The Isocitrate Dehydrogenase Phosphorylation Cycle

IDENTIFICATION OF THE PRIMARY RATE-LIMITING STEP

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In *Escherichia coli*, the branch point between the Krebs cycle and the glyoxylate bypass is regulated by the phosphorylation of isocitrate dehydrogenase (IDH). Phosphorylation inactivates IDH, forcing isocitrate through the bypass. This bypass is essential for growth on acetate but does not serve a useful function when alternative carbon sources, such as glucose or pyruvate, are present. When pyruvate or glucose is added to a culture growing on acetate, the cells respond by dephosphorylating IDH and thus inhibiting the flow of isocitrate through the glyoxylate bypass. In an effort to identify the primary rate-limiting step in the response of IDH phosphorylation to alternative carbon sources, we have examined the response rates of congeneric strains of *E. coli* which express different levels of IDH kinase/phosphatase, the bifunctional protein which catalyzes this phosphorylation cycle. The rate of the pyruvate-induced dephosphorylation of IDH was proportional to the level of IDH kinase/phosphatase, indicating that IDH kinase/phosphatase was primarily rate-limiting for dephosphorylation. However, the identity of the primary rate-limiting step appears to depend on the stimulus, since the rate of dephosphorylation of IDH in response to glucose was independent of the level of IDH kinase/phosphatase.

EXPERIMENTAL PROCEDURES

MATERIALS—[γ-32P]ATP was obtained from Du Pont-New England Nuclear. Restriction endonucleases and T7 DNA ligase were products of Bethesda Research Laboratories. Affi-Gel Blue was purchased from Bio-Rad. All other reagents were the purest grades available.

GROWTH MEDIA AND BACTERIAL STRAINS—The minimal acetate medium employed was the MOPS-based medium described by Neidhardt et al. (22) containing 2% sodium acetate, 0.5 mM L-histidine, and 200 μg/ml ampicillin. L broth contained 1% bacto Tryptone, 1% NaCl, 0.5% yeast extract, and when appropriate, 200 μg/ml ampicillin.

The primary strains used in this study, DEK2010 and DEK2011, have the same genetic background (i.e. are congeneric) but are *aceK* and *aceK1*, respectively (10). As a result of the mutation in *aceK*, DEK2011 fails to express detectable IDH kinase/phosphatase. In the experiments described below, these strains harbored plasmid pDL20, which expresses *aceK* (see below).

Plasmid Construction—Except as noted, plasmids were manipulated by standard procedures (23).

Plasmid pDL9 expresses *aceK* from the strong tac promoter (11). To produce a derivative of this plasmid which would express IDH kinase/phosphatase below the wild-type level, we fused pDL9 with pMC7 (24), a plasmid which expresses the gene encoding the lac repressor, *lacI*. A similar method was employed by Clegg and Koshland (25) to regulate the expression of a clone of *cheY*. Plasmids pDL9 and pMC7 were digested with BamHI, mixed, and ligated with T7 DNA ligase. The ligation mixture was transformed into *E. coli* strain MM294A (lac, *recA52*), and transformants were selected on L broth supplemented with 200 μg/ml ampicillin and 80 μg/ml X-gal. Plasmid DNA was isolated from white colonies and the identity of the plasmid was confirmed by restriction mapping. (Unfused pMC7 would not have been recovered in this procedure because it does not confer ampicillin resistance.) The product of this fusion is designated pDL20.
**Rate-limiting Step of a Phosphorylation Cycle**

Protein Purification—Isocitrate dehydrogenase was purified by a modification of the method of Garnak and Reeves (6) from *E. coli* strain Y1090 harboring pTK509, a multicopy plasmid which expresses the gene encoding IDH (10). Phospho-IDH was prepared in vitro using purified IDH kinase/phosphatase (8). Following the reaction, IDH kinase/phosphatase and dephospho-IDH were removed by chromatography on Affi-Gel Blue.

The purification of IDH kinase/phosphatase has been described previously (15). This protein was purified from *E. coli* strain Y1090 harboring pBDL9, a plasmid which expresses aceK (14).

