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# Regulation of the Pyruvate Kinase from *Alcaligenes eutrophus H 16 in vitro* and *in vivo*

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*Abstract.* The biosynthesis of the enzyme pyruvate kinase (E.C. 2.7.1.40) of *Alcaligenes eutrophus* (*Hydrogenomonas eutropha*) *H 16* was influenced by the carbon and energy source. After growth on gluconate the specific enzyme activity was high while acetate grown cells exhibited lower activities (340 and 55 μmoles/min · g protein, respectively).

The pyruvate kinase from autotrophically grown cells was purified 110-fold. The enzyme was characterized by homotropic cooperative interactions with the substrate phosphoenolpyruvate, the activators AMP, ribose-5-phosphate, glucose-6-phosphate and the inhibitor ortho-phosphate. In addition to phosphate ATP caused inhibition but in this case non-sigmoidal kinetics was obtained. The half maximal substrate saturation constant $S_{0.5}$ for phosphoenolpyruvate in the absence of any effectors was 0.12 mM, in the presence of 1 mM ribose-5-phosphate 0.07 mM, and with 9 mM phosphate 0.67 mM. The corresponding Hill values were 0.96, 1.1 and 2.75. The ADP saturation curve was hyperbolic even in the presence of the effectors, the $K_m$ value was 0.14 mM ADP.

When the known intracellular metabolite concentrations in *A. eutrophus H 16* were compared with the regulatory sensitivity of the enzyme, it appeared that under the conditions *in vivo* the inhibition by ATP was more important than the regulation by the allosteric effectors.

*Key words:* Pyruvate Kinase – Allosteric Regulation – Regulation *in vivo* – *Alcaligenes eutrophus H 16.*

The facultative autotrophic hydrogen bacterium *Alcaligenes eutrophus H 16* is able to grow by fixing carbon dioxide via the Calvin-Bassham-cycle (Hirsch *et al.*, 1963) or to grow on fructose using the Entner-Doudoroff pathway; the enzymes of glycolysis are only used for glucogenesis (Gottschalk *et al.*, 1964). The $C_3$-intermediates of the glycolytic pathway are metabolites that are common to the pathways of autotrophic metabolism, fructose or gluconate degradation, and glucogenesis from $C_2$, $C_3$ or $C_4$ carboxylic acids as growth substrates. Depending on the carbon and energy source the different pathways are suppressed either by repression or by enzyme inhibition. For example, the key enzyme of the Calvin cycle, phosphoribulokinase (Abdelal and Schlegel, 1974), and that of the Entner-Doudoroff-pathway, glucose-6-phosphate dehydrogenase (Blackkolb and Schlegel, 1968; Tunail and Schlegel, 1972; Bowien *et al.*, 1974), are subject to regulation by sugar and adenosine phosphates and by nicotinamide nucleotide. In a facultative autotroph rather effective regulatory mechanisms are also expected to be involved in the enzyme reactions dealing with phosphoenolpyruvate or pyruvate, *i.e.* the thermodynamically irreversible reactions of the pyruvate kinase and the phosphoenolpyruvate carboxylase on the one hand and the energy requiring reactions of the phosphoenolpyruvate carboxykinase and of the phosphorylation of pyruvate (Frings and Schlegel, 1971a, b) on the other hand.

The kinetic properties of pyruvate kinase have already been studied in many prokaryotic and eukaryotic organisms and the regulatory patterns of the enzyme obtained from heterotrophic and autotrophic organisms were interpreted with regard to the special type of metabolism. However, there are few data (Collins and Thomas, 1974) indicating the significance of the observed regulatory properties of the pyruvate kinase *in vivo*.

This investigation concerns the regulation of pyruvate kinase (E.C. 2.7.1.40) in *Alcaligenes eutrophus H 16*. The enzyme was partially purified from autotrophically grown cells and studied with respect to its mode of regulation. The experimental results provided a clear picture of regulatory control of pyruvate kinase *in vitro*, and the situation *in vivo* is discussed in the light of measured metabolite concentrations.

