Phosphorylation of Isocitrate Dehydrogenase of *Escherichia coli*

Abstract. *Addition of acetate to a stationary phase culture of* Escherichia coli *in glycerol mineral salts medium containing phosphorus-32-labeled orthophosphate results in rapid loss of isocitrate dehydrogenase activity and concomitant incorporation of phosphorus-32 into the enzyme. This is the first example of protein phosphorylation in a bacterium in which the endogenous substrate for the protein kinase has been identified.*

Protein phosphorylation in recent years has become recognized as an important regulatory mechanism in many eukaryotic organisms and in some animal viruses (*1*). In contrast, there have been few reports on protein kinase and protein phosphorylation in bacteria, and in no instance has a specific substrate for a bacterial protein kinase been identified (*2*).

Kuo and Greengard (*3*) first reported the presence of protein kinase in *Escherichia coli*. Subsequently, protein kinase activity has been observed in various oral strains of streptococci (*4*) and in *E. coli* (*5*). The most conclusive evidence for protein kinase-catalyzed phosphorylation in bacteria was presented by Rahnsdorf *et al.* (*6*), who observed phosphorylation of several proteins in *E. coli* K12, but only in cells infected with bacteriophage T7. Both bacterial and viral proteins were phosphorylated, and the gene for protein kinase was mapped in the early region of the phage genome. The lack of conclusive reports on the presence of protein kinase activity in bacteria concurs with the finding that bacteria contain much less phosphoserine and phosphothreonine than is found in eukaryotic organisms (*7*). In this report, we describe the phosphorylation in vivo of nicotinamide-adenine dinucleotide phosphate ($NADP^+$)-specific isocitrate dehydrogenase (E.C. 1.1.1.42) of *E. coli* during adaptation to acetate utilization.

The phosphorylation was carried out with two 100-ml cultures of *E. coli* K12 grown in a low-phosphate medium (*8*) containing 0.1 percent glycerol. When the cells entered stationary phase, indicating exhaustion of the limiting glycerol, rifampicin (200 μg/ml) was added to the culture to prevent further RNA synthesis. After 10 minutes 1 mCi of [^{32}P]orthophosphoric acid was added, and incubation was continued for 5 minutes. One of the cultures was then made 25 m*M* in sodium acetate. The decrease in specific activity of isocitrate dehydrogenase was followed by removing 10-ml samples from the culture at 30-minute intervals. Preparation of crude extracts, enzyme assays, and protein determinations were carried out as described previously (*9*). Figure 1 shows the growth curve and the specific activity of isocitrate dehydrogenase.

One hour after the addition of acetate, the remainder of the culture in each flask was treated as described in the legend of Fig. 1. Samples of the crude extract were then heated with 2 percent sodium dodecyl sulfate (SDS) at 95°C for 15 minutes and subjected to polyacrylamide slab gel electrophoresis (*10*). The incorporation of [^{32}P]orthophosphate into isocitrate dehydrogenase was demonstrated by autoradiography of the dried gel, using Kodak X-Omat film. As shown in Fig. 2, we observed a major band of radioactivity with an electrophoretic mobility identical to that of isocitrate dehydrogenase.

Identical samples of the crude extracts were treated with proteinase K, deoxyribonuclease I, and ribonuclease I, respectively, before the SDS treatment. As shown in Fig. 2, proteinase K treatment resulted in the loss of the radioactive band corresponding to isocitrate dehydrogenase, whereas the labeled material at the top of the gel was lost only after deoxyribonuclease treatment. Treatment of the extracts with ribonuclease had no demonstrable effect. From these results, we conclude that the ^{32}P-labeled material that coelectrophoresed with isocitrate dehydrogenase is indeed a phosphorylat-

