THE FORMATION AND CATABOLISM OF METHYLGLYOXAL DURING GLYCOLYSIS IN *ESCHERICHIA COLI*

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1. Introduction

Methylglyoxal was once believed to be involved in glucose catabolism as a component of the so-called non-phosphorylating glycolysis [1]. When phosphorylated compounds were identified as intermediates in glucose breakdown the formation of methylglyoxal was considered to be due to non-enzymic side-reactions and thus of little or no importance [2]. In this paper we show that *E. coli* contains enzymes which convert dihydroxyacetone phosphate (DHAP) to pyruvate via methylglyoxal and D-lactate. This sequence provides a by-pass of the normal glycolytic reactions for the conversion of DHAP to pyruvate and as such represents a combination of the phosphorylated and non-phosphorylated pathways of glucose breakdown (scheme 1).

2. Materials and methods

The organisms used were the *E. coli* K12 derivatives K10, [3], K1 [4], P10 [5], AA200 [6], K2.1t [4] K2. 1.20 and *E. coli* B. Cultures were grown aerobically at 37° on minimal media [7] containing the appropriate carbon sources at 25 mM concentration. Cells were harvested in the late logarithmic phase of growth (approx. 0.5 mg dry wt./ml) by centrifugation at 4°. They were resuspended in 10 mM tris-HCl buffer pH 7.4, disrupted by exposure to ultrasonic oscillations then centrifuged at 2000 g for 5 min at 4° and the supernatant retained. Soluble protein was measured colorimetrically [8].

For the conversion of DHAP to methylglyoxal the reaction mixture contained, in 1 ml, sodium maleate buffer pH 6.6 (25 μmoles), DHAP (2 μmoles) and *E.*
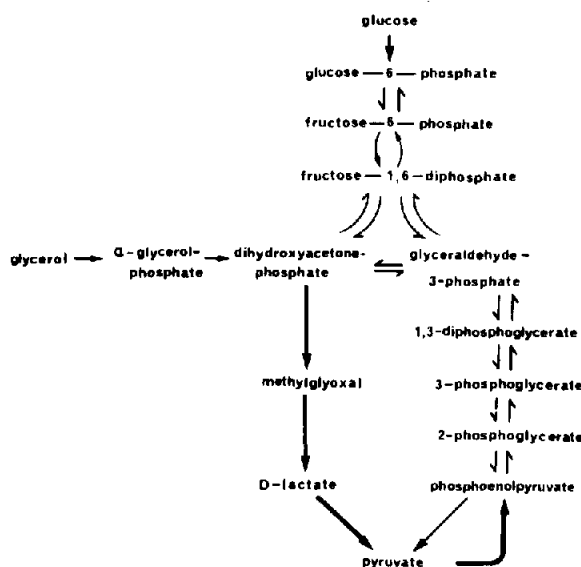
Scheme 1.

coli protein (100–150 μg). The methylglyoxal formed was measured enzymatically with glyoxalase 1 [9] or colorimetrically with 2, 4-dinitrophenylhydrazine (2, 4-DNP). In the latter assay, a 1 ml sample containing 0.02–0.10 μmole methylglyoxal was added to 0.33 ml of 2, 4-DNP reagent (0.1% 2, 4-DNP in 2 N HCl), incubated at 30° for 15 min then 1.67 ml of 10% NaOH added. The absorbance at 550 nm was measured 15 min later. In this assay 0.1 μmole of methylglyoxal gave an absorbance of 1.64 units. For the conversion of methylglyoxal to lactate the reaction mixture contained in 1ml, sodium phosphate buffer pH 6.8 (100 μmoles), reduced glutathione pH 6.8 (2 μmoles), methylglyoxal (0.5 μmole) and *E. coli*

protein (300 μg). The removal of methylglyoxal was measured using the 2, 4-DNP method. Lactate oxidase was measured in a 1 ml reaction mixture containing sodium phosphate buffer pH 6.8 (100 μmole), 2, 6-dichlorophenolindophenol (DCPIP) (0.05 μmole), KCN (1 μmole), phenazine methosulphate (0.66 μmole), D- or L-lactate (10 μmoles) and *E. coli* protein (50–100 μg). In this assay the absorbance of 0.1 μmole of DCPIP at 600 nm was 1.6 units. All assays were carried out at 30°.

