ISOLATION OF MUTANTS OF ESCHERICHIA COLI LACKING NAD- AND NADP-LINKED MALIC ENZYME ACTIVITIES

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SUMMARY

When Escherichia coli utilizes C_4-dicarboxylic acids as sole sources of carbon and energy for growth, phosphoenolpyruvate is synthesized by means of phosphoenolpyruvate carboxykinase or by sequential action of malic enzyme and phosphoenolpyruvate synthetase. Chemical mutagenesis of a strain of E. coli K-12 lacking phosphoenolpyruvate carboxykinase activity yielded a mutant which grows very slowly on malate-mineral medium and lacks NAD-linked malic enzyme activity as well as phosphoenolpyruvate carboxykinase activity. A revertant of this double mutant which possesses elevated levels of NADP-linked malic enzyme activity grows well on malate-mineral medium. Further mutagenesis of this revertant produced a mutant which grows very slowly on malate-mineral medium and lacks NADP-linked malic enzyme activity as well as phosphoenolpyruvate carboxykinase and NAD-linked malic enzyme activities.

INTRODUCTION

Phosphoenolpyruvate (PEP) is required for gluconeogenesis as well as for the synthesis of pentoses and certain amino acids when microorganisms are grown on a C_4-dicarboxylic acid, such as malate or succinate, as the sole source of carbon and energy (1). It has previously been reported by this laboratory that Escherichia coli possess two separate pathways for the synthesis of PEP from C_4-dicarboxylic acids, each of which can function independently or in concert to supply the PEP requirements of cells growing in malate-mineral medium (2). One well-known pathway involves the ATP-dependent decarboxylation of oxalacetate by PEP carboxykinase (EC 4.1.1.32) to yield PEP and carbon dioxide, while the other route requires the production of pyruvate from C_4-dicarboxylic acids, with its subsequent conversion to PEP via the action of PEP synthetase, as shown in Fig. 1. The enzyme most likely to be involved in this decarboxylation of C_4-dicarboxylic acids to pyruvate was postulated to be malic enzyme, which E. coli contains as both NAD- and

NADP-linked species (EC 1.1.1.38 and EC 1.1.1.40, respectively)(3). The function of malic enzyme in eukaryotic organisms has been extensively investigated, but relatively little is known about its physiological significance in prokaryotes (4, 5). In the present communication, we describe the isolation of mutants of E. coli lacking NAD- and NADP-linked malic enzyme activities. The properties of these mutants provide further evidence that malic enzyme, in conjunction with PEP synthetase, is indeed involved in the synthesis of PEP from C_4-dicarboxylic acids by this bacterium.

MATERIALS AND METHODS

The bacterial strain used in this study, a derivative of Escherichia coli K-12, has been previously described (2). For enzyme studies cells were grown in S-2 mineral medium (6) containing 0.4% carbon source at 37°C. In all growth curves, 1.0 ml of a stationary phase culture grown in 0.3% lactate S-2 medium was centrifuged at room temperature, resuspended in 1.0 ml of fresh S-2 medium, and added to 9.0 ml of one-half strength S-2 containing 0.2% carbon source. Growth curves were performed as described previously (2). Solid media consisted of one-half strength S-2 mineral medium containing 0.2% carbon source solidified with 1.5% agar.

Mutagenesis was performed with N methyl-N'-nitro-N-nitrosoguanidine (7). Mutagenized cultures were grown in lactate-mineral medium overnight at 37°C with shaking. Aliquots of these cultures were subjected to penicillin selection (7) in order to isolate mutants incapable of growth in mineral medium containing malate and acetate as a combined carbon source. Transduction using Plvir was carried out as described by Miller (7). PEP carboxykinase, NAD- and NADP-linked malic enzymes were assayed as described previously (2). Cell free extracts were prepared for enzyme assays by sonication of cell suspensions in 0.1 M pH 7.8 Tris-HCl containing 10 mM $MgCl_2$ and 5 mM mercaptoethanol.

RESULTS

A strain of E. coli K-12 lacking PEP carboxykinase activity, designated as strain 1122, was chemically mutagenized, and outgrowth and segregation of the surviving bacteria was accomplished in lactate-mineral medium, in order to prevent the proliferation of any possible PEP synthetase mutants. An aliquot of the lactate culture was subjected to penicillin selection in mineral medium containing both malate and acetate as carbon sources, and a mutant which grows very slowly on malate was isolated by this procedure. This mutant, designated strain 1249, contains PEP synthetase activity, as evidenced by its ability to grow well in lactate-mineral medium, but lacks NAD-linked malic enzyme activity as well as PEP carboxykinase activity, while

retaining wild type levels of NADP-linked malic enzyme activity (Table 1).

