer pH 7.0 (40 μmoles), DHAP (0.75 μmoles), reduced glutathione pH 6.8 (1.65 μmoles), glyoxalase I (Sigma Type III, sufficient to catalyse the condensation of 0.8 μmole of methylglyoxal per min) and bacterial protein. The increase in absorbance at 240 nm was measured at 30° on a recording spectrophotometer. For assay 3 the incubation mixture at 30° contained, in 0.5 ml: iminazole buffer pH 7.0 (20 μmoles), DHAP (0.6 μmole), and bacterial protein. Samples (0.1 ml) were withdrawn at known times, transferred to cuvettes containing 2 mM orthophosphate (to inhibit further enzyme action) and the DHAP content estimated enzymatically [4].

3. Results

3.1. *The partial purification of methylglyoxal synthase*

The methylglyoxal synthase used in many of the experiments reported here was a partially purified preparation obtained from *E. coli* K12.

Frozen cells of glycerol-grown *E. coli* CA 244 were thawed in 20 mM tris-HCl–1 mM EDTA–0.1 M KCl buffer pH 8.0, disrupted by passage through a French pressure-cell and centrifuged at 15,000 *g* for 3 hr at 4° and the precipitate discarded. All the subsequent operations were carried out at 0–4° unless stated otherwise. The supernatant was treated with protamine sulphate (1 mg of protamine sulphate for each 10 mg of bacterial protein), centrifuged at 15,000 *g* for 30 min and the precipitate discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant to give 55% saturation and the precipitate obtained was collected by centrifugation. The precipitate was dissolved in 50 mM iminazole buffer pH 7.0 and dialysed against several changes of the iminazole buffer. The dialysed fraction was held at 67.5° for 2 min, cooled

Table 1
Stoicheiometry of methylglyoxal and Pi production from DHAP.

	DHAP added (μmoles)	DHAP used (μmoles)	Pi produced (μmoles)	Methylglyoxal produced (μmoles)
Expt. 1	0.7	0.47	0.47	0.43
Expt. 2	1.4	0.89	0.81	0.79

in ice and the heavy precipitate was removed by centrifuging at 9000 *g* for 15 min. The resulting supernatant was then fractionated with $(NH_4)_2SO_4$ and the fraction precipitating between 40–45% saturation was retained.

This fraction was essentially free of triosephosphate isomerase and fructose diphosphate aldolase and the specific activity of the methylglyoxal synthase (7.3 μmoles methylglyoxal formed per min/mg protein) was approx 25-fold greater than that of the crude extract.

3.2. *Stoicheiometry of the methylglyoxal synthase reaction*

To determine the stoicheiometry of the methylglyoxal synthase reaction an incubation mixture was set up containing iminazole buffer pH 7.0 (45 μmoles), DHAP (0.7 or 1.4 μmole) and the purified enzyme (3.5 μg of protein) in a final volume of 1.0 ml. A similar mixture containing heat-inactivated enzyme was used as a control. After 30 min incubation at 30° the reaction mixtures were cooled in ice and subsequently analysed for DHAP [4], Pi [5] and methylglyoxal [1]. A molar absorbance coefficient of 4.48×10^4 was assumed for methylglyoxal 2,4-dinitrophenylhydrazone in alkaline solution [6]. As can be seen from table 1, each equivalent of DHAP utilised gave rise to one equivalent each of Pi and methylglyoxal. Hence the methylglyoxal synthase reaction can be written:

$$\text{DHAP} \longrightarrow \text{Pi} + \text{methylglyoxal}$$

Although several attempts have been made to demonstrate the reversibility of this reaction, none has yet been successful.

