Phosphorylation of isocitrate dehydrogenase in *Escherichia coli* mutants with a non-functional glyoxylate cycle

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The phosphorylation of NADP-specific isocitrate dehydrogenase in an isocitrate lyase and in a malate synthase mutant of *Escherichia coli* has been investigated. The results clearly demonstrate that isocitrate dehydrogenase may undergo an acetate-induced phosphorylation in organisms which do not have a functional glyoxylate cycle. This observation, together with those reported in *Salmonella typhimurium*, suggest that the current notion concerning the interrelationship between the glyoxylate cycle and the reversible phosphorylation of NADP-isocitrate dehydrogenase in microbial physiology should be re-evaluated, and that phosphoenolpyruvate may be a key factor in the regulation of the reversible covalent modification of this enzyme in vivo.

Isocitrate dehydrogenase *Phosphorylation* Escherichia coli *Glyoxylate cycle* *Mutant* *Phosphoenolpyruvate* *Malate synthase* *Isocitrate lyase*

1. INTRODUCTION

The reversible inactivation of NADP-isocitrate dehydrogenase (EC 1.1.1.42) was originally described in *Escherichia coli* in [1,2] following growth of the organism in a medium containing limiting glucose as the sole carbon source. It was suggested by these workers that the decrease in isocitrate dehydrogenase activity observed under these conditions provided the cell with a means to utilize the acetate which had accumulated in the medium. This occurred as the enzymes of the glyoxylate by-pass, isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2), became derepressed and an alternate pathway for the metabolism of isocitrate became available. It is well known that during growth on acetate the tricarboxylic acid and glyoxylate cycles must function simultaneously, and that isocitrate occurs at the branch point of these two cycles [3,4].

Our laboratory subsequently reported that in *E. coli* the reversible inactivation of isocitrate dehydrogenase may occur as a result of the reversible covalent phosphorylation of the enzyme [5–8]. The enzyme was also later shown to be subject to covalent phosphorylation in *Salmonella typhimurium* [9–11].

The view has been generally accepted that the covalent modification of isocitrate dehydrogenase which occurs in vivo provides the cell with a regulatory mechanism to partition the metabolism of isocitrate through two competing metabolic pathways, both of which are essential during growth on acetate [5–12]. The data presented here suggest that this interpretation should be re-evaluated, since mutants of *E. coli* which are unable to utilize the glyoxylate cycle because of a deficiency in either isocitrate lyase or in malate synthase are, nevertheless, able to catalyze the phosphorylation of isocitrate dehydrogenase in vivo in response to the addition of acetate to the medium.

2. EXPERIMENTAL

2.1. *Organism*

For these studies, two mutants were employed. *E. coli* DV21A05 (*Hfr, glu1, aceB, glc,* B_1^-) is a

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well-characterized malate synthase-deficient mutant which is devoid of both malate synthase A, which permits growth on acetate, and malate synthase G, which is essential for growth on glycolate as the sole carbon source [13]. *E. coli* strain AH_2 (*F⁻, glu1, aceA, his⁻, B₁⁻*) is an isocitrate lyase-deficient mutant which has also been characterized in Vanderwinkel's laboratory [13]. These mutants were both kindly supplied by Dr Edgard Vanderwinkel at CERIA, Institut Emil Gryzon (Brussels).

2.2. *Materials*

The materials and reagents used in these experiments have been described in [8]. The [^{32}P]orthophosphoric acid (carrier-free) in HCl-free water was from New England Nuclear.

2.3. *Enzyme inactivation*

Strain DV21A05 was grown in a 1-l culture flask containing 100 ml of mineral salts medium [7] containing 1 μg/ml of thiamin and 0.3% succinate as the sole carbon source in a New Brunswick shaker at 300 rev./min at 37°C. Strain AH_2 was grown under the same conditions in medium containing, in addition, 100 μg/ml of L-histidine. Cell proliferation was measured using a Klett-Summerson colorimeter with a 660 nm filter. Aliquots of 10 ml were removed from the culture for assay as the cells entered the stationary phase of growth. The remainder of the culture was divided into two 40-ml aliquots and placed into 300-ml culture flasks. The experimental flask was supplemented with 0.25% acetate, while no addition was made to the control. The flasks were placed in the incubator at 37°C and shaking continued at 300 rev./min. The initial samples, and those removed at the times indicated in fig.1, were centrifuged at 12061 × *g* for 10 min at 4°C. The cell pellets were suspended in 2 ml of 0.1 M potassium phosphate buffer (pH 7.0) and sonicated in an ice-water bath for 5 min. Cell debris was removed by centrifugation at 48246 × *g* for 15 min at 4°C. The extracts were assayed for isocitrate dehydrogenase activity and protein as in [7].

