TWO ROUTES FOR SYNTHESIS OF PHOSPHOENOLPYRUVATE FROM C_4-DICARBOXYLIC AICDS IN ESCHERICHIA COLI

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SUMMARY

Mutants of E. coli defective in both phosphoenolpyruvate carboxykinase and phosphoenolpyruvate synthetase are unable to use C_4-dicarboxylic acids such as succinate and malate as carbon and energy sources for growth. Revertants that have restored function for either one of these enzymes can grow in a malate-mineral medium, but at a reduced rate compared with the growth of wild-type cells. E. coli appears to use two pathways for synthesis of phosphoenolpyruvate from C_4-dicarboxylic acids. One of these involves decarboxylation of oxalacetate catalyzed by phosphoenolpyruvate carboxykinase. The second pathway makes use of the combined action of malic enzyme and phosphoenolpyruvate synthetase.

INTRODUCTION

When microorganisms are grown with a C_4-dicarboxylic acid such as succinate, furmarate, or malate as the sole carbon source they must convert this acid to phosphoenolpyruvate (PEP), which is required for gluconeogenesis as well as for synthesis of pentoses and certain amino acids (1). Phosphoenolpyruvate carboxykinase (PEPCK), which readily decarboxylates oxalacetate to PEP in the presence of a suitable nucleoside triphosphate (2), is generally considered to be the enzyme responsible for net synthesis of PEP during growth with C_4-dicarboxylic acid carbon sources (1, 3, 4). A mutant of E. coli lacking a functional PEPCK was reported to be able to grow at slow rates on C_4-dicarboxylic acids (3). Another mutant of E. coli was described as being deficient in PEPCK activity and unable to grow with any of the tricarboxylic acid cycle intermediates as a carbon source (4), but this strain was subsequently shown to possess at least two mutations which affect C_4-dicarboxylic acid utilization but not PEPCK activity (5). It is the purpose of the present communication to demonstrate that in addition to the PEPCK route to PEP formation from C_4-dicarboxylic

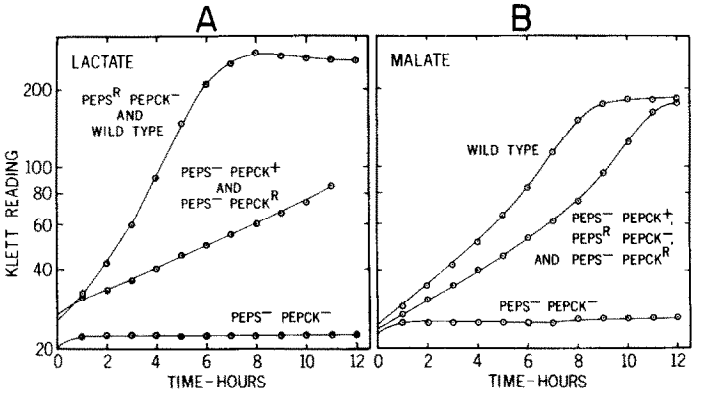

Fig. 1. Growth of wild-type and mutant strains in A. lactate-mineral medium; B. malate-mineral medium. Since several of the curves overlapped each other, only one set of experimental points is shown for each of these cases.

acids, E. coli can also synthesize PEP by a pathway that includes malic enzyme and phosphoenolpyruvate synthetase (PEPS).

MATERIALS AND METHODS

The bacterial strain used in these studies, E. coli K37 (a Sm^r derivative of W3102 galK2), was obtained from D. I. Friedman. Cells were grown in M9 mineral medium (6) containing 0.2% or 0.5% carbon source or on one-half strength S2 mineral agar (7) containing 0.2% carbon source at 37 C. In all growth curves 0.5 ml of stationary phase culture grown in 0.5% glycerol was inoculated into 9.5 ml of malate- or lactate-mineral medium and incubated with shaking in a water bath and turbidity was measured in a Klett-Summerson colorimeter (66 filter). The plotted values shown in Fig. 1 are corrected for the departure from linearity of the absorbance observed with dense suspensions.

