Regulation at the Phosphoenolpyruvate Branchpoint in *Azotobacter vinelandii*: Pyruvate Kinase

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Pyruvate kinase (EC 2.7.1.40) from *Azotobacter vinelandii* responds sharply to the adenylate energy charge, with a decrease in activity at high values of charge, as expected for an enzyme of an adenosine triphosphate-regenerating sequence. Glycolytic intermediates, especially glucose 6-phosphate, fructose 6-phosphate, and fructose-1,6-diphosphate, strongly stimulate the reaction and overcome the inhibition caused by high values of energy charge. Thus, the properties of this enzyme depend on interaction between energy charge and the concentrations of hexose phosphates. The properties of pyruvate kinase, together with those of phosphoenolpyruvate carboxylase, aspartokinase, and citrate synthase, seem adapted to provide appropriate partitioning of phosphoenolpyruvate between competing pathways in response to metabolic needs.

In many bacterial species, phosphoenolpyruvate is a major metabolic branchpoint at which carbon is partitioned between oxidation for adenosine triphosphate (ATP) regeneration and the replenishment of the pools of primary metabolites that are depleted in the cell's biosynthetic activities. The preceding article (15) reported some regulatory properties of phosphoenolpyruvate carboxylase from *Azotobacter vinelandii*. This paper reports observations on pyruvate kinase [phosphoenolpyruvate + adenosine diphosphate (ADP) ↔ pyruvate + ATP; EC 2.7.1.40] from the same organism, and discusses how the properties of the two enzymes may interact in vivo to control the partitioning of phosphoenolpyruvate between degradative and anaplerotic sequences in response to the momentary needs of the cell.

**MATERIALS AND METHODS**

**Reagents.** All nucleotides and substrates were commercial products. The counter ion for buffers was potassium. Other solutions were neutralized with tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (TES; pH 6.8), 15 mM MgCl₂, 0.2 mM reduced nicotinamide adenine dinucleotide (NADH), 10 mM phosphoenolpyruvate, 2 mM ADP, 10 µM of lactate dehydrogenase (3 units), and a suitably diluted pyruvate kinase preparation. Assay mixture II, used for study of effects of modifiers, was similar except for lower concentrations of phosphoenolpyruvate (0.1 mM), ADP (0.1 mM), and MgCl₂ (10 mM). In energy charge experiments, assay II was used except that mixtures of adenine monophosphate (AMP), ADP, and ATP (or of AMP and ATP with adenylate kinase) were added to produce the desired value of charge. In all cases, the reaction mixture, including lactate dehydrogenase, was incubated at 25 C for 3 to 5 min to remove contaminating pyruvate from the phosphoenolpyruvate preparation. The reaction was initiated by addition of enzyme. The rate observed when either phosphoenolpyruvate or ADP was omitted was negligible when enzyme preparations that had been purified through the high-speed centrifugation step were used. One unit of activity is defined as the amount of enzyme that catalyzes the production of 1 µmole of pyruvate per min under the conditions of assay I.

**Purification of ATP.** All of the commercial ATP preparations that we tested contained 2 to 3% ADP. This contamination is especially undesirable in energy charge experiments on pyruvate kinase, since it causes grossly erroneous results at a nominal charge value of 1.0. The contaminating ADP was removed by a procedure based on that of Moffatt (17). A 20 mM solution of commercial ATP was applied to a diethylaminoethyl

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eluted with \((\text{DEAE})\) and purified into a triethylammonium bicarbonate gradient. The eluting salt was removed by lyophilization and the purified ATP was converted to the sodium salt by use of a Dowex-50 cation-exchange column. The mole fraction of ADP, about 7 \times 10^{-4}, was low enough to cause no interference in energy charge experiments.

**RESULTS**

**General properties of enzyme.** Dilution of pyruvate kinase with buffer resulted in severe inactivation. After 100-fold dilution, about 90% of the enzyme's activity was lost within 5 min. Addition of dithiothreitol, glycerol, or phosphoenolpyruvate, but not ADP, stabilized the enzyme. Accordingly, 10 mM dithiothreitol and 30% glycerol were used in column purification steps, and 30% glycerol was routinely included in the buffer (50 mM TES) with which the enzyme preparation was diluted.