Glycogen synthesis was measured by the previously published method [10]. The chemicals and biochemicals used were of the highest purity commercially available and only the methylglyoxal was further purified (by passage through a Dowex-1-chloride form column) before use.

3. Results

The existence of a partial glycolytic by-pass was suggested by the properties of *E. coli* mutants devoid of triose phosphate isomerase (TPI) activity. As expected such mutants were unable to form glycogen from lactate but, surprisingly, they still made glycogen from glycerol [11]. With 1-^{14}C-glycerol the specific radioactivity of the glycogen formed was identical to that of the wild-type cells, indicating that in the mutant both parts of the glucose moiety of the glycogen still came from glycerol. The TPI$^-$-mutants also produced an oxo-compound from glycerol [11] which accumulated in the medium. This oxo-compound was isolated as its 2, 4-dinitrophenylhydrazone which was identified as methylglyoxal 2, 4-dinitrophenylhydrazone by the following criteria: it was not extracted from ethyl acetate solution on treatment with aqueous 1 M sodium carbonate indicating that it was not acidic; it gave a melting-point of 305–307° which was not depressed on admixture with authentic methylglyoxal 2, 4-dinitrophenylhydrazone (m. p. 305–307°); its infrared spectrum was identical to that of the authentic compound as was its visible spectrum in alkaline solution (A$_{max}$ 550 nm). Finally, the free oxo-compound gave a positive reaction when assayed with glyoxalase 1 [9].

The TPI$^-$-mutants also accumulated methylglyoxal to 0.3 mM during growth on compounds such as glucose and gluconate. When methylglyoxal (final concentration 0.3 mM) was added to wild-type cells growing

Table 1
Glycogen synthesis by various *E. coli* mutants.

Mutant	Glycogen formed (μg 2 hr/mg dry wt.bacteria)* from:	
	Glucose	Glycerol
K2. 1t (*pps*)	41.5	27.1
K2. 20 (*tpi*)	55.8	24.0
K2. 1.20 (*pps, tpi*)	24.0	0.0

* Lactate + ribose-grown cells were used

on glucose or gluconate it was catabolised at a rate of 30 nmoles per min per mg protein and growth was not affected. However, 1 mM methylglyoxal completely inhibited growth [12].

The formation and utilization of methylglyoxal provided a possible explanation for the synthesis of glycogen from glycerol in the absence of TPI. Thus the methylglyoxal formed from glycerol could be converted to lactate via the glyoxalase reaction [13] and since lactate can be converted to pyruvate and then to glyceraldehyde-3-phosphate (G-3-P) in the TPI$^-$-mutants [11] this glycolytic by-pass plus gluconeogenesis would provide a pathway from glycerol to G-3-P which did not require TPI activity (see scheme 1).

The properties of the double mutant K2. 1.20 which lacks phosphoenolpyruvate synthetase (PPS) [10] and TPI and thus is blocked in both pathways from glycerol to G-3-P supports this view. Thus whereas the TPI$^-$-mutant (K2.20) and the PPS$^-$-mutant (K2.1t) form glycogen from glycerol the strain lacking both of these enzymes (K2. 1.20) was unable to do so (table 1). This shows that PPS is essential for glycogen synthesis from glycerol in the absence of TPI and that pyruvate must be formed from glycerol for this process.

Further support for the proposed glycolytic by-pass has come from the demonstration that extracts of *E. coli* contain enzymes which can convert DHAP to pyruvate via methylglyoxal and D-lactate. Many years ago it was suggested that methylglyoxal may be formed from glyceraldehyde [1] and more recently it was claimed that methylglyoxal was formed from G-3-P by extracts of a strain of *E. coli* [14]. However, since TPI$^-$-mutants formed methylglyoxal readily from compounds such as glycerol or rhamnose whose catabolism generates DHAP [15, 16] but failed to do so

from compounds such as lactate or glucuronate which can be converted to G-3-P [11, 17] it seemed that DHAP rather than G-3-P was the precursor of the methylglyoxal. This suggestions has been confirmed by the discovery of a novel enzyme which catalyses the formation of methylglyoxal from DHAP. The enzyme is present in all the *E. coli* K12 strains tested (K10, P10, K1, AA200) and in *E. coli* B, the specific activity in glucose-grown K10 being 150 nmoles of methylglyoxal formed per min per mg protein. The specificity of the enzyme was determined using extracts devoid of TPI activity so that no interconversion of G-3-P and DHAP could occur. In such extracts neither G-3-P, dihydroxyacetone or glyceraldehyde could replace DHAP for methylglyoxal formation. The formation of methylglyoxal was enzymic since neither whole cells nor boiled cell-free extracts catalysed the reaction.