Enzyme Assays—All assays were performed at 37 °C.

The activity of IDH phosphatase was measured by following the release of \[^{32}P\]phosphate from \[^{32}P\]phospho-IDH, as described previously (15). The standard reaction conditions were 25 mM MOPS, pH 7.5, 250 pM NADP, 500 pM DL-isocitrate, 1 mM MgCl<sub>2</sub>, 100 mM NaCl, 2 mM dithiothreitol, 0.5 mM EDTA, 5 mM 3-phosphoglycerate, 1 mM DL-isocitrate, and 2 mg/ml bovine serum albumin. One unit of IDH phosphatase is defined as the amount of enzyme required to achieve 50% dephosphorylation of the phospho-IDH in 1 min.

Isocitrate dehydrogenase was assayed spectrophotometrically by monitoring the reduction of NADP at 340 nm. The reaction mixture contained 25 mM MOPS, pH 7.5, 250 mM NADP, 500 mM DL-isocitrate and 5 mM MgCl<sub>2</sub> in a final volume of 1 ml. One unit of isocitrate dehydrogenase will produce 1 pmol of NADPH/min under these conditions.

Protein concentration was determined by the method of Lowry et al. (29) using bovine serum albumin as a standard.

Determination of the Fractional Phosphorylation of IDH—The fraction of IDH which was present in the active, dephosphorylated form was determined by measuring the increase in IDH activity that resulted from treatment of the cell-free extract with IDH phosphatase. Parallel samples contained the extract, 25 mM MOPS, pH 7.5, 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM dithiothreitol, 2.5 mM 3-phosphoglycerate, 30 mM pyruvate, and 1 mM DL-isocitrate. In addition, one of the samples contained 1 μg of purified IDH kinase/phosphatase and 1 mM ADP. ADP was included because IDH phosphatase has an absolute requirement for ATP or ADP (15). The samples were incubated at 37 °C, and aliquots were withdrawn periodically and assayed for IDH activity to ensure that complete dephosphorylation had been achieved. Incubation was without effect on the IDH activity of the sample that lacked ADP and IDH kinase/phosphatase.

Response of IDH Phosphorylation to Alternative Carbon Sources—Starter cultures were grown to stationary phase on LB broth supplemented with 200 μg/ml ampicillin and were used to inoculate 250-ml cultures of acetate medium. Following overnight growth in a gyratory shaker at 37 °C, the cultures were harvested by centrifugation, resuspended in 500 ml of fresh medium to an A<sub>600</sub> of 0.2, and returned to the incubator for one generation (~3 h). Samples (250 ml) of each culture were then removed, the cells were harvested by centrifugation, and the samples were stored at −70 °C for the determination of IDH phosphatase and total IDH activities. Glucose or pyruvate was added to the remainder of the cultures to 50 or 40 mM, respectively, and the cultures were returned to the gyratory incubator. At time intervals before and after the addition of the alternative carbon source, 9-ml samples were withdrawn, mixed with 1 ml of 10 mg/ml bovine serum albumin, chilled in a saline ice bath and immediately disrupted by sonication. The sonicated samples were stored at −70 °C until the end of the time courses and were then assayed for IDH activity. The observed IDH activities were corrected for growth of the culture as determined by measuring turbidity at 650 nm. This correction did not exceed 20%. The fractional phosphorylation of the samples taken at the initial and final time points were also determined, as described above.

To determine the total IDH activity, samples were thawed by resuspension in 25 mM MOPS, pH 7.5, 1 mM EDTA, 2 mM 2-mercaptoethanol and disrupted by sonication. Cellular debris was removed by centrifugation at 15,000 × g for 20 min. The total level of IDH was determined following dephosphorylation with IDH phosphatase, as described above.

**RESULTS**

During growth on acetate, strain DEK2010, which is aceK<sup>+</sup>, maintained ~20% of its IDH in the active, dephosphorylated form (Table I). Addition of pyruvate to this culture resulted in a rapid increase in IDH activity (Fig. 2). By the end of the experiment, the fraction of IDH in the active form had increased to 87%.