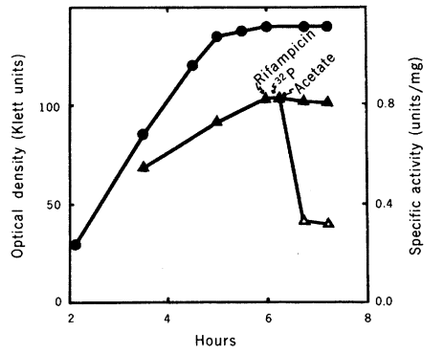

Fig. 1. Phosphorylation in vivo of isocitrate dehydrogenase of *E. coli* K12 grown on limiting glycerol. Two 100-ml cultures were grown on a low-phosphate medium (*8*) with 0.1 percent glycerol and thiamine HCl (0.03 μg/ml). The cultures were incubated at 37°C with shaking at 300 rev/min. At the beginning of stationary phase, rifampicin was added to a final concentration of 200 μg/ml. Ten minutes later 1 mCi of [^{32}P]orthophosphoric acid was added to each culture, and 5 minutes after that one of the cultures was made 25 m*M* in sodium acetate (no acetate was added to the control culture). The decrease in specific activity of isocitrate dehydrogenase was followed by removing samples of 10 ml from the culture at 30-minute intervals. One hour after the addition of acetate, the remainder of the culture in each flask was harvested by centrifugation at 10,000*g* for 10 minutes at 4°C. The pellets were suspended in 2 ml of 0.01*M* potassium phosphate buffer, *p*H 7.5, containing 0.5*M* NaCl and 2 m*M* $MgCl_2$. The cells were disrupted by sonication for 5 minutes in an ice bath. Crude cell-free extracts were obtained by centrifugation at 45,000*g* for 40 minutes at 4°C. The crude extracts were assayed for isocitrate dehydrogenase as reported earlier (*9*). (●) Growth curve, (▲) isocitrate dehydrogenase specific activity in the absence of added acetate, and (△) isocitrate dehydrogenase specific activity after the addition of the 25 m*M* acetate.

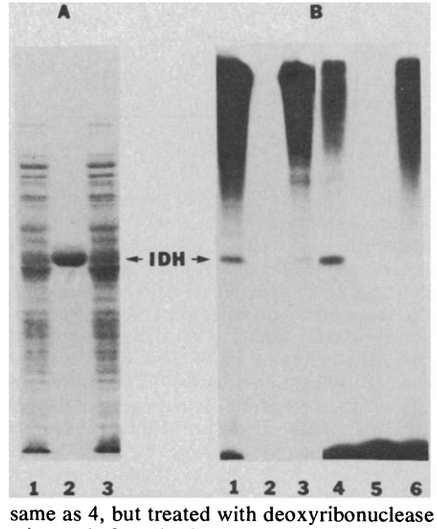

Fig. 2. Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis of crude extracts prepared as described in the legend of Fig. 1. The supernatant was equilibrated in a sample buffer solution containing 0.0625*M* tris-HCl, *p*H 6.8, SDS (2 percent), glycerol (10 percent), bromophenol blue (0.001 percent), and 2-mercaptoethanol (5 percent) and heated at 95°C for 15 minutes. Samples of 20 μl containing 200 μg of protein and having a ^{32}P counting rate of 5×10^3 to 6×10^3 count/min were electrophoresed for $2\frac{1}{2}$ hours at 70 mA. The gel was stained with fast green and dried. Autoradiography was carried out for 24 hours using Kodak X-Omat R film. (A) Protein stain (fast green), (B) autoradiogram. Gels represent the following (gels 1 to 3 are the same in A and B): 1, extract of *E. coli* K12 1 hour after the addition of acetate; 2, purified isocitrate dehydrogenase (*IDH*); 3, extract of *E. coli* K12 control (no acetate added); 4, same as 1, but from a different culture; 5, same as 4, but treated with deoxyribonuclease I (70 μg/ml, containing protease activity) for 15 minutes before the SDS treatment; and 6, same as 1, but treated with proteinase K (70 μg/ml) for 15 minutes before the SDS treatment.

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ed protein and that under the conditions of this experiment it is a major phosphorylated protein. Preliminary studies in vitro with crude extracts obtained from cells grown under similar conditions indicate that the kinase is dependent on adenosine triphosphate (*11*).

Partial purification of the phosphorylated isocitrate dehydrogenase was accomplished by applying a 2-ml sample of the crude extract to a Sephadex G-150 column. As shown in Fig. 3A, a peak of radioactivity was observed coincident with the elution of isocitrate dehydrogenase activity from the column. The combined active fractions were concentrated and subjected to SDS polyacrylamide slab gel electrophoresis, as described above. All radioactivity present in this partially purified enzyme preparation coelectrophoresed with isocitrate dehydrogenase (not shown).

The products of protein kinase–catalyzed phosphorylation are typically phosphoesters in which the phosphoryl group is linked to the hydroxyl group of a seryl or threonyl residue (*12*). The *p*H stability of the phosphorylated isocitrate dehydrogenase was studied by using the partially purified enzyme, as described by Anthony and Spector (*13*). Our results indicate that the phosphate-protein bond of the phosphorylated isocitrate dehydrogenase is stable at low or neutral *p*H and unstable at high *p*H (Fig. 3B). This type of *p*H stability profile is characteristic of phosphoserine or phosphothreonine (*14*).

The regulatory role of mitochondrial nicotinamide-adenine dinucleotide (NAD^+)-specific isocitrate dehydrogenase (E.C. 1.1.1.41) has been studied extensively. This enzyme is known to be regulated by the intracellular concentration of nucleotides and of citrate (*15*). In contrast, detailed knowlege of the regulatory role of $NADP^+$-specific isocitrate dehydrogenase is lacking. This enzyme, isolated from various sources, has been shown to be inhibited in vitro by purine nucleoside triphosphates (*16*) and is subject to concerted inhibition by glyoxylate and oxalacetate (*17*).