Transductants of this double mutant that grow well on malate-mineral medium were constructed by using the generalized transducing phage P1vir grown on wild type cells. These transductants can be categorized into two distinct classes. One type, as exemplified by strain 1261, has regained wild type levels of PEP carboxykinase activity, and still lacks NAD-linked malic enzyme activity (Table 1). The other class of transductant, as represented

Table 1

Specific activities of PEP carboxykinase, NAD-, and NADP-linked malic enzyme in cell-free extracts of lactate-grown cultures

Strain	Genotype	PEPCK CPM/mg x 10^{-3}	NAD-ME Δ OD_{340}/mg/min	NADP-ME Δ OD_{340}/mg/min
Wild Type	$PEPCK^+$, $NAD\text{-}ME^+$, $NADP\text{-}ME^+$	90.3	.041	.055
1122	$PEPCK^-$, $NAD\text{-}ME^+$, $NADP\text{-}ME^+$	6.5	.047	.058
1249	$PEPCK^-$, $NAD\text{-}ME^-$, $NADP\text{-}ME^+$	6.8	<.007	.069
1261	$PEPCK_T^+$, $NAD\text{-}ME^-$, $NADP\text{-}ME^+$	93.3	<.005	.060
1263	$PEPCK^-$, $NAD\text{-}ME_T^+$, $NADP\text{-}ME^+$	6.4	.046	.054
1252	$PEPCK^-$, $NAD\text{-}ME^-$, $NADP\text{-}ME_R^+$	6.3	<.006	1.251
1321	$PEPCK^-$, $NAD\text{-}ME^-$, $NADP\text{-}ME^-$	4.7	<.007	0

PEPCK = PEP carboxykinase; NAD-ME = NAD-linked malic enzyme, NADP-ME = NADP-linked malic enzyme.

The designations $PEPCK_T^+$ and $NAD\text{-}ME_T^+$ represent activities obtained by transduction of strains in which the corresponding activities were originally absent. The designation $NADP\text{-}ME_R^+$ represents a revertant activity obtained by mutation of a strain in which the corresponding activity was originally present at normal wild type levels.

by strain 1263, has acquired wild type levels of NAD-linked malic enzyme and still lacks PEP carboxykinase activity (Table 1). Thus, it is evident that either PEP carboxykinase or NAD-linked malic enzyme, but not both, is necessary for wild type growth of E. coli in malate-mineral medium (Fig. 2A).

A number of spontaneous revertants of the double mutant strain 1249 that grow well on malate-mineral medium were shown to contain considerably elevated levels of the NADP-linked malic enzyme, while still lacking both PEP carboxykinase and NAD-linked malic enzyme activities (Table 1). These revertants, subjected to the same mutagenesis and selection procedures described above, yielded mutants which grow very slowly on malate-mineral medium (Fig. 2B). Enzymatic analysis of one of these mutants, designated strain 1321, revealed that this organism contained little or no NADP-linked malic enzyme activity, in addition to lacking both PEP carboxykinase and NAD-linked malic enzyme activities (Table 1). This triple mutant grows at a rate comparable to wild type on lactate-mineral medium.

DISCUSSION

E. coli has been shown to employ two different mechanisms for the synthesis of PEP from C_4-dicarboxylic acids (2). One pathway involves the ATP-dependent action of PEP carboxykinase upon oxalacetate (Fig. 1). The other route utilizes PEP synthetase to convert pyruvate, produced from C_4-dicarboxylic acids, to PEP (Fig. 1). The enzyme responsible for the decarboxylation of C_4-dicarboxylic acids to pyruvate was postulated to be malic enzyme (2). This enzyme has been reported to be present in a number of different bacterial and fungal genera as either or both NAD-linked and NADP-linked species (4).

Evidence for the physiological role of malic enzyme is available for only a few microorganisms. The malic enzyme of Bacillus subtilis, which can utilize either NAD or NADP as a cofactor, has been implicated in a "pyruvate shunt" mechanism, in conjunction with pyruvate carboxylase, for supplementing the synthesis of oxalacetate by malic dehydrogenase during growth of this

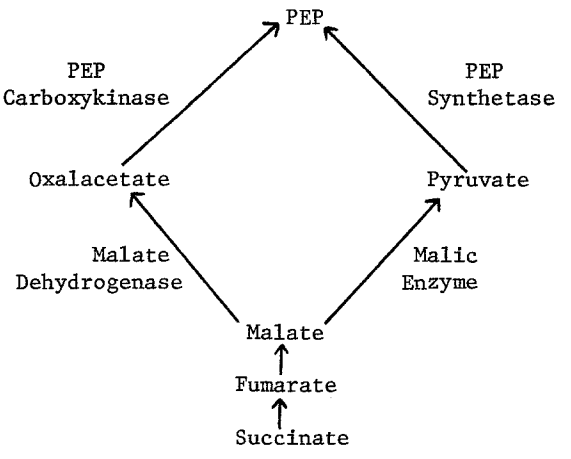

Fig. 1. Alternative pathways for synthesis of PEP from C_4-dicarboxylic acids operative in E. coli.