To determine if IDH kinase/phosphatase catalyzed the primary rate-determining step in the response of the IDH phosphorylation cycle to pyruvate, we compared the rate of the response of DEK2010 with that of a congenic strain which underproduced IDH kinase/phosphatase. This congenic strain was constructed by introducing plasmid pDL20, which expressess IDH kinase/phosphatase at a low level, into strain DEK2011, which lacks this enzyme due to a mutation in the chromosomal allele of aceK (10). The response rates were estimated from the slopes of the initial portions of the response curves (Fig. 2 and Table I). The 5-fold difference in the cellular levels of IDH kinase/phosphatase exhibited by these strains correlated with a 3.7-fold difference in the rates of the response. (As discussed below, the 3.7-fold difference between these response rates probably represents the lower limit of the effect of the cellular level of IDH kinase/phosphatase.) The near-proportionality of the response rate with the cellular level of IDH kinase/phosphatase indicates that IDH kinase/phosphatase is at least partially rate-limiting for the dephosphorylation of IDH under these conditions.

In addition to the cellular level of IDH kinase/phosphatase, the relative rates at which the IDH phosphorylation cycles of these strains responded to pyruvate may have been influenced by the concentration of phospho-IDH. The total level of IDH (i.e. phospho-IDH + dephospho-IDH) in DEK2010 was ~25% less than that in DEK2011 (p < 0.001; the number of independent determinations was 9 for DEK2010 and 6 for DEK2011) (Table I). The phosphorylation system appears to have compensated for this difference (p = 0.02), maintaining essentially the same concentration of active, dephospho-IDH in both strains. (The ability of the IDH phosphorylation cycle to compensate for differences in the cellular level of IDH has been reported previously (10).) Although the concentration of dephospho-IDH in these strains was relatively constant, the concentration of phospho-IDH in DEK2010 was ~30% less than that in DEK2011 (p < 0.001). This difference in concentration could effect the velocities of IDH phosphatase (V<sub>pmax</sub>) and IDH kinase (V<sub>kine</sub>), and thus the response rate (V<sub>resp</sub>).

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1 These experiments were performed with DEK2010 harboring plasmid pLD20 so that this strain would be congenic with the strain which underproduced IDH kinase/phosphatase, DEK2011 harboring the same plasmid.
TABLE I
Dephosphorylation of IDH

<table>
<thead>
<tr>
<th>Strain*</th>
<th>None</th>
<th>Pyruvate</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IDH phosphate</td>
<td>IDH total</td>
<td>Fraction of IDH in dephospho form</td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
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<td>----------------------------------</td>
</tr>
<tr>
<td>DEK2010(pDL20)</td>
<td>19 ± 2*</td>
<td>2.8 ± 0.2</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>DEK2011(pDL20)</td>
<td>3.8 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>0.17 ± 0.01</td>
</tr>
</tbody>
</table>

*The indicated carbon source was added to a mid-log phase culture growing on minimal acetate medium.
*IDH activity measured following dephosphorylation with IDH phosphatase.
*The fractional phosphorylation of IDH was determined by measuring IDH activity before and after dephosphorylation with IDH phosphatase.
*The effective IDH activity during growth on acetate was estimated from the total IDH activity and the fractional phosphorylation of this enzyme.
*The response rate was defined as the initial slope of the plot of IDH activity versus time as determined by linear regression analysis.
*The strains employed were DEK2010 (aceK+) and DEK2011 (aceK1), each harboring plasmid pDL20 which expresses aceK at a low level.
*Standard error of the mean.