2.4. *In vivo phosphorylation*

^{32}P-Labeling was carried out using a 50-ml culture which was grown in a low phosphate medium containing 1 μg/ml of thiamine and 0.3% succinate as the carbon source [7]. As the cells entered the stationary growth phase, the culture was supplemented with 0.25% acetate and 1 mCi of [^{32}P]orthophosphate (carrier-free). The culture was incubated at 37°C with shaking at 300 rev./min for an additional hour after which the cells were harvested as in section 2.3. Cell-free extracts were prepared as in [8].

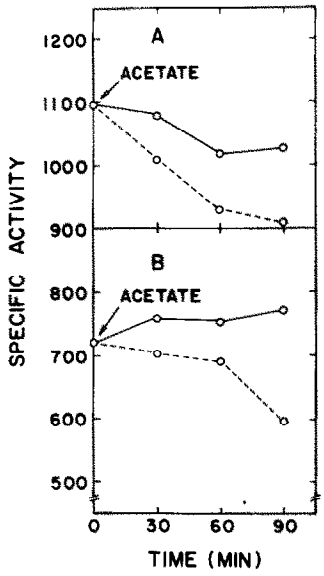

Fig.1. In vivo inactivation of isocitrate dehydrogenase in stationary phase cultures. Specific activity is expressed as μmol of NADP reduced.min^{-1}.mg protein^{-1}, in the presence (○---○) or absence (○——○) of acetate. (A) isocitrate lyase mutant; (B) malate synthase mutant.

2.5. *Immunoprecipitation*

The ^{32}P-labeled isocitrate dehydrogenase was immunoprecipitated from a 1-ml aliquot of the extract by the addition of 100 μl of crude antisera raised in rabbits against purified isocitrate dehydrogenase as in [7]. The immunoprecipitates were washed and examined following SDS gel electrophoresis and autoradiography as in [8].

3. RESULTS

3.1. *Inactivation of isocitrate dehydrogenase*

The data in fig.1A,B demonstrate the acetate-induced inactivation of isocitrate dehydrogenase in *E. coli* mutants devoid of isocitrate lyase or malate synthase activity, respectively. It can also be noted

that the activity of isocitrate dehydrogenase in extracts prepared from the isocitrate lyase mutant is considerably higher than that found in the malate synthase mutant when the cultures are harvested during the early stationary growth phase.

The extent to which isocitrate dehydrogenase is inactivated in the mutants when induced by acetate is much less than that previously observed in wild-type cells [6–8]. As reported in [10], however, there is a rapid turnover of the phosphate group in phosphorylated isocitrate dehydrogenase and the degree of inactivation one observes represents a steady state. Thus, the relative activities of the kinase and phosphatase catalyzing the reversible phosphorylation of isocitrate dehydrogenase determine the activity observed in extracts of cells obtained following the induction of these enzymes.

3.2. *In vivo phosphorylation of isocitrate dehydrogenase by [^{32}P]orthophosphate*

The data presented in fig.2 demonstrate conclusively that isocitrate dehydrogenase undergoes an in vivo phosphorylation in mutants of *E. coli* which do not have a functional glyoxylate cycle. The gels shown in fig.2A,B were strained for protein and show a heavily strained band in the immunoprecipitate (lane B) which corresponds in electrophoretic mobility to purified isocitrate