3.3. *Inhibition of methylglyoxal synthase activity*

When the time-course for the methylglyoxal synthase reaction was measured the initial linear rate of methylglyoxal formation was found to decline rapidly. To see whether this was due to the accumulation of an inhibitory end-product the effect of methylglyoxal and Pi on the initial rate of reaction was determined. When tested at 1 mM concentration in assay 3, methylglyoxal had no effect but Pi was found to be a potent inhibitor. Subsequently, various phosphate-containing compounds were tested at 1 mM concentration in either assay 1 or assay 2 for their effect on methylglyoxal synthase. Under these conditions, Pi gave virtually 100% inhibition whereas α-glycerophosphate, fructose-1,6-diphosphate, fructose-6-phosphate, fructose-1-phosphate, glucose-6-phosphate, 6-phosphogluconate, ribose-5-phosphate, ATP, AMP, NAD^+ and NADH had little or no effect, causing from 0–10% inhibition. However, 3-phosphoglycerate (3-PGA) and phospho*enol*pyruvate (PEP) were more effective giving 50% and 70% inhibition respectively and inorganic pyrophosphate (PPi) was almost as effective as Pi causing 95% inhibition. The inhibition by PPi was apparently not due to its chelating action since 4 mM EDTA had no effect on the enzyme. Nor was the inhibition due to the conversion of PPI to Pi since there was no hydrolysis of the Pi under the assay conditions (assay 2, purified enzyme).

The mode of action of some of these inhibitors is discussed in the next section.

3.4. *Kinetic studies of methylglyoxal synthase*

When methylglyoxal synthase activity was measured at various concentrations of DHAP and the initial rates plotted by the method of Lineweaver and Burk [7], a straight line was obtained, the K_m for DHAP being 0.5 mM (fig. 1). However, when the experiments were repeated in the presence of the inhibitor Pi a quite different result was observed. The line

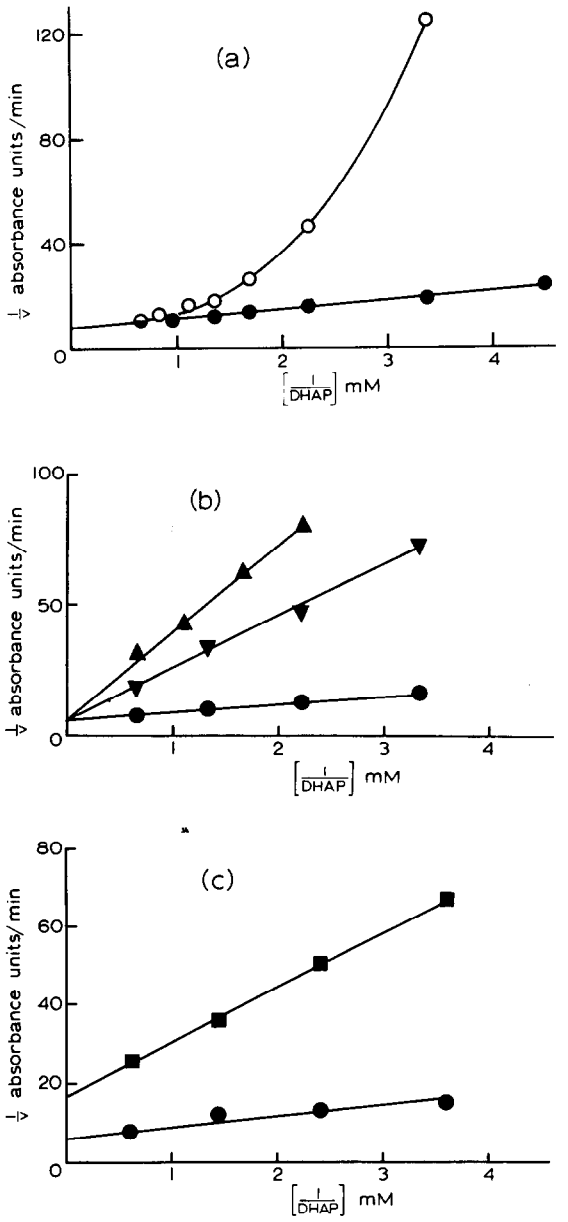

Fig. 1. The inhibition of methylglyoxal synthase. The coupled spectrophotometric assay (see text) was used with various concentrations of DHAP in the presence or absence of inhibitor. No inhibitor ●——●; plus 0.3 mM Pi ○——○ fig. 1a; plus 0.2 mM (▼——▼) or 0.4 mM (▲——▲) PPi fig. 1b; plus 1 mM PEP ■——■ fig. 1c.