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organism on malate (8). The isolation of mutants of B. subtilis lacking malic enzyme by means of penicillin selection in malate-mineral medium has established the essential role of this enzyme during growth of this spore-forming organism on malate (9). The NADP-linked malic enzyme of Aspergillus nidulans appears to be essential to cells growing on glutamate or acetate for the production of pyruvate (10). Single gene mutants of this fungus lacking NADP-linked malic enzyme activity fail to grow on the above-mentioned precursors of Krebs cycle intermediates, despite the presence of PEP carboxy-kinase. The low level of pyruvate kinase present in acetate grown mycelia implies that this organism is not able to synthesize adequate amounts of pyruvate via the combined actions of PEP carboxykinase and pyruvate kinase during growth on acetate or glutamate, and thus relies primarily on malic enzyme to accomplish this task. Preliminary evidence for the possible involvement of a NADP-linked malic enzyme in CO_2 fixation has been obtained for a Micrococcus species (11). E. coli is somewhat unusual in that it contains both NAD- and NADP-linked species of malic enzyme, which are present at high levels during growth of this organism on malate or succinate mineral media (12)

The synthesis and activities of these two enzymes are controlled by a number of different intermediary metabolites, as evidenced by data obtained both *in vivo* and *in vitro* (12,13,14,15).

The fact that a double mutant of *E. coli* lacking both PEP carboxykinase and NAD-linked malic enzyme activities grows very slowly on malate-mineral medium (Fig. 2A) serves to verify the hypothesis that NAD-linked malic enzyme is the primary means by which pyruvate is produced from C_4-dicarboxylic acids, in the absence of PEP carboxykinase activity (2). Substantial quantities of pyruvate must be formed from malate, in the absence of PEP carboxykinase, because pyruvate is required not only for PEP synthesis but also as a source of acetate, which is necessary for the oxidative energy yielding function of the tricarboxylic acid cycle. The double mutant 1249 still contains wild type levels of the NADP-linked malic enzyme (Table 1) which, by itself, does not seem to suffice to allow wild type growth of *E. coli* on malate-mineral medium (Fig. 2A). This is in accordance with the concept that NADPH is used primarily for the reductive steps in biosynthesis and does not readily supply electrons to oxygen via the electron transport system present in *E. coli* (2). Indeed, the very slow growth of the double mutant 1249 on malate is most probably not due to the NADP-linked malic enzyme activity present in this organism, since the triple mutant strain 1321, lacking NADP-linked malic enzyme activity as well as NAD-linked malic enzyme and PEP carboxykinase activities, grows similarly to strain 1249 in malate-mineral medium (Fig. 2A,2B). The enzymatic mechanism which enables these two mutants to grow at all on malate may be due to leakiness in one or more of the mutations or to the presence of low levels of oxalacetic decarboxylase, which has been reported to be present in a number of widely varying bacterial genera (16,17,18) including *Salmonella* species (19,20). This enzyme itself has never been detected in *E. coli*, although both the NAD-linked malic enzyme and the NADP-linked malic enzyme of this organism have been reported to possess oxalacetate decarboxylating activity (13,21).

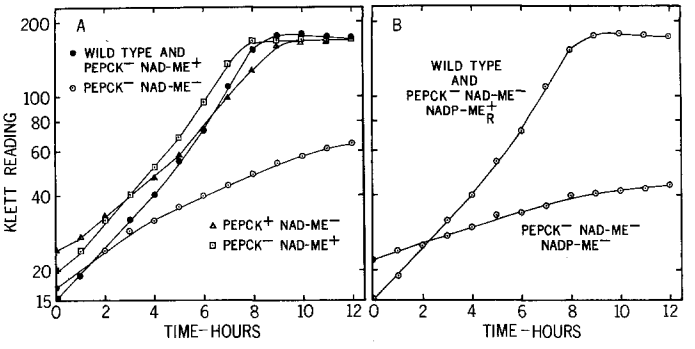

Fig. 2. Growth of wild type and mutant strains described in Table 1 on malate-mineral medium. When two curves overlapped each other, only one set of experimental points is shown. A. ● - strains 925 and 1122; ⊙ - strain 1249; △ - strain 1261; ⊡ - strain 1263. B. Top curve - strains 925 and 1252; Bottom curve - strain 1321.

The ability of strain 1252 to grow on malate-mineral medium appears to be the result of the elevated level of the NADP-linked malic enzyme (Table 1), possibly because of the increased rate of decarboxylation of oxalacetate due to the derepressed NADP-linked malic enzyme present in this strain.

The results of the present study confirm our previous finding (2) that there are two separate pathways for the synthesis of PEP from C_4-dicarboxylic acids operative in E. coli and that the NAD-linked malic enzyme plays a major role in one of these pathways (Fig. 1). The function of the NADP-linked malic enzyme in normal wild type cells is most probably the generation of reducing power for biosynthesis, although other enzymes such as NADP-linked isocitrate dehydrogenase, NADP-linked glucose-6-phosphate dehydrogenase, and pyridine nucleotide-linked transhydrogenases can also serve to supply NADPH. The fact that mutants lacking NADP-linked malic enzyme activity grow well on lactate-mineral medium indicates that NADP-linked malic enzyme is dispensable under these conditions. The role of the NAD-linked malic enzyme has been shown to be the decarboxylation of large amounts of malate, to yield substrate quantities of pyruvate for use both in gluconeogenesis as well as in oxidative energy yielding metabolism.

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