## *Materials and Methods*

*Organism and Growth Conditions. Alcaligenes eutrophus* strain *H 16* (*Hydrogenomonas eutropha H 16*) ATCC 17699, DSM Göttingen 428 (Wilde, 1962), was grown autotrophically with 80% $H_2$, 10% $O_2$ and 10% $CO_2$ in a 10 l Biostat-Fermenter

Table 1. Partial purification of the pyruvate kinase from autotrophically grown *Alcaligenes eutrophus H 16*

| Purification step | Volume (ml) | Protein (mg/ml) | Specific activity (μmol/min/g) | Total activity (μmol/min) | Recovery (%) | Purification (-fold) |
|---|---|---|---|---|---|---|
| Crude extract | 190 | 26 | 74 | 360 | 100 | 1 |
| Protamine sulfate | 212 | 16.8 | 97.4 | 346 | 96 | 1.33 |
| Ammonium sulfate 30–40% saturation | 8 | 73 | 778 | 285 | 79 | 10.7 |
| Sephadex G-200 chromatography | 52 | 2.1 | 2655 | 290 | 80 | 36.2 |
| Hydroxylapatite chromatography | 4 | 2.1 | 8370 | 70 | 23 | 112 |

(B. Braun, Melsungen, Germany) in the mineral medium described by Schlegel *et al.* (1961). For heterotrophic growth 1 l shaken fluted Erlenmeyer flasks were used, and the mineral medium contained 0.5% fructose, sodium-gluconate, sodium-pyruvate or 0.2% sodium-acetate instead of $NaHCO_3$. The cells were harvested in the exponential growth phase, washed twice in saline and stored in 10 mM potassium-phosphate buffer, pH 7.2, containing 5 mM $MgSO_4$ at – 20° C (1 g wet weight/4 ml buffer).

*Preparation of Extract and Partial Enzyme Purification.* The frozen cell suspension was thawed, and after addition of DNase, disrupted using a French Press (4° C; 70 kp/cm$^2$). Cell debris was removed by centrifugation (20000 *g*, 15 min, 4° C) and the membrane bound NADH oxidase by ultracentrifugation (100000 *g*, 90 min, 4° C). Specific enzyme activities in extracts were measured in portions freed of small molecules on a Sephadex G-25 column.

The pyruvate kinase showed a reversible loss of activity on exposure to cold. A rapid inactivation also occurred at enzyme concentrations below 4 μmoles/min · ml. This loss of activity on dilution could be prevented by a PEP concentration of 1 mM; the addition of 1 mM PEP to the inactivated enzyme partially restored its activity. The allosteric inhibitor ortho-phosphate (2 mM) also stabilized the enzyme, but the activators AMP, ribose-5-phosphate and glucose-6-phosphate accelerated the loss of activity at low enzyme concentration.

For the partial purification of pyruvate kinase (see Table 1) from frozen (– 20° C) extract of autotrophically grown cells (26 mg protein/ml) a 4% protamine sulfate solution was added at 4° C to a final concentration of 0.5% (w/v). The sediment was removed by centrifugation for 20 min at 20000 *g* and 4° C. The supernatant fluid was subject to an ammonium sulfate fractionation procedure (solid salt; 15 min equilibration time). The 30–40% ammonium sulfate fraction was dissolved in 10 mM potassium-phosphate buffer, pH 7.2, containing 5 mM $MgSO_4$, 0.05% sodium-azide and was then dialyzed three times for 1 hr at 20° C against a 50-fold volume of the same buffer. The dialyzed enzyme preparation was centrifuged and the pellet was discarded. To avoid loss of enzyme activity due to dilution in the next steps phosphoenol-pyruvate (PEP) was added to a final concentration of 1 mM. The enzyme was then chromatographed at room temperature on Sephadex G-200, normal, particle size 40–120 μm in a 2.5 × 90 cm column which was equilibrated with the dialysis buffer containing 1 mM PEP. From the G-200 column the pyruvate kinase fractions were pooled and used for hydroxyl-apatite adsorption chromatography. The column (1.6 × 25 cm) was equilibrated with the same buffer. The enzyme was eluted at room temperature with a linear gradient of 10–60 mM potassium-phosphate, pH 7.2 (containing $MgSO_4$, PEP and sodium-azide). The pyruvate kinase fractions were pooled, concentrated by means of a Diaflo chamber (Amicon corporation, U.S.A.) and two volumes of glycerol were added.

