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The Isocitrate Dehydrogenase Phosphorylation Cycle: Regulation and Enzymology

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Abstract Isocitrate dehydrogenase (IDH) of *Escherichia coli* is regulated by phosphorylation and dephosphorylation. This phosphorylation cycle controls the flow of isocitrate through the glyoxylate bypass, a pathway which bypasses the CO_2 evolving steps of the Krebs' cycle. IDH is phosphorylated at a single serine which resides in its active site. Phosphorylation blocks isocitrate binding, thereby inactivating IDH. The IDH phosphorylation cycle is catalyzed by a bifunctional protein kinase/phosphatase. The kinase and phosphatase reactions appear to be catalyzed at the same site and may share some catalytic steps. A variety of approaches have been used to examine the IDH phosphorylation cycle in the intact organism. The picture which has emerged is one of an exquisitely sensitive and flexible system which is capable of adapting efficiently to the environment both inside and outside the cell. © 1993 Wiley-Liss, Inc.

Key words: phosphorylation, dephosphorylation, isocitrate, glyoxylate bypass, Krebs' cycle

Protein phosphorylation is one of the most pervasive control mechanisms in biological systems [Edelman et al., 1987; Cohen, 1989]. Although this regulatory strategy had been extensively characterized in eukaryotes, it has only recently been identified in prokaryotes [Garnak and Reeves, 1979; Cozzone, 1988; Wang and Koshland, 1991]. The first prokaryotic phosphorylation cycle to be identified was that which regulates isocitrate dehydrogenase (IDH) of *Escherichia coli* [Garnak and Reeves, 1979; Bennett and Holms, 1975].

The IDH phosphorylation cycle controls the flow of isocitrate through the glyoxylate bypass (Fig. 1). The glyoxylate bypass, which is found in plants and microorganisms, is essential for growth on carbon sources such as acetate or fatty acids because it allows the net conversion of acetyl-CoA into metabolic intermediates by preventing the quantitative loss of the entering carbon as CO_2 in the Krebs' cycle [Kornberg, 1966]. During growth on acetate, ca. 75% of the IDH is converted to the inactive, phosphorylated form, inhibiting the Krebs' cycle and thus forcing isocitrate through the bypass [LaPorte et al., 1984; Borthwick et al., 1984].

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STRUCTURAL BASIS OF THE INHIBITION OF IDH

IDH is phosphorylated at a single position: serine 113 [Borthwick et al., 1984; Thorsness and Koshland, 1987]. Unlike many phosphorylated enzymes, the phosphorylated form of IDH is completely inactive [Borthwich et al., 1984; LaPorte and Koshland, 1983]. Conversion of serine 113 to aspartate also inactivated this enzyme, suggesting that inactivation resulted from the introduction of a negative charge at this position [Thorsness and Koshland, 1987]. This conclusion was supported by X-ray crystallography [Hurley et al., 1990a,b, 1989]. Serine 113 is in the active site of IDH and forms a hydrogen bond with isocitrate in the active, dephosphorylated enzyme. Phosphorylation of IDH blocks binding of isocitrate by disrupting this hydrogen bond and by introducing steric and electrostatic repulsion between the phosphate group and isocitrate [Dean et al., 1989; Dean and Koshland, 1990].

ENZYMOLOGY OF THE IDH PHOSPHORYLATION CYCLE

The phosphorylation and dephosphorylation of IDH are catalyzed by a single, bifunctional protein: IDH kinase/phosphatase [LaPorte and Koshland, 1982], which is the product of a single

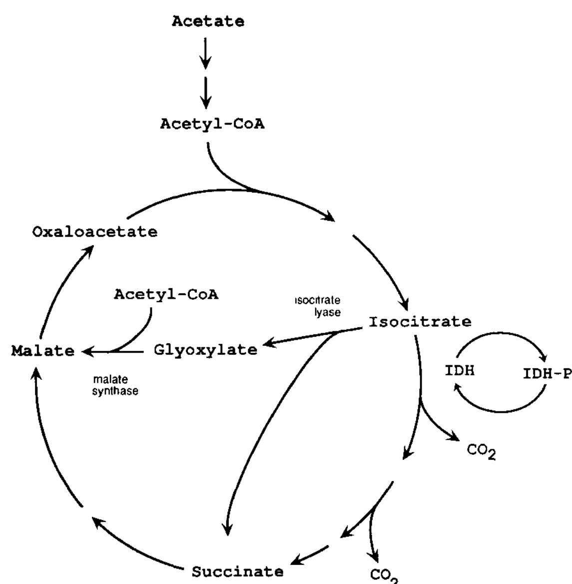

Fig. 1. The Krebs' cycle and the glyoxylate bypass. The glyoxylate bypass is composed of isocitrate lyase and malate synthase. IDH and IDH-P indicate the dephosphorylated and phosphorylated forms of isocitrate dehydrogenase, respectively.

gene, aceK [LaPorte and Chung, 1985; Klumpp et al., 1988]. The kinase and phosphatase activities reside on the same polypeptide and appear to be catalyzed at the same active site (see below).