Mutants were obtained after treatment with N-methyl-N'-nitro-N-nitroso-guanidine (8). The penicillin selection technique (6) was used to isolate mutants capable of growing in a glucose- but not in a lactate-mineral medium (9). Strain H21-13, obtained in this way, was shown to lack PEPS (9). Further mutagenesis of strain H21-13 and selection for mutants unable to grow in a mineral medium contain acetate and malate as a combined carbon source, but still able to grow in a glucose-mineral medium, resulted in the isolation of strain H3-5,

which was shown to be defective in PEPCK as well as in PEPS activity. Revertants of strain H3-5 able to grow in malate- or lactate-mineral medium were obtained by mutagenesis with diethyl sulfate by the method of Iyer and Szybalski (10). PEPCK was assayed manometrically by measurement of the rate of CO_2 evolution from oxalacetate in the presence of ATP (11), and also by incorporation of $^{14}CO_2$ into non-labelled oxalacetate (4). The isotope exchange reaction was terminated by addition of 0.1 ml of the reaction mixture to 0.4 ml of ice-cold 5% trichloroacetic acid, the acidified mixture was gassed for 1.5 min with CO_2, 1.5 ml of 0.5 M tris buffer, pH 8.4, was then added, followed after mixing by 0.1 ml of freshly prepared 1 M sodium borohydride to reduce residual oxalacetate to malate. This solution was permitted to stand at room temperature for 30 min at which time 0.1 ml aliquots were pipetted onto glass fiber filters in scintillation vials. After drying the open vials for 1 hr at 90 C 10 ml of a toluene based scintillation fluid was added to each and the samples were counted in a Beckman Model LS-230 scintillation counter. NAD- and NADP-linked malic enzymes were assayed according to the method of Yamaguchi et al. (12) using extracts of malate-grown wild-type cells which had been subjected to the first purification step described by these authors (12). Cell-free extracts for enzyme assays were prepared by sonication of cell suspensions in 0.2 M tris-maleate buffer, pH 6.8.

RESULTS

The specific activities of PEPCK and PEPS in wild-type and mutant strains of E. coli K37 are shown in Table 1. It is of interest to note that the specific activity of PEPS obtained from malate-grown wild-type cells is very similar to the specific activity of this enzyme obtained from lactate-grown cells and contrasts markedly with the lower specific activity observed when glycerol is the growth substrate. This finding is consistent with the concept that an appreciable amount of intracellular pyruvate is formed when malate is the carbon source for growth.

Mutants lacking either PEPCK or PEPS grow well on malate or succinate plates.

Table I

Specific activities of PEPS and PEPCK in extracts from wild-type and mutant strains of E. coli K37

Strain	Genotype	Carbon source	PEPS μmoles PEP/mg/hr*	PEPCK μl CO_2/mg/min*	PEPCK CPM/mg*
W-T	$PEPS^+$, $PEPCK^+$	lactate	2.23	-	-
		malate	1.99	6.65	80,600
		glycerol	0.68	2.08	38,200
H21-13	$PEPS^-$, $PEPCK^+$	glycerol	0.05	2.61	38,900
H3-5	$PEPS^-$, $PEPCK^-$	glycerol	0.05	0	4,600
H8	$PEPS_r^+$, $PEPCK^-$	glycerol	0.71	0	5,800
H14	$PEPS^-$, $PEPCK_r^+$	glycerol	0	-	35,000

*These values are the differences between activities observed in the presence and in the absence of ATP.

The designations $PEPS_r^+$ and $PEPCK_r^+$ represent revertant activities obtained by mutation of strains in which the corresponding activities were originally absent.

The double mutant, strain H3-5, which essentially lacks both of these activities, however, is unable to use the C_4-dicarboxylic acids as carbon sources for growth. Two classes of revertants of this double mutant were found. One type of revertant, exemplified by strain H14, which can grow with malate but not with lactate as the carbon source, was shown to have regained PEPCK activity. Revertants able to grow with either malate or lactate as the carbon source, such as strain H8, had regained only PEPS activity. This last finding strongly implies that one route for gluconeogenesis from C_4-dicarboxylic acids in E. coli must proceed through pyruvate as an intermediate in PEP formation.

When growth of mutants lacking either PEPCK or PEPS in malate-mineral medium was compared with growth of wild-type cells in the same medium it was

found that each mutant grows at a somewhat reduced rate (Fig. 1B). Mutant H8, which is defective only in PEPCK activity, grows at the wild-type rate in lactate-mineral medium, as expected (Fig. 1A). By contrast, mutants lacking only PEPS activity grow at an extremely slow rate in lactate-mineral medium (Fig. 1A). This slow rate of growth most probably results from utilization of acetate, formed from pyruvate by action of pyruvate dehydrogenase in the relatively large culture inoculum. In this case C_4-dicarboxylic acids are synthesized through operation of the glyoxylate cycle and PEP synthesis makes use of PEPCK which is present in strains H21-13 and H14. The fact that the double mutant lacking both PEPCK and PEPS is unable to grow at all in lactate-mineral medium (Fig. 1A) serves to verify this hypothesis.