Activity of pyruvate kinase is optimal at about pH 6.3 to 6.8, and falls sharply at pH values below 6. Decline in activity at higher pH values is gradual, and about 80% of maximal activity is retained at pH 8. In isoelectric focusing experiments (14), pyruvate kinase activity was found in a zone at pH 4.5, indicating this to be the effective isoelectric point of the protein.

Guanine nucleotides can replace adenine nucleotides in the reaction catalyzed by *Azotobacter* pyruvate kinase. Guanosine diphosphate (GDP) can serve as substrate, and guanosine monophosphate, like AMP, is stimulatory. The rabbit muscle enzyme can phosphorylate GDP and inosine diphosphate (7, 10).

**Effects of cations.** Curves of velocity as a function of Mg\(^{2+}\) concentration are sigmoidal, and a Hill plot had a slope of 1.8. Half-maximal velocity was observed when the concentration of Mg\(^{2+}\) was 3.1 mM. Like the corresponding enzymes from *Escherichia coli* (16) and *Brevibacterium flavum* (18), pyruvate kinase from *A. vinelandii* does not require a monovalent cation, and neither K\(^+\) nor NH\(_4\)\(^+\) at concentrations up to 100 mM affects the activity of the enzyme. These bacterial enzymes differ in this respect from pyruvate kinases from eukaryotic organisms (7).

**Effects of modifiers.** Pyruvate kinase from *A. vinelandii* is strongly stimulated by hexose phosphates and by AMP, and inhibited by inorganic orthophosphate (P\(_i\)) (Table 1). Little is known of the extent and significance of variation in the concentration (or chemical potential) of P\(_i\), in vivo. The strong inhibition of pyruvate kinase by P\(_i\) (90% at 2 mM) cannot be overcome by addition of MgCl\(_2\) or by increasing the concentration of lactate dehydrogenase, the coupling enzyme. It is largely abolished if 5 mM Na\(_2\)SO\(_4\) or (NH\(_4\))\(_2\)SO\(_4\) is added, or if higher concentrations of substrates are used (assay mixture I). The inhibition by P\(_i\) was not studied further.

Under the conditions described in Table 1, little or no effect was observed on the addition of glucose, glucose-1-P, ATP, citrate, DL-threoisocitrate, \(\alpha\)-ketoglutarate, L-glutamate, succinate, fumarate, L-malate, L-aspartate, or L-asparagine, all at 2 mM, or of 3',5'-cyclic AMP or acetyl-SO\(_4\) at 100 \muM. Because no metabolic role for fructose-1-P in *Azotobacter* is known and because the activation by 3-phosphoglyceric acid is relatively weak, glucose-6-P, fructose-6-P, and fructose-1,6-diphosphate were chosen for further study.

The rate of the pyruvate kinase reaction at low concentrations of substrates (assay mixture II) is shown in Fig. 1 as a function of the concentrations of the positive modifiers. The apparently hyperbolic shape of the curves for fructose-6-P, glucose-6-P, and AMP was confirmed by double reciprocal plots, in which \((v-v_o)\)' \(\rightarrow\), where \(v_o\) is velocity in the absence of modifier, was plotted against \((\text{modifier})\). All three modifiers gave linear plots extrapolating to the same value of \(V_{max}\). Values of \((\text{M})_{0.5}\) (concentration of modifier required for 50% of its maximal effect) estimated from these plots are: fructose-6-P, 0.1 mM; glucose-6-P, 0.36 mM; AMP, 0.5 mM. The curve for fructose diphosphate in Fig. 1 is clearly sigmoidal, and a Hill plot was linear with a slope of 2 (Fig. 2). Half-maximal effect was seen at 0.83 mM fructose diphosphate. Fructose diphosphate has been previously shown to be a positive effector for yeast pyruvate kinase, with a Hill slope of 2 (11).