The enzyme preparation was free from NADH oxidase, ATPase and adenylate kinase. Stabilized by 66% glycerol it showed no loss of activity within 2 months when stored at – 20° C. At saturating substrate concentrations and at 1.4 μg protein per ml reaction mixture the course of the reaction was linear with time for more than 15 min. Between 1 and 7 μg protein per ml mixture the reaction rate was proportional to enzyme concentration. The partially purified pyruvate kinase showed no sensitivity to cold, when it was stored in the presence of glycerol. Furthermore, there were no differences in activity, when the reaction was started either with the enzyme or with ADP after preincubation in the assay mixture containing PEP, indicating the preparation to consist of a fully active enzyme.

*Determination of Pyruvate Kinase.* The activity of pyruvate kinase was determined in a photometer (PL4; Zeiss, Oberkochen, FRG) connected to a Vitatron recorder (The Netherlands), measuring NADH oxidation (365 nm, 1 cm path length) in the coupled reaction with lactate dehydrogenase modified from Beisenherz *et al.* (1953). The reaction mixture contained 50 mM tris (hydroxymethyl)-aminomethane (TRIS)-HCl buffer, pH 7.4; 6 mM $MgSO_4$; 0.23 mM NADH-disodium salt; 1.6 mM PEP tricyclohexylammonium salt; 0.7 mM ADP-disodium salt; and 13 μg lactate dehydrogenase (550 units per mg) in a final volume of 1.00 ml. This mixture showed no alterations within 12 hrs at 30° C.

For the determination of the specific enzyme activities in the crude extract the mixture contained 2 mM AMP as activator of the enzyme. When tests were done with crude extract as the enzyme source, the extract was kept for at least 1 hr at room temperature before use and was then preincubated in the reaction mixture in the presence of PEP for 3–5 min before the reaction was started with ADP. For tests with the partially purified pyruvate kinase the reaction was started by the addition of the enzyme.

*Determination of NADH Oxidase, ATPase and Adenylate Kinase.* NADH oxidase in the crude extract was calculated from the control reaction in absence of the pyruvate kinase substrates. The ATPase and the adenylate kinase were tested in the same assay after Bergmeyer (1970). It contained in a final volume of 3.00 ml; 50 mM TRIS-HCl buffer, pH 7.4; 0.5 mM AMP-disodium salt; 1.1 mM ATP-disodium salt; 12 mM $MgSO_4$; 48 mM KCl; 0.23 mM NADH-disodium salt; 0.8 mM PEP-tricyclohexylammonium salt; 0.1 mg pyruvate kinase; 40 μg lactate dehydrogenase. For measuring the ATPase the extract was added to the mixture in the absence of AMP. After 2–3 min the adenylate kinase reaction was started by the addition of AMP.

*Protein Determination.* Crude extract protein was determined according to the method of Beisenherz *et al.* (1953). During the enzyme purification the protein was determined according to the method of Lowry *et al.* (1951).

Table 2. Specific activities of the pyruvate kinase from *Alcaligenes eutrophus H 16* after growth on various carbon sources

| Growth on | Specific activities (μmoles/min/g protein) |
|---|---|
| $H_2 + O_2 + CO_2$ | 150 |
| Fructose | 174 |
| Gluconate | 340 |
| Pyruvate | 96 |
| Acetate | 55 |

*Chemicals.* All enzymes and biochemical substrates were purchased from Boehringer Mannheim GmbH, Germany. Protamine sulfate was obtained from Sigma Chemical Company, St. Louis, U.S.A.; Sephadex G-25 and G-200 from C. Roth, Karlsruhe, Germany; hydroxylapatite from Bio-Rad Laboratories, Richmond, U.S.A.; all other chemicals from E. Merck, Darmstadt, Germany.