obtained in the double reciprocal plot (fig. 1a) was a parabola rather than a straight line, indicating that the response to DHAP was now apparently cooperative. This view was confirmed when the data were plotted using the Hill equation [8] and a straight line with a slope of 2.9 was obtained. Although PPi was also a potent inhibitor its mode of action was quite distinct from that of Pi. As can be seen from fig. 1b, PPi was apparently a competitive inhibitor. The mechanism of inhibition by PEP was different again since both K_m and V_{max} were affected in this case, fig. 1c.

Although these results were obtained using the partially purified methylglyoxal synthase, identical results for Pi inhibition were obtained when crude preparations of methylglyoxal synthase were used.

3.5. *The occurrence of methylglyoxal synthase*

Since methylglyoxal synthase was formed by *E. coli* K10 under various growth conditions (table 2), it was apparently a constitutive enzyme. In an attempt

Table 2
Activity of methylglyoxal synthase in various bacteria.

Bacterium	Specific activity (μmoles/min/mg protein)
Escherichia coli K10 (glucose-grown)	0.174
Escherichia coli K10 (pyruvate-grown)	0.190
Escherichia coli K10	0.142
Aerobacter aerogenes SB 8	0.100
Serratia marcescens D 106	0.043
Salmonella typhimurium LT-2	
Proteus vulgaris M 13	0.064
Proteus rettgeri M 9	0.020
Erwinia uredovora D 1080	0.055
Bacillus subtilis	0.00
Pseudomonas ovalis	0.00
Streptococcus faecalis C 117	0.00
Azotobacter vinelandii	0.00

Extracts were prepared from nutrient broth-grown cells unless indicated otherwise and the enzyme estimated using assay 2.

to assess its metabolic significance a variety of bacteria were grown in nutrient broth and tested for the enzyme. As can be seen from table 2, it was present in all the Enterobacteriaceae tested but none of the other bacteria employed had activity when assayed under the same conditions.

4. Discussion

The presence of methylglyoxal synthase in all the Enterobacteriaceae suggests that it is of significance in their metabolism. Further, the inhibition by Pi and the relief of this inhibition in an allosteric manner by DHAP suggests that methylglyoxal synthase may be involved in the regulation of glycolysis.

Although methylglyoxal synthase is present in *E. coli* at a reasonable activity it is possible that the normal intracellular concentrations of Pi and DHAP may prevent it being fully active. However, any factor which raised the DHAP concentration or decreased the Pi concentration would tend to de-inhibit the enzyme. Any circumstance which both decreased the Pi concentration and raised the DHAP concentration would be particularly effective in this respect. Thus, if glycolysis was restricted by reduced availability of Pi, glyceraldehyde-3-phosphate dehydrogenase would be inhibited and as a consequence the triosephosphate concentration would rise. In this situation methylglyoxal synthase could function liberating Pi and generating an oxidisable substrate through the further action of the glyoxalase system on the methylglyoxal [1]. The Pi could be utilised by glyceraldehyde-3-phosphate dehydrogenase thus allowing glycolysis to function again. Equally, energy could be obtained oxidatively and the glycolytic block imposed by the lack of Pi would be bypassed [1].

The physiological significance of the inhibition by PEP, 3-PGA and PPi is less clear. For glycolysis inhibition by PEP and 3-PGA would reinforce the effect of Pi since the glycolytic formation of PEP and 3-PGA is dependent on the availability of Pi. However, since methylglyoxal synthase is formed constitutively in *E. coli* the inhibition by PEP and 3-PGA may serve to prevent the breakdown of DHAP formed under gluconeogenic conditions.

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