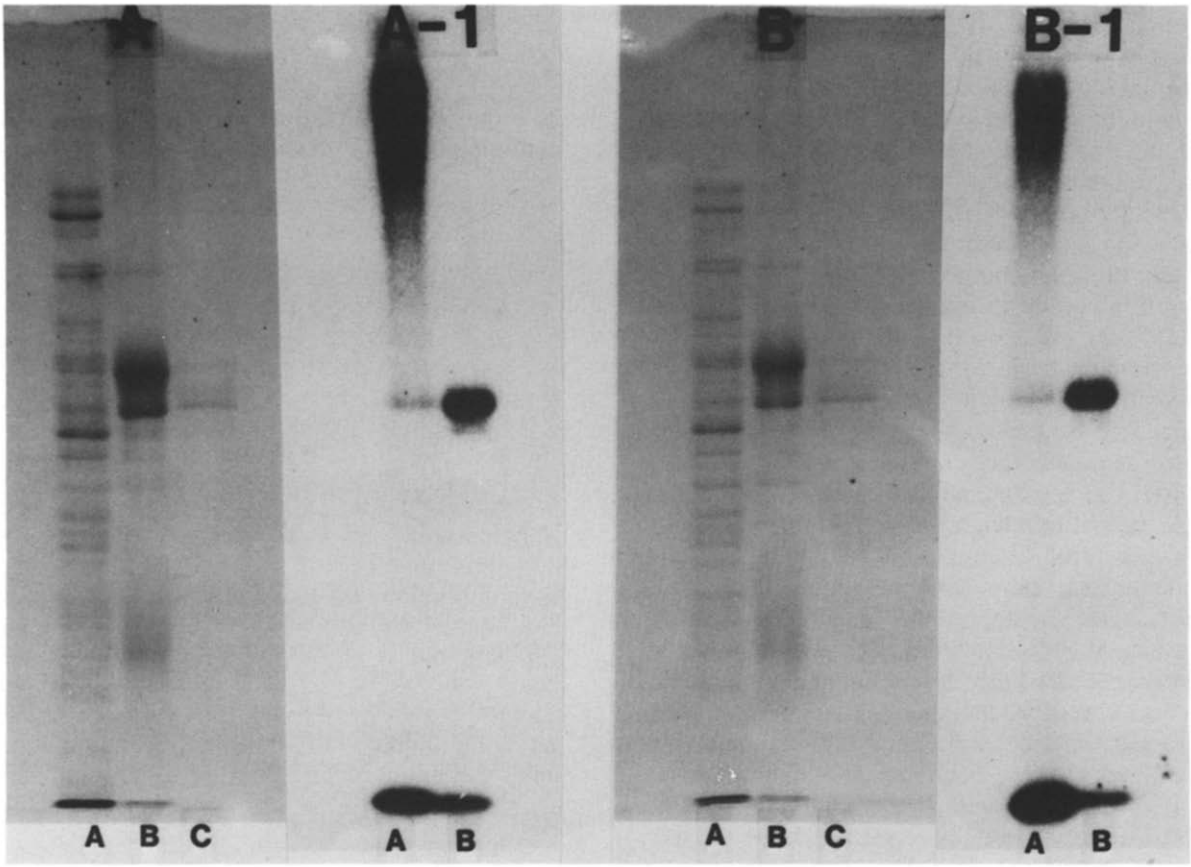

Fig.2. SDS–polyacrylamide gel electrophoresis of crude sonic extracts and immunoprecipitates of isocitrate dehydrogenase from ^{32}P-labeled cells. Panel A, protein stain of gel containing samples from isocitrate lyase mutant. Lane (A) contains 38 μg of crude sonic extract; (B) contains 52 μg of immunoprecipitate; (C) contains 5 μg of purified isocitrate dehydrogenase. Panel A-1 is an autoradiograph of the same gel. Panel B, protein stain of gel containing samples from malate synthase mutant. Lane: (A) contains 34 μg of crude sonic extract; (B) contains 40 μg of immunoprecipitate; (C) contains 5 μg of purified isocitrate dehydrogenase. Panel B-1 is an autoradiograph of the same gel.

dehydrogenase shown in lane C. When these gels were dried and exposed to X-ray film, the autoradiograms (fig.2A-1 and 2B-1) show that isocitrate dehydrogenase had become phosphorylated during the in vivo experiment in the presence of [^{32}P]orthophosphate.

4. DISCUSSION

The data presented in fig.1 and 2 demonstrate that in *E. coli* DV21A05, a mutant devoid of malate synthase activity, and in *E. coli* AH_2, a mutant devoid of isocitrate lyase activity, grown in a medium containing succinate as the sole carbon source, NADP-isocitrate dehydrogenase undergoes an acetate-induced phosphorylation with a concomitant loss of catalytic activity. This study, employing mutants in which the glyoxylate cycle is not functional, provides convincing evidence that such cells are, nevertheless, able to catalyze an acetate-dependent phosphorylation of isocitrate dehydrogenase.

In addition, authors in [10] reported the acetate-induced phosphorylation of isocitrate dehydrogenase in acetate-grown *S. typhimurium* and further observed that the in vivo phosphorylation also could be induced in these cells by either ethanol, a-methylglucoside or deoxyglucose. Ethanol can be oxidized to acetate in this organism and, therefore, might be considered analogous to acetate as an inducer of the phosphorylation. Although a-methylglucoside and deoxyglucose are rapidly transported into these cells, it is well known that they are non-metabolizable [10].

These recent observations necessitate a re-evaluation of the currently accepted notion concerning the interrelationship between the glyoxylate cycle and the reversible covalent phosphorylation of NADP-isocitrate dehydrogenase in microbial metabolism. Clearly, the kinase catalyzing the ATP-dependent phosphorylation of isocitrate dehydrogenase in vivo is not dependent upon a functional glyoxylate cycle. Of numerous compounds which have been examined for their ability to increase the phosphorylation of ICDH in vivo, only acetate, a-methylglucoside and deoxyglucose have been shown to be effective, and the latter two are non-metabolizable. The known inducers do, however, have in common the fact that they are rapidly phosphorylated via the phosphoenolpyruvate–glucose phosphotransferase system at the expense of phosphoenolpyruvate (PEP). The phosphorylation of isocitrate dehydrogenase induced by acetate could also be envisioned as a result of the lowering of the intracellular concentration of PEP by the rapid conversion of oxalacetate to citrate, thereby preventing the re-synthesis of PEP from oxalacetate by phosphoenolpyruvate carboxykinase. Thus, perhaps the intracellular level of PEP may be a key factor in the regulation of the phosphorylation of isocitrate dehydrogenase, but additional studies are clearly needed before its regulatory role can be fully evaluated.

ACKNOWLEDGEMENT

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