In *E. coli* and most other microorganisms only the $NADP^+$-specific isocitrate dehydrogenase is present. In these organisms, isocitrate dehydrogenase is a key enzyme in one of the main energy-producing pathways, the tricarboxylic acid cycle. The activities of isocitrate dehydrogenase and of the first enzyme of the glyoxylate bypass, isocitrate lyase (E.C. 4.1.3.1), regulate the flow of isocitrate into either the tricarboxylic acid cycle or the glyoxylate cycle.

Burke and Reeves (*18*) recently observed that cultures of *E. coli* K12 exhibit a specific drop in isocitrate dehydrogenase activity in response to growth conditions that elicit glyoxylate cycle activity, such as adaptation to growth on acetate as the sole carbon source. Bennett and Holms (*19*) earlier reported a similar inactivation of isocitrate dehydrogenase in other strains of *E. coli* and in other enteric bacteria. When metabolism via the glyoxylate cycle ceases, isocitrate dehydrogenase activity recovers in the absence of protein synthesis de novo. These observations suggest the existence of a reversible inactivation mechanism for the regulation of isocitrate dehydrogenase. The studies presented in this report suggest that this mechanism may involve a protein kinase.

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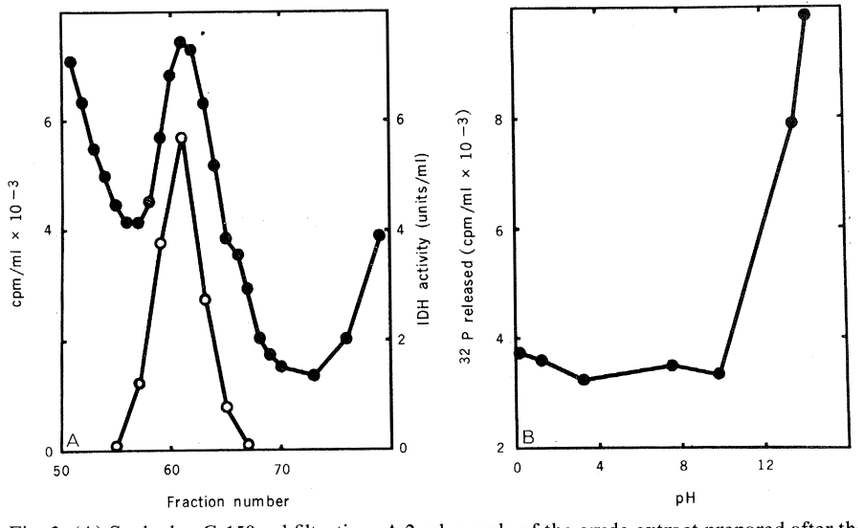

Fig. 3. (A) Sephadex G-150 gel filtration. A 2-ml sample of the crude extract prepared after the addition of acetate (Fig. 1) was applied to a Sephadex G-150 column (2.5 by 90 cm) and eluted with 0.1*M* sodium phosphate buffer, *p*H 7.5, 2 m*M* $MgCl_2$, and 0.02 percent sodium azide at a flow rate of 9 ml/hour. Fractions of 3 ml were collected. (●) Radioactivity profile, (○) isocitrate dehydrogenase activity. Fractions containing high isocitrate dehydrogenase activity were combined and concentrated. All radioactivity present in these fractions coelectrophoresed with isocitrate dehydrogenase (data not shown). When crude extract from a control culture without acetate was passed through the column under identical conditions, no ^{32}P peak was observed corresponding to the isocitrate dehydrogenase peak. (B) Effect of *p*H on the decomposition of phosphorylated isocitrate dehydrogenase. Partially purified isocitrate dehydrogenase (50 μl) was added to 50 μl of 0.2*M* buffer and incubated at 37°C for 100 minutes. The tubes were then chilled in ice for 5 minutes. Five microliters of 0.1*M* potassium phosphate (*p*H 2.87) and 5 μl of bovine serum albumin (1 mg/ml) were added as carriers. Cold 1*N* perchloric acid (25 μl) was added and the tubes were centrifuged for 6 minutes at 12,000*g* in an Eppendorf microcentrifuge 3200. Samples (50 μl) of the supernatant were withdrawn for counting. The buffers (final concentration, 0.1*M*) were *p*H 0.1, 1*N* HCl; *p*H 1.2, HCl-KCl; *p*H 3.2, KH_2PO_4; *p*H 7.5, KH_2PO_4-K_2HPO_4; *p*H 9.8, glycine NaOH; *p*H 13.5, 0.2*N* KOH; and *p*H 14.2, 1*N* KOH.

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20. We thank W. F. Burke for helpful assistance during the preparation of this manuscript. This work was supported by NSF grant PCM 77-00172.

30 August 1978; revised 6 November 1978