DISCUSSION

Synthesis of PEP by microorganisms growing with C_4-dicarboxylic acids, or other tricarboxylic acid cycle intermediates, as carbon and energy sources has been considered to take place exclusively by action of PEPCK (1, 3, 4):

$$\text{succinate} \xrightarrow{-2H} \text{fumarate} \xrightarrow{+H_2O} \text{malate} \xrightarrow{-2H} \text{oxalacetate} \xrightarrow[\text{PEPCK}]{+ATP} \text{PEP} \qquad 1.$$

Our findings confirm the brief report of Kornberg (3) that mutants of E. coli lacking PEPCK are still able to grow with C_4-dicarboxylic acids as carbon sources, although at reduced rates (Fig. 1B). The fact that a double mutant lacking PEPS in addition to PEPCK is unable to grow with C_4-dicarboxylic acids as carbon sources strongly suggests that a second pathway for PEP synthesis from these acids may involve the action of PEPS with pyruvate as the immediate precursor of PEP. Such a pathway could make use of the NAD-linked malic enzyme which is known to be present in E. coli (12):

$$\text{malate} \xrightarrow[\text{malic enz.}]{+NAD^+} \text{pyruvate} \xrightarrow[\text{PEPS}]{+ATP} \text{PEP} \qquad 2.$$

Since the growth rates in malate-mineral medium of mutants lacking either PEPCK or PEPS are less than the growth rate of wild-type cells in this medium (Fig. 1B), it would appear that both pathways (Equations 1 and 2) contribute

to PEP synthesis during growth with C_4-dicarboxylic acids as sources of carbon and energy. Further evidence for this role of PEPS comes from the finding that the specific activity of PEPS in malate-grown cells is very similar to the specific activity of this enzyme in lactate-grown cells (Table I). In addition, we have confirmed that extracts of E. coli K37 contain both NAD- and NADP-linked malic enzymes (12). The specific activity of PEPCK in E. coli has been shown to be high in cells grown with C_4-dicarboxylic acid carbon sources and to be repressed when growth takes place in the presence of glucose (4, 13, 14). Similar findings have also been made for the malic enzymes in E. coli (15).

Although each of the malic enzymes may play a role in PEP synthesis in E. coli by the pathway outlined in equation 2, it seems most likely that the NAD-linked enzyme is of major significance for gluconeogenesis. Since pyruvate, formed during growth with malate, is largely oxidized to acetate by pyruvate dehydrogenase, only a relatively small proportion of pyruvate derived from malate will be used for PEP synthesis. NADH formed by action of the NAD-linked malic enzyme is readily oxidized by the electron transport system in E. coli with rapid regeneration of NAD^+. The amount of malate oxidized by the NADP-linked malic enzyme will be limited by the requirement for NADPH for biosynthesis. In fact, it would appear that the primary function of the NADP-linked malic enzyme may be to supply this required NADPH when cells grow with C_4-dicarboxylic acids as carbon sources.

It has been reported that two spontaneous mutants of Salmonella typhimurium lacking PEPCK cannot grow with succinate as the carbon source (11). These mutants have been demonstrated to also lack α-ketoglutarate dehydrogenase activity and are undoubtedly deletions since it has not been possible to obtain revertants which are able to grow in a succinate-mineral medium (11). The extent of these deletions is not known, but it is possible that one or both of the malic enzymes may also be deleted in the mutant strains making it impossible for these organisms to use the second pathway for PEP synthesis (Equation 2). Microorganisms such as yeast (16), Neurospora (17), certain

bacteria such as Rhodopseudomonas spheroides (18), and Bacillus species (19) appear to lack PEPS and convert pyruvate to PEP by the combined action of pyruvate carboxylase and PEPCK (20). In these cases only the pathway outlined in Equation 1 can be used to form PEP from C_4-dicarboxylic acids. It is, therefore, not surprising to find that single step mutants of such organisms which have defective PEPCK are unable to grow with C_4-dicarboxylic acids as growth substrates (17, 19).

It has been considered previously that PEPS in E. coli functions only when cells grow with pyruvate, lactate, alanine, or any other compound which is degraded to pyruvate, as a source of carbon and energy (9). The results of the present study suggest that PEPS also has a gluconeogenic function in E. coli growing with C_4-dicarboxylic acids as carbon sources.

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