All four of the positive modifiers increase the affinity of the enzyme for phosphoenolpyruvate and have little or no effect on \(V_{max}\). This is shown for fructose-6-P and fructose-diphosphate in Fig. 3. Curves for both glucose-6-P and AMP were very similar to that for fructose-6-P and are omitted. Although it is barely evident on the scale of Fig. 3, fructose diphosphate does not alter the sigmoidal shape of the phosphoenolpyruvate response curve, whereas each of the other three modifiers converts the curve to a hyperbola. This difference in effect is more clearly indicated by means of Hill plots (Fig. 4), which also demonstrate the changes in \((S)_{0.5}\) (substrate concentration giving half-maximal velocity) caused by the modifiers. All modifiers were added at 3 mM, which is a nearly saturating concentration for each of them.

Similar experiments were performed to test for possible effects of the modifiers on the affinity of the enzyme for the other substrate, ADP. The ADP response curve is hyperbolic, and is not changed significantly by any of the modifiers in the presence of excess phosphoenolpyruvate.
TABLE 1. Effects of modifiers on the rate of the reaction catalyzed by pyruvate kinase from Azotobacter vinelandii

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>(1.0)</td>
</tr>
<tr>
<td>Adenosine monophosphate</td>
<td>14</td>
</tr>
<tr>
<td>P_i</td>
<td>0.1</td>
</tr>
<tr>
<td>3-P-glycerate</td>
<td>2.2</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>20</td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>23</td>
</tr>
<tr>
<td>Fructose-di-P</td>
<td>10</td>
</tr>
<tr>
<td>Fructose-I-P</td>
<td>19</td>
</tr>
</tbody>
</table>

* Assay mixture II was used. Each modifier was present at a final concentration of 2 mM. Tabulated numbers are reaction rates relative to that of the control assay lacking additions.

Thus, the modifiers seem to be specific in affecting only affinity for phosphoenolpyruvate, and neither $V_{max}$ nor affinity for ADP.

The effects of AMP and of the hexose phosphates are not additive (Fig. 5). In the presence of 5 mM of any of the three hexose phosphate modifiers, the addition of AMP has almost no effect. This result might be taken to suggest that the binding sites are sufficiently close so that binding of the modifiers is mutually exclusive. This picture is not easy to reconcile, however, with the previously noted observation that fructose diphosphate differs from the other three modifiers both in yielding a sigmoidal activation curve and in not causing a decrease in the order of the phosphoenolpyruvate response curves.

Response to adenylate energy charge. The pyruvate kinase reaction produces 1 mole of ATP directly for each mole of phosphoenolpyruvate consumed. Much more importantly in an aerobic organism like Azotobacter, this reaction is a necessary step in the oxidation of carbohydrate and the regeneration of ATP by electron transport phosphorylation. Thus, pyruvate kinase should give an R-type response (2) to variation in adenylate energy charge (6)—that is, its activity should be maximal at low values of charge, and should
decrease with increasing charge, especially in the physiological charge range above 0.75.

In preliminary experiments, response to adenylate energy charge was similar at total adenylate pool levels (AMP + ADP + ATP) ranging from 2 to 6 mM, which probably includes the physiological range. A value of 3 mM was chosen for further work. Magnesium ion was used at 10 mM because this concentration approaches saturation. No good estimate of the chemical potential of Mg²⁺ in vivo seems to be available. Since the modifiers and energy charge mainly affect the affinity of the enzyme for phosphoenolpyruvate, their effects are best seen under conditions where the concentration of phosphoenolpyruvate is rate-limiting—at or below the (S)₀.₅ value for this substrate. Such a value of phosphoenolpyruvate is also to be preferred on physiological grounds, since it is very likely that the concentration of a metabolite in vivo approximates the effective (S)₀.₅ values of the enzymes that catalyze its further reaction (1, 9, 20) or is somewhat lower (4). A phosphoenolpyruvate concentration of 0.1 mM was accordingly used in energy charge experiments. For similar reasons, it seemed desirable to measure the effects of the activators at their (M)₀.₅ values or lower, as well as at nearly saturating concentrations. Preliminary experiments at an energy charge of 0.9 gave the following values of (M)₀.₅ and (M)₂₀, respectively (all expressed as millimolarity): fructose-6-P, 0.11, 0.04; glucose-6-P, 0.40, 0.18; fructose diphosphate, 1.0, 0.4. These values guided our choice of concentrations to be used in energy charge experiments.

