

# Cyclic AMP-independent phosphorylation of *Escherichia coli* isocitrate dehydrogenase

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The phosphorylation of NADP-specific isocitrate dehydrogenase in a wild-type and in an adenylate cyclase deletion mutant of *Escherichia coli* has been investigated. The results obtained clearly indicate that cyclic AMP is not required for the phosphorylation reaction *per se*, nor is it for the synthesis or possible activation of the phosphoprotein kinase in this organism. This data are in contrast to results observed in *Salmonella typhimurium*, and indicate that important differences exist in the phosphorylation of the isocitrate dehydrogenase in these two organisms.

<i>Cyclic AMP</i>	<i>Phosphorylation</i>	<i>Isocitrate dehydrogenase</i>	<i>Escherichia coli</i>
	<i>Protein kinase</i>	<i>Mutant</i>	

## 1. INTRODUCTION

The regulation of enzyme activity by reversible covalent phosphorylation has been reviewed most recently in [1]. Although this mechanism of modulating the activity of enzymes is well known in eukaryotic systems, it has been reported only recently in bacteria and, at this time, NADP-isocitrate dehydrogenase (EC 1.1.1.42) is the only one of several proteins phosphorylated in bacteria which has been identified [2–8].

Krebs and Beavo [1] divided the regulatory protein kinases known to occur in eukaryotic systems into two classes – those in which activity is controlled by cyclic nucleotides, and those under specific metabolite control. Here, we have used a mutant of *Escherichia coli* which lacks adenylate cyclase to corroborate *in vivo* the report [2] that the *in vitro* phosphorylation of isocitrate dehydrogenase in *E. coli* is not dependent on cyclic AMP. Further, in contrast to *Salmonella typhimurium* [6], we have shown that the synthesis of the protein catalyzing the phosphorylation of isocitrate dehydrogenase in *E. coli*, is not dependent upon cyclic AMP.

## 2. EXPERIMENTAL

### 2.1. Organism

For these studies, *E. coli* KC-13 was employed. This is a well-characterized adenylate cyclase deletion mutant (*F<sup>-</sup>gal<sup>-</sup>cya-2*) which was kindly supplied by Dr E.L. Kline at Clemson Univ. [9].

### 2.2. Materials

RNase and DNase were purchased from Sigma. [<sup>32</sup>P]-Orthophosphoric acid (carrier-free) in HCl-free water was from New England Nuclear. Sources of other reagents and equipment have been described [4].

### 2.3. Enzyme inactivation

Cells were grown in 500 ml culture flasks containing 100 ml mineral salts medium [4] containing 0.1% glucose as the carbon source in a New Brunswick shaker at 300 rev./min at 37°C. 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 in late log phase, and 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 culture was supplemented with 0.25% acetate, while no additions were 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  $12061 \times g$  for 10 min at 4°C. The cell pellets were suspended in 2 ml 0.1 M potassium phosphate buffer (pH 7.0) and sonicated in an ice-water bath for 10 min. Cell debris was removed by centrifugation at  $48246 \times g$  for 15 min at 4°C. The extracts obtained were assayed for isocitrate dehydrogenase activity and protein as in [4].

#### 2.4. *In vivo phosphorylation*

$^{32}\text{P}$ -Labeling was carried out using a 50 ml culture which was grown in a low phosphate medium [4] containing 0.1% glucose. As the cells entered stationary growth phase, the culture was supplemented with 0.25% acetate and 1 mCi [ $^{32}\text{P}$ ]orthophosphate (carrier-free). The culture was incubated at 37°C with shaking at 300 rev./min for an additional h after which the cells were harvested as in section 2.3. The  $^{32}\text{P}$ -labeled cells were suspended in 2 ml phosphate-buffered saline containing 5 mM NaF and 0.1% NaN<sub>3</sub> and sonicated as described earlier. Cell debris was removed by centrifugation at  $48246 \times g$  for 30 min at 4°C. Nucleic acids in the extract were digested by the addition of 501 of DNase (2 mg/ml), 501 of RNase (2 mg/ml), 501 of 0.5 M MgCl<sub>2</sub> and incubation at 26°C for 30 min. The extract was then applied to a Sephadex PD-10 column equilibrated in phosphate-buffered saline and the fractions containing enzyme activity combined.