DISCUSSION
The glyoxylate bypass (Fig. 1) is essential for growth when acetate or fatty acids are the sole carbon source. The behavior of this pathway during growth on acetate and its response to the addition of glucose have been extensively characterized (10, 11, 13, 21). During growth on acetate, 28% of the isocitrate is diverted from the Krebs cycle through the glyoxylate bypass. Under these conditions, the intracellular concentration of free isocitrate (~80 μM) is nearly saturating of IDH (Km for isocitrate = 8 μM). In contrast, this concentration is in the pseudo-first order range for isocitrate lyase (Km = 600 μM). This situation results in an ultrasensitivity of the glyoxylate bypass flux to the maximum velocity of IDH, a phe-
nomenon termed the "branch point effect." Operation of the glyoxylate bypass is thus highly dependent on the phosphorylation and consequent inhibition, of IDH. Addition of glucose to a culture growing on acetate results in the dephosphorylation of IDH. This 4-fold increase in IDH activity, combined with a 5-fold decrease in the rate of isocitrate production, produces a precipitous drop in the steady-state level of isocitrate. Since the intracellular level of isocitrate is in the pseudo-first order range of isocitrate lyase, the decrease in isocitrate levels that result from the addition of glucose yields a proportional inhibition of the glyoxylate bypass, reducing the flux through this pathway to an undetectable level. Theoretical considerations indicate that the ~4-fold increase in IDH activity which results from dephosphorylation would, by itself, yield a 20-fold decrease in the flux through the glyoxylate bypass, a consequence of the branch point effect.

The observation that the rate of pyruvate-induced dephosphorylation of IDH is proportional to the cellular level of IDH kinase/phosphatase suggests that IDH phosphatase catalyzes the primary rate-limiting step in the response of this phosphorylation cycle. However, the role of IDH kinase/phosphatase in determining this response rate appears to depend on the stimulus, since this protein does not appear to be rate-limiting in the response of IDH phosphorylation to glucose.

Theoretical studies have made it clear that the concept of a unique rate-limiting step is an oversimplification (11, 27, 28). Each component (e.g. enzyme, transport protein) in a metabolic system possesses some ability to influence the overall behavior of that system. However, the capacity of most of these components to control the overall system is usually of little significance. Rather, the capacity for control is maintained by a small subset of these components and often by a single component. We have employed the term "primary rate-determining step" to refer to a step which exerts the greatest influence on the rate of a process, even though that step may not be the unique determinant of this rate.

The insensitivity of the rate of glucose-induced dephosphorylation to the level of IDH kinase/phosphatase suggests that the phosphorylation cycle maintained a quasi-steady state throughout the response. The rate of glucose-induced dephosphorylation thus appears to be limited by the rate at which the effectors of IDH kinase/phosphatase change. IDH kinase and IDH phosphatase are controlled by a wide variety of metabolites. Isocitrate, 3-phosphoglycerate, pyruvate, AMP, ADP, oxaloacetate, α-ketoglutarate, and phosphoenolpyruvate activate IDH phosphatase and inhibit IDH kinase, providing a mechanism for sensitivity enhancement termed a "multistep effect." In contrast, NADP, citrate, fructose 6-phosphate, and glyoxylate inhibit IDH kinase but have little effect on IDH phosphatase activity (8, 12). The physiological significance and possible roles of these metabolites remain uncertain. It seems likely that pyruvate is partially responsible for the glucose-induced dephosphorylation of IDH, since its cellular level increases upon addition of glucose (29). However, this observation does not automatically suggest a net dephosphorylation, since the cellular concentrations of 3-phosphoglycerate and isocitrate are reduced under these conditions. Furthermore, pyruvate is a relatively modest effector of IDH kinase/phosphatase within its physiological concentration range: 1 to 2 mM. For example, in experiments in which we allowed a steady state to be established between IDH kinase and IDH phosphatase in vitro, 50% dephosphorylation of IDH required 30 mM pyruvate. It thus appears that the concentration of pyruvate needed to achieve the ~90% dephosphorylation resulting from glucose addition would require pyruvate concentrations ~100-fold greater than those obtained in vivo. Thus, while the increase in the concentration of pyruvate probably contributes to the glucose-induced dephosphorylation of IDH, the overall process appears to be considerably more complex.