Kinetic analyses of the products of mutant alleles of *aceK* have provided evidence for a single active site on IDH kinase/phosphatase. The products of one class of alleles retained IDH kinase activity but suffered reductions in IDH phosphatase by factors of 200 to 400 [Ikeda and LaPorte, 1991]. The kinase, phosphatase, and an intrinsic ATPase activity (see below) of these proteins all exhibited striking reductions in their affinity for phospho-IDH even though they retained affinity for dephospho-IDH. This observation suggests that there is a single binding site for phospho-IDH, which is common to the kinase and phosphatase activities.

An unusual feature of IDH phosphatase is its absolute dependence on either ATP or ADP for activity (see below). A kinetic analysis of the mutant proteins described in the preceding paragraph provided an unexpected insight into the organization of the ATP binding sites of IDH kinase/phosphatase. The mutation in one of these proteins caused a 10-fold increase in the Michaelis constants of IDH kinase, IDH phosphatase and the ATPase activities for ATP

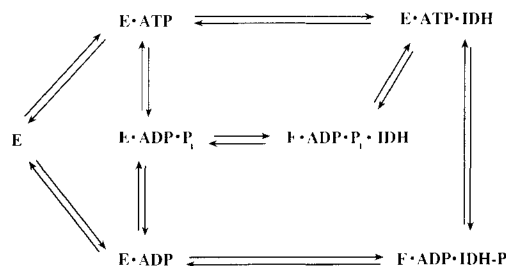

Fig. 2. Model for the catalytic mechanism of IDH kinase/phosphatase. Details are discussed in the text.

(Ikeda TP and LaPorte DC, manuscript in preparation). This parallel effect suggests that these activities share a common ATP binding site.

Site-directed mutagenesis has also provided insight into the organization of the ATP binding sites. Examination of the sequence of IDH kinase/phosphatase identified a single sequence which resembled the consensus for ATP binding sites [Klumpp et al., 1988]. A mutation which converted the "invariant" lysine of this site to a methionine virtually eliminated both IDH kinase and IDH phosphatase activities without destabilizing the protein [Stueland et al., 1989]. This parallel effect is consistent with the proposal that these activities share a binding site for ATP.

Affinity labeling of IDH kinase/phosphatase with an ATP analogue has also provided insight into the ATP binding sites [Varela and Nimmo, 1988]. This analogue yielded parallel inhibition of IDH kinase and IDH phosphatase and, in a preliminary experiment, appeared to label a single peptide. These results support the suggestion that IDH kinase and IDH phosphatase share a binding site for ATP.

Our working model proposes that the IDH kinase and IDH phosphatase reactions occur in the same active site and that the phosphatase reaction results from the back reaction of IDH kinase tightly coupled to ATP hydrolysis (Fig. 2). According to this model, the phosphatase reaction requires the formation of a ternary complex between IDH kinase/phosphatase, ADP and phospho-IDH. The phosphate group of phospho-IDH is then transferred to ADP (the kinase back reaction) and then to water (an ATPase reaction). This sequence of steps results in the net dephosphorylation of phospho-IDH (the phosphatase reaction) [LaPorte et al., 1989; Stueland et al., 1987].

This model is supported by a variety of experimental observations. i) IDH phosphatase activity exhibits an absolute requirement for either ATP or ADP [LaPorte and Koshland, 1982] but is not activated by nonhydrolyzable analogues of ATP [LaPorte et al., 1989]. ii) IDH kinase/phosphatase catalyzes an intrinsic ATPase activity which is not dependent on IDH. The ATPase activity appears to be retained even when the protein substrate, IDH, is bound [Stueland et al., 1987]. iii) Several observations indicate that the IDH kinase and IDH phosphatase reactions occur at the same active site (see above).