#### 2.5. *Immunoprecipitation*

The  $^{32}\text{P}$ -labeled isocitrate dehydrogenase was immunoprecipitated from a 1 ml aliquot of the extract by addition of 1001 of crude antisera raised in rabbits against purified isocitrate dehydrogenase as in [3] and incubated at 37°C for 30 min. Carrier unlabeled purified isocitrate dehydrogenase (51 containing 10 g protein) was added and incubation continued at 37°C for an additional 30 min. The immunoprecipitate was collected by centrifugation for 2 min in a Brinkman Eppendorf micro-centrifuge. The pellet was resuspended in 701 of twice

concentrated SDS buffer [10], and heated at 95°C for 15 min. SDS-polyacrylamide gel electrophoresis and autoradiography were conducted as in [3].

### 3. RESULTS

#### 3.1. *Inactivation of isocitrate dehydrogenase*

The data in fig. 1 demonstrate the acetate induced inactivation of isocitrate dehydrogenase. The specific activity of the enzyme decreased from 0.41–0.14 within 1 h after the addition of 0.25% acetate to the culture. In the control culture, to which no acetate was added, the enzyme activity decreased slightly during the first 30 min after the culture was divided, and then increased to 0.50 after an additional 60 min incubation. These results are similar to observations in [3,4] employing *E. coli* K-12.

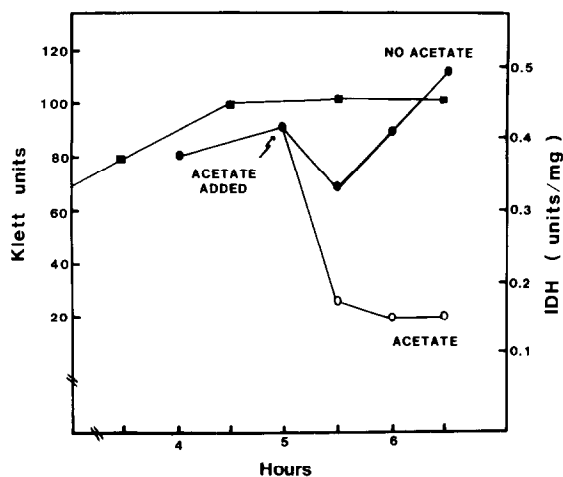


Fig. 1. *In vivo* inactivation of isocitrate dehydrogenase: cell growth (■---■); specific activity (mol NADP reduced. min<sup>-1</sup>. mg protein<sup>-1</sup>) in the presence (○---○) or absence (●---●) of acetate.

#### 3.2. *Purification of [ $^{32}\text{P}$ ]isocitrate dehydrogenase*

Following *in vivo* phosphorylation, the enzyme was immunoprecipitated from partially purified sonic extracts and examined by electrophoresis in SDS gels. The gel shown in fig. 2A was stained for protein and shows a heavily stained band in the immunoprecipitate (lane C) which corresponds in electrophoretic mobility to purified isocitrate dehydrogenase shown in lane D. When this gel was