In contrast to the results obtained with glucose, IDH phosphatase appeared to be the primary determinant of the rate of pyruvate-induced dephosphorylation of IDH. This observation indicates that the phosphorylation cycle was not in quasi-steady state during the response to pyruvate. Although it is tempting to conclude that the effectors responsible for dephosphorylation respond more rapidly to pyruvate than to glucose, it seems quite possible that pyruvate-induced dephosphorylation of IDH results primarily from increases in the intracellular concentration of pyruvate. Since pyruvate was added to 40 mM and this compound is actively transported in E. coli (30), the cell may achieve the intracellular concentrations of this metabolite needed to produce net dephosphorylation of IDH. If a sufficient concentration of intracellular pyruvate could be achieved relatively rapidly, IDH phosphatase would be expected to be primarily rate-limiting in the pyruvate-induced dephosphorylation of IDH.

The possibility that intracellular pyruvate plays a primary role in the pyruvate-induced dephosphorylation of IDH is suggested by the observation that a mutant lacking phosphoenolpyruvate synthase and pyruvate dehydrogenase still responded to pyruvate (31). Apparently, the concentrations of the effectors responsible for the dephosphorylation of IDH still respond to pyruvate addition despite the absence of the enzymes that are primarily responsible for converting pyruvate into the metabolites of the central pathways. This observation must be interpreted with a degree of caution, however, since the absence of phosphoenolpyruvate synthase and pyruvate dehydrogenase may have resulted in a much higher steady-state concentration of intracellular pyruvate than would be obtained in a wild-type cell. Furthermore, the absence of pyruvate dehydrogenase and phosphoenolpyruvate synthase is likely to have had pleiotropic effects on the metabolism of this mutant strain. In a similar experiment, Lowry et al. (29) examined the effects of glucose addition to minimal acetate cultures of a mutant deficient in phosphoenolpyruvate synthase and phosphoenolpyruvate carboxylase and of a congenic wild-type strain. These strains exhibited a variety of striking differences in the effects of glucose on the concentrations of metabolic intermediates. For example, the cellular levels of ATP and ADP increased 2-fold in the wild-type strain but decreased by 50% in the mutant. Clearly, differences in the cellular concentration of metabolites which control IDH kinase/phosphatase will affect the phosphorylation state of IDH. These observations are, however, difficult to interpret in terms of simple causes and effects because of the complex, interdependent relationships of the components of intermediary metabolism. However, despite these caveats, the most likely interpretation of the observation that mutants deficient in phosphoenolpyruvate synthase and pyruvate dehydrogenase exhibit pyruvate-induced dephosphorylation of IDH is that intracellular pyruvate contributes substantially to this response.

* The concentration of pyruvate employed in these experiments (40 mM) is below that generally used in minimal growth medium. Although there is some variation between laboratories, carbon sources other than glucose are typically present at 1% in minimal media. For sodium pyruvate, this is equivalent to 90 mM.

9 C. S. Stueland, K. Gorden, and D. C. LaPorte, unpublished result.
The rate of response to a stimulus is a fundamental characteristic of any regulatory system. In the case of covalent regulation, the potential advantages of a rapid response must be balanced against the need to minimize futile cycling (32). It seems appropriate for IDH kinase/phosphatase to catalyze the primary rate-limiting step in the response of the IDH phosphorylation cycle to the addition of at least some alternative carbon sources to a culture growing on acetate. The precipitous addition of a large amount of an alternative carbon source is, after all, a situation that is not likely to occur often in nature. The rate of the IDH phosphorylation cycle is probably sufficient for an optimal response to more gradual changes in the extracellular environment. In contrast, many phosphorylation cycles must respond quite rapidly to a stimulus. In these systems, it seems quite possible that the phosphorylation cycle will not be rate-limiting. The response rates of phosphorylation cycles have been extensively studied for a wide variety of systems. However, to our knowledge, the pyruvate-induced dephosphorylation of IDH represents the first system for which the primary rate-limiting step of a phosphorylation cycle has been experimentally determined in vivo.

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REFERENCES