The response of *A. vinelandii* pyruvate kinase to variation in energy charge is shown in Fig. 6. For comparison, the composition of the adenylate pool as a function of energy charge is indicated by the broken lines. Because ADP is a substrate, the rate of the reaction is necessarily zero at charge values of 0 and 1, where the adenylate pool contains no ADP. It is clear, however, that the experimental curve does not depend merely on the concentration of ADP, which is symmetrical with a maximum value at a charge of 0.5. The decrease in rate with increase in energy charge, beginning at a charge value of about 0.1, clearly reflects the decreasing concentration of the positive modifier AMP, and the consequent decrease in affinity of the enzyme for phosphoenolpyruvate. The velocity of an enzymatic reaction is a function of (S)/(S)₀.₅—that is, of the concentration of the substrate and the affinity of the enzyme for the substrate. In the classical approach in vitro, this ratio varies only as a consequence of variation in (S), since (S)₀.₅ (or Kₘ) is, or is considered to be, constant. In the experiment reported in Fig. 6, on the other hand, (S) is constant and the degree of saturation of the enzyme by phosphoenolpyruvate, and hence the rate of the reaction, changes solely as a consequence
highly active remain evidently in increase charge is 6 above about 0.75. Curves the charge energy those charge and consequently with which charge to vivo. Modification of the charge was essentially saturating curve C, each was added. For curve D, each modifier was added at an essentially saturating concentration; for curve C, each was added at a level that would be half-saturating in the absence of the other modifiers; for curve B, each was added at its (M)0.25, or quarter-saturating level; and curve A was obtained with no modifiers added.

Figures like Fig. 7 and 8 should be reliable in reflecting the interactions of modifiers with energy charge, but they are somewhat arbitrary in that a single concentration of substrate (in this case, phosphoenolpyruvate) was used. The af-
finity of the enzyme for phosphoenolpyruvate is probably a more fundamental variable. Figure 9 shows $(S)_0.5$ for phosphoenolpyruvate as a function of energy charge in the absence of modifiers and in the presence of saturating levels of all three. These two lines are the boundaries of a fan-shaped family of curves. It is evident that at concentrations of the modifiers approximating their $(M)_0.25$ values, when the $(S)_0.5$ curve would be about midway between the two curves of Fig. 9, the affinity of pyruvate kinase for phosphoenolpyruvate would be a sensitive function of both energy charge and hexose phosphatase concentration.

**Determination of energy charge in A. vinelandii cells.** For all enzymes that have been shown to respond to energy charge, the steep regions of the response curves are in the charge range above 0.75, with the midpoint usually near 0.85. Most of the published analytical values for nucleotide concentrations in vivo indicate that the charge value in living cells of a variety of types is well stabilized in the region between about 0.8 and 0.95. This summary of published values will be documented in a forthcoming paper. For comparison with the results of Fig. 6 to 9, it was desirable to determine the energy charge in growing *A. vinelandii* cells. A 200-ml culture was grown in a 1-liter Erlenmeyer flask with rapid shaking at 28 to 30 C. Samples (1 ml) were removed at intervals and rapidly added to 0.2 ml of 35% cold HClO₄ or to 1 ml of boiling ethanol. Adenine nucleotides were estimated in the resulting solutions by the firefly luciferase assay essentially as described by Pradet (19). By using a scintillation counter, the level of ATP in the solution was estimated by the light emitted when a sample was added to a luciferin-luciferase preparation. Incubation with pyruvate kinase (rabbit muscle) and excess phosphoenolpyruvate converted ADP to ATP, allowing determination of the sum of these two nucleotides. On incubation of another sample with pyruvate kinase, phosphoenolpyruvate, and adenylate kinase (rabbit muscle), both AMP and ADP were converted to ATP, and the total adenine nucleotide pool level could then be determined. The concentrations of the individual nucleotides were determined by difference and used to calculate the energy charge of the adenylate pool from the defining equation: energy charge = $(ATP + \text{ADP})/(ATP + \text{ADP} + \text{AMP})$.

The results (Fig. 10) show that during most of the growth period the energy charge of *A. vinelandii* cells is constant, within experimental error, at a value between 0.8 and 0.9. This indicates that we are justified in considering values in this range to be physiological when analyzing curves such as those presented in Fig. 6 to 9.