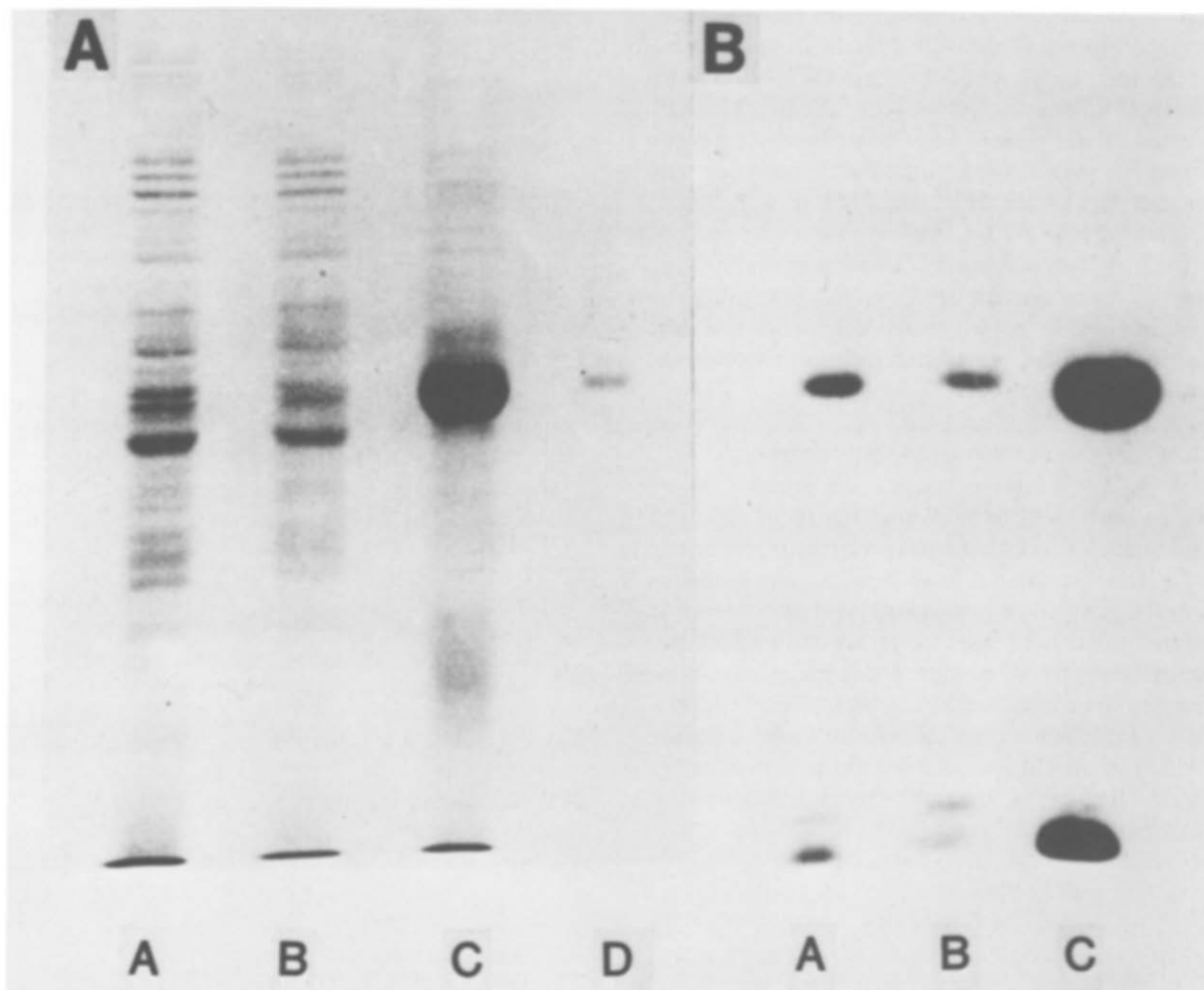


Fig. 2. SDS-polyacrylamide gel electrophoresis of samples taken during purification of isocitrate dehydrogenase from <sup>32</sup>P-labeled cells (see text): (A) stained for protein; (B) autoradiograph of the same gel. Lane (A) contains 53 µg crude sonic extract; (B) contains 34 µg extract eluted from the Sephadex PD-10 column; (C) contains 84 µg immunoprecipitate; (D) contains 6 µg purified isocitrate dehydrogenase.

dried and exposed to X-ray film, the autoradiogram (fig. 2B) shows that isocitrate dehydrogenase had become phosphorylated during the *in vivo* experiment in the presence of [<sup>32</sup>P]orthophosphate.

#### 4. DISCUSSION

##### 4.1. *In vivo* inactivation or phosphorylation of isocitrate dehydrogenase

The acetate-induced inactivation and concomitant phosphorylation of isocitrate dehydrogenase in *E. coli* KC-13 is similar to that in *E. coli* K-12

[3,4]. It is of particular significance, however, that the phosphorylation occurs in *E. coli* KC-13 which is a well-characterized mutant which is devoid of adenylate cyclase and hence contains no cyclic AMP. The results obtained in this study clearly indicate that cyclic AMP is not required for the phosphorylation reaction *per se*, nor is it required for the synthesis or possible activation of the phosphoprotein kinase in this organism.

These data are in sharp contrast to those in [6] concerning the phosphorylation of isocitrate dehydrogenase in an adenylate cyclase mutant (*cya*<sup>-</sup>)

of *S. typhimurium*. In this mutant, isocitrate dehydrogenase is not phosphorylated in vivo unless the culture is supplemented with cyclic AMP during growth. These investigators conclude that this observation could be due to the lack of a protein component for the phosphorylation rather than dependence of the kinase on cyclic AMP.

There appear to be important differences between *S. typhimurium* and *E. coli* in either the synthesis or the activation of the kinase catalyzing the phosphorylation or isocitrate dehydrogenase but additional studies are needed before this observation can be understood.

#### 4.2. Proteolysis of isocitrate dehydrogenase

In fig. 2B, a low- $M_r$  radioactive protein can be seen in lanes A–C which migrates at or near the dye-front. We had observed this protein previously and concluded that it is a peptide which results from the action of a protease present in crude sonic extracts which acts upon isocitrate dehydrogenase. The proteolysis of isocitrate dehydrogenase (not shown) can be inhibited by the inclusion of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) in all buffers, or by the passage of crude extracts through an agarose–hemoglobin column [11].

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