REGULATION OF THE GLYOXYLATE BYPASS BY IDH PHOSPHORYLATION

The role of the IDH phosphorylation cycle is to regulate the branch point between the glyoxylate bypass and the Krebs' cycle during growth on acetate or fatty acids (see above). Inhibition of IDH by phosphorylation causes an increase in the steady-state level of isocitrate, thereby increasing the velocity of isocitrate lyase. Mutant strains which are deficient in IDH kinase failed to grow on acetate, suggesting that the phosphorylation of IDH is required for use of the glyoxylate bypass [LaPorte et al., 1985].

IDH kinase/phosphatase also controls the glyoxylate bypass during transitions between carbon sources. For example, addition of a preferred carbon source (e.g., glucose or pyruvate) to a culture growing on acetate renders the glyoxylate bypass unnecessary. Under these conditions, the cell shuts this pathway down by dephosphorylating IDH. Inhibition of the bypass results because the activation of IDH draws isocitrate through the Krebs' cycle. The resulting decrease in isocitrate concentration yields a proportional decrease in the velocity of isocitrate lyase, the first step in the glyoxylate bypass [LaPorte et al., 1984].

WHAT CONTROLS THE IDH PHOSPHORYLATION CYCLE?

A variety of metabolites have been identified which effect IDH kinase/phosphatase in vitro. These metabolites activate IDH phosphatase and inhibit IDH kinase [LaPorte and Koshland, 1983; Nimmo and Nimmo, 1984]. During growth on acetate, it appears that isocitrate and 3-phosphoglycerate participate in the control of the IDH phosphorylation cycle. They probably act as general indicators of the levels of metabolic intermediates and thus of the need for isocitrate to be directed to the glyoxylate bypass. For example, depletion of these metabolites would result in increased phosphorylation of IDH, forcing more isocitrate through the bypass. However, isocitrate and 3-phosphoglycerate are not responsible for the dephosphorylation of IDH which results from the addition of preferred carbon sources such as glucose, since their levels fall under these conditions. Dephosphorylation of IDH during these metabolic transitions is probably promoted, at least in part, by pyruvate, since the level of this metabolite rises dramatically upon addition of glucose [Lowry et al., 1971; el-Mansi et al., 1986].

Although the glyoxylate bypass can provide metabolic intermediates, the Krebs' cycle is more efficient in providing energy. The cell must, therefore, precisely balance the flux of isocitrate between these competing pathways during growth on acetate. The energy requirements of the cell appear to be monitored through AMP. Like the other metabolites discussed above, AMP activates IDH phosphatase and inhibits IDH kinase. AMP is a particularly attractive choice for monitoring the energy needs of the cell because, if the adenylate kinase reaction is at equilibrium, the level of AMP will vary as the square of the ADP concentration. For the IDH phosphorylation cycle, an increase in AMP, signalling a depletion of cellular energy, would yield a net dephosphorylation of IDH, diverting more isocitrate through the Krebs' cycle. A surplus of energy, indicated by a low level of AMP, would have the opposite effect.

The mutations which selectively inhibited IDH phosphatase (see above) have profoundly altered the response of both IDH kinase and IDH phosphatase to pyruvate, 3-phosphoglycerate, and AMP. The affinities of these effectors for IDH kinase/phosphatase are decreased by a factor of ca. 10, while the relative activations of IDH phosphatase are increased 5- to 50-fold (Ikeda and LaPorte, manuscript in preparation). These mutations correspond to a 113 amino acid region of this 578 residue protein (Fig. 3) [Ikeda et al., 1992]. Our working model proposes that this region of the protein acts as a regulatory domain which controls a conformational equilibrium between kinase and phosphatase forms of the protein.

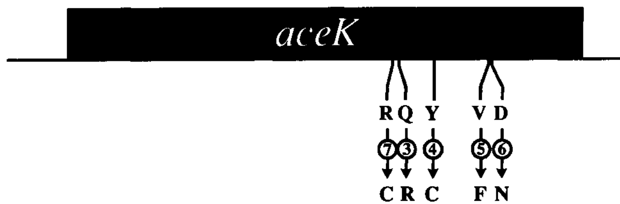

Fig. 3. Mutations which selectively inhibit IDH phosphatase. The locations of the mutations and the corresponding amino acid substitutions are indicated. Allele numbers are shown.