The conversion of methylglyoxal to lactate by *E. coli* cells was first reported by Neuberg and Gorr [18], and subsequently Still [19] showed that *E. coli* has a soluble glyoxalase system. We have confirmed these results with our strains of *E. coli* and have shown that as for glyoxalases from other sources the product of the reaction was D-lactate [20]. For this *E. coli* K10 cells in 50 mM sodium/potassium phosphate buffer pH 6.8 were incubated at 30° with 5 mM methylglyoxal. When essentially all the methylglyoxal had been utilised the cells were removed by centrifugation and the supernatant analysed by the method of Barker and Summerson [21]. This showed an almost quantitative formation of lactate from methylglyoxal but this lactate failed to react with muscle L-lactate dehydrogenase (EC 1.1.1.27) [22]. That it was D-lactate was confirmed by its ready reaction with D-lactate oxidase prepared from a strain of *E. coli* devoid of L-lactate oxidase activity [23]. Whilst washed suspensions of whole-cells utilized methylglyoxal at a rate of 50 nmoles per min per mg protein cell-free extracts showed only 25% of this rate. Such extracts converted methylglyoxal to pyruvate rather than to lactate but since they also converted D-lactate (but not L-lactate) to pyruvate it seems likely that D-lactate was formed initially from methylglyoxal.

Although *E. coli* contains a NAD-linked D-lactate dehydrogenase [24] and flavin-linked D- and L-lactate oxidases [25] only the flavin-linked enzymes are involved in the conversion of lactate to pyruvate *in vivo* [24]. The D-lactate oxidase is formed constitutively in *E. coli* [25] and extracts from glucose-grown K10

oxidised 205 nmoles of D-lactate per min per mg protein. Thus all the enzymes necessary for the by-pass sequence are present in glucose-grown K10 cells.

There is evidence that the by-pass sequence operates in both mutant and wild-type cells. For instance mutants which lack both TPI and glucose-6-phosphate dehydrogenase grow slowly (mean generation time 4—5 hr) on glucose minimal medium and methylglyoxal is produced. At the end of growth when all the glucose has been utilised neither methylglyoxal nor lactate can be detected in the medium. This implies that the DHAP formed glucose has been catabolised via the by-pass reactions. More significantly small amounts of methylglyoxal have been detected in the medium during growth of the wild-type strains K1 and K10 on glucose-6-phosphate. Once again no methylglyoxal was detected after growth had ceased. However, no methylglyoxal was observed during growth of the wild-type strains on glucose.

4. Discussion

Evidence has been presented for the existence and operation in *E. coli* of a sequence of reactions which convert DHAP to pyruvate via methylglyoxal and D-lactate thus by-passing the normal glycolytic reactions from DHAP to pyruvate. The only novel enzyme involved is one which forms methylglyoxal from DHAP. This reaction appears to be complex involving a phosphate-dependent dehydration and internal oxido-reduction. It bears some resemblance to the vitamin B_{12}-dependent dioldehydratases [26] but no studies on the reaction mechanism have yet been made.

Although all the necessary enzymes are present in wild-type cells little is known about the normal operation or physiological significance of this sequence. Since it by-passes both substrate-linked phosphorylations of glycolysis it would seem to be energetically unfavourable for glucose degradation. However, two other possible functions for the by-pass can be envisaged. It may play a regulatory role by preventing the accumulation of high concentrations of phosphorylated glycolytic intermediates which are known to inhibit growth [3] or it may produce the D-lactate which might be needed for the process of amino acid and β-galactoside transport into *E. coli* [27, 28]. Attempts are underway to assess these various possibilities.

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