**DISCUSSION**

The importance of phosphoenolpyruvate as a metabolic branchpoint is illustrated in Fig. 11. Glycolysis and much of the citrate cycle are anaerobic pathways, contributing both to regeneration of ATP and to the replenishment of pools of intermediates that are depleted by biosynthesis. Pyruvate kinase, like the other en-
zymes in these sequences, is amphibolic in function, but the reaction catalyzed by phosphoenolpyruvate carboxylase serves only to replenish pools of intermediates. The citrate cycle oxidizes acetyl-SCoA; entry of oxalacetate replaces oxalacetate or other intermediates of the cycle that have been consumed in biosynthetic processes, but cannot contribute to regeneration of ATP. It is thus appropriate that activity of the carboxylase should be regulated in part by simple negative feedback response to the concentration of aspartate. The positive modifier effect of acetyl-SCoA must in part play a similar role, since a fall in the concentration of oxalacetate will decrease the rate of the citrate synthase reaction. As a consequence, the concentration of acetyl-SCoA will tend to increase, and the modifier action of this compound on phosphoenolpyruvate carboxylase will lead to an increase in the rate of oxalacetate production. Such regulatory interactions have been previously discussed for bacterial phosphoenolpyruvate carboxylases (8) as well as for pyruvate carboxylases from eukaryotic organisms (13, 21, 22).

As appears to be the general situation at metabolic branch-points, the adenylate energy charge may be expected to profoundly affect the partitioning of phosphoenolpyruvate between the kinase and carboxylase reactions. It is evidently desirable that an increase in the energy status of the cell, as reflected in a rise in the energy charge, should cause an increase in the relative amount of phosphoenolpyruvate that is converted to oxalacetate for use in biosyntheses and a corresponding decrease in the amount converted to pyruvate. Several regulatory effects contribute to this desirable result.

(i) As a consequence of the direct modulation of pyruvate kinase reported in this paper, an increase in energy charge will decrease the rate of the kinase reaction and increase the concentration of phosphoenolpyruvate, since the main effect of energy charge is on the affinity of the enzyme for phosphoenolpyruvate (Fig. 9). This in-
crease in concentration of the common substrate will increase the rate of the carboxylase reaction.

(ii) If the enzymes of Azotobacter are similar in regulatory properties to those of other organisms that have been studied, indirect effects of variation in energy charge on partitioning at the phosphoenolpyruvate branchpoint will reinforce the primary effect on pyruvate kinase. Citrate synthases from various organisms are strongly affected by the energy charge (3); an increase in the charge causes a marked increase in (S)0.5 for acetyl-SCoA. The resulting increase in concentration of acetyl-SCoA will lead to an increase in the affinity of phosphoenolpyruvate carboxylase for phosphoenolpyruvate, thus increasing the fraction of the phosphoenolpyruvate flux that is directed toward oxalacetate. Acetyl-SCoA is also a biosynthetic precursor. Any tendency toward depletion of the acetyl-SCoA pool will be resisted by this same effect on phosphoenolpyruvate carboxylase; since the carboxylase will compete less strongly, more phosphoenolpyruvate would be used for regeneration of acetyl-SCoA.

(iii) An increase in charge increases the affinity of the lysine-sensitive aspartokinase of E. coli for aspartate (12). If the Azotobacter enzyme responds similarly, an increase in charge will cause a decrease in the concentration of aspartate, with a consequent tendency to increase the rate of carboxylation of phosphoenolpyruvate.

Thus, although phosphoenolpyruvate carboxylase is not directly modulated by adenylate energy charge, it seems that an increase in charge will cause an increase in affinity of this enzyme for phosphoenolpyruvate because of the indirect effects mediated through variations in the concentrations of acetyl-SCoA and aspartate.

The effects observed in this and the preceding paper (15), as well as previously recorded responses of related enzymes, seem adapted to interact in such a way as to assure that the partitioning of phosphoenolpyruvate between the kinase and carboxylase reactions will faithfully reflect the momentary needs of the cell for ATP and for biosynthetic intermediates.

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LITERATURE CITED