SENSITIVITY AMPLIFICATION AND THE GLYOXYLATE BYPASS

The regulation of the glyoxylate bypass appears to be exquisitely sensitive to the metabolic state of the cell. This conclusion is supported by a variety of theoretical analyses and by experiments performed in vitro. A high degree of sensitivity amplification is achieved by simultaneous activation of IDH phosphatase and inhibition of IDH kinase by its effectors. This type of sensitivity amplification is termed a multistep effect. The IDH phosphorylation cycle also appears to be subject to zero-order ultrasensitivity, a form of sensitivity amplification which occurs in covalent regulatory systems when the concentration of the interconvertible protein (in this case IDH) exceeds the Michaelis constants of the converter enzymes [LaPorte and Koshland, 1983; Goldbeter and Koshland, 1984]. A third mechanism of sensitivity enhancement, termed a branch point effect, results from the profound difference in the affinities of IDH and isocitrate lyase for isocitrate (Michaelis constants of 8 and 600 mM, respectively) [LaPorte et al., 1984]. As a result of the branch point effect, the flux of the glyoxylate bypass is strikingly sensitive to the phosphorylation state of IDH. These three mechanisms for sensitivity amplification combine to produce a system in which very subtle changes in metabolic signals have the potential for producing profound changes in the flux through the glyoxylate bypass.

ADAPTATION TO THE INTRACELLULAR ENVIRONMENT

In addition to responding to changes in the external environment, the IDH phosphorylation cycle must be capable of adapting to the conditions which prevail inside the cell. For example, the cellular level of IDH can vary by at least a factor of two between different strains of *E. coli*. The effect of this difference in IDH levels would be amplified by the branch point effect (see above), potentially preventing growth on acetate. However, the IDH phosphorylation cycle responds to these differences by altering the fractional phosphorylation of IDH so that a constant level of IDH activity is maintained under these growth conditions. To test the limits of this adaptational process, we used a clone of *icd* (the gene encoding IDH) to increase the level of IDH expression by 15-fold. The phosphorylation system was able to efficiently compensate for this increase, converting all of the excess IDH into the inactive, phosphorylated form. As a result, these cells maintained the same level of IDH activity as a wild-type strain even though the total level of this enzyme had increased by more than an order of magnitude [LaPorte et al., 1985]. It seems likely that this response to the cellular level of IDH resulted because the control of IDH kinase/phosphatase by a variety of metabolites provides a particularly effective feedback control mechanism.

IS PRECISE CONTROL OF IDH PHOSPHORYLATION REALLY NECESSARY?

The tolerance of *E. coli* to variation in IDH activity was tested using mutant strains which had defects in IDH kinase/phosphatase. The effect of excess IDH activity was determined by comparison of strains with null mutations in *aceK*. A 2-fold increase in IDH activity reduced the growth rate on acetate while a 4-fold increase yielded nearly complete inhibition of growth [LaPorte et al., 1985]. The minimum level of IDH required for growth on acetate was determined by coexpression of wild-type *aceK* with an allele of *aceK* whose product had selectively lost IDH phosphatase activity (see above). The cells were able to tolerate a 50% reduction in IDH activity without apparent effect on their growth rate. However, further reductions in IDH activity inhibited growth, with arrest occurring when this level dropped to 15% of the wild-type [Ikeda and LaPorte, 1991]. Thus, the striking precision exhibited by the IDH phosphorylation cycle in vivo is not absolutely essential. However, in nature, even small differences in growth rate can yield dramatic differences during competition between organisms for limiting resources.

BALANCING ENVIRONMENTAL SENSITIVITY AND "FUTILE" CYCLING

A regulatory system which employs covalent control must balance the benefits of a rapid response with the need to minimize the loss of cellular energy which results from the cyclic modification of the target protein. Insight into the minimum level of IDH kinase/phosphatase required for growth on acetate came from an unexpected source. Although mutation of the consensus ATP binding site reduced the IDH kinase and IDH phosphatase activities by factors of at least 100 in vivo and in vitro (see above), this protein retained sufficient kinase activity to support growth on acetate. It thus appears that wild-type IDH kinase/phosphatase is maintained in massive excess over the level required for steady state phosphorylation of IDH [Stueland et al., 1989].

What is the function of this "excess" IDH kinase/phosphatase? A likely function would be to allow rapid responses to changes in the available carbon source. For example, when pyruvate is added to cultures growing on acetate, the cells dephosphorylate IDH, increasing its activity and thus inhibiting the flux of isocitrate through the glyoxylate bypass (see above). The ability to control the expression of wild-type IDH kinase/phosphatase using a clone of *aceK*$^{+}$ provided a method for determining the effect of the level of this protein on the response rate. The rate of the pyruvate-induced dephosphorylation of IDH was proportional to the level of IDH kinase/phosphatase [Stueland et al., 1988]. Thus, the level of IDH kinase/phosphatase is not excessive during metabolic transitions. Under these conditions, this enzyme represents the primary rate limiting step.

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