## *Results*

### Variation of Enzyme Activity with Growth Substrate

The specific activities of the enzyme after growth on various carbon sources are shown in Table 2. The specific activity of pyruvate kinase in the cells of *Alcaligenes eutrophus H 16* was dependent on the growth substrate. After autotrophic growth or growth on fructose or gluconate the enzyme level was three to seven times higher than in acetate grown cells.

### General Characteristics of the Partially Purified Enzyme

The pyruvate kinase had a temperature optimum of 45° C, and an activation energy of 10.3 kcal/mol (Arrhenius plot). The pH dependency of the enzyme at saturating substrate concentrations and in the absence of effectors showed a broad plateau between pH 6.4 and 7.7 in 40 mM imidazole-HCl and 50 mM TRIS-HCl buffer, and an optimum at pH 6.6.

The effect of a number of metabolites on the activity of the pyruvate kinase was tested at saturating (1.6 mM) as well as nonsaturating (0.24 mM) PEP concentrations. The metabolites each tested singly at 2 mM, were such as glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, ribose-5-phosphate, sedoheptulose-1,7-diphosphate, 2- and 3-phosphoglycerate, 6-phosphogluconate, citrate, isocitrate, malate, fumarate, succinate, 2-oxoglutarate, AMP, ATP, $K^+$, $Na^+$, $NH_4^+$ and ortho-phosphate. ATP and ortho-phosphate were observed to inhibit the pyruvate kinase. The inhibition by ATP could not be alleviated, but the inhibition by 9 mM phosphate was overcome by 2 mM AMP, ribose-5-phosphate and glucose-6-phosphate.

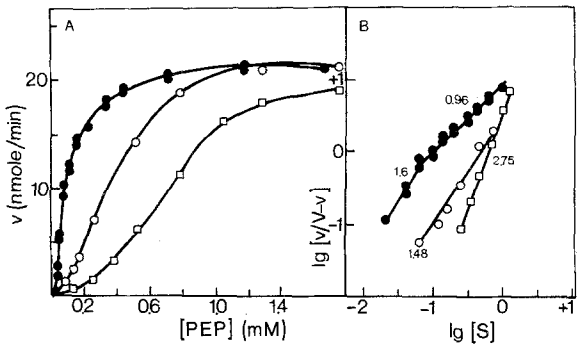


Fig. 1 A and B. PEP saturation curves of the pyruvate kinase from *Alcaligenes eutrophus H 16* in the absence and presence of the inhibitor ortho-phosphate. The reaction mixture is described in "Materials and Methods"; 3.5 μg of the purified enzyme was used. (A) (●) in the absence of effectors, (○) with 3 mM phosphate, (□) with 9 mM phosphate. (B) Hill plot of the data from (A)

Table 3. Kinetics data of activators of pyruvate kinase[a]

| Activator | Half maximal saturation constant (mM) | Hill-coefficient | Maximum activation (%) | |
|---|---|---|---|---|
| | | | Without ortho-phosphate | With 9 mM ortho-phosphate |
| AMP | 0.166 | 1.30 | 10 | 360 |
| Ribose-5-phosphate | 0.446 | 1.52 | 25 | 430 |
| Glucose-6-phosphate | 3.20 | 1.44 | 10 | 350 |

[a] All reaction mixtures contained 0.24 mM PEP and 9 mM ortho-phosphate.

### Kinetics Data of the Enzyme

The substrate(PEP)-saturation curves of the 110-fold purified pyruvate kinase as well as those of the crude extract were sigmoidal. The half maximal substrate saturation constant $S_{0.5}$ of the purified enzyme for PEP equalled 0.12 mM, the Hill coefficient $n_H$ was 0.96 (Fig. 1).

The inhibition of the pyruvate kinase by ortho-phosphate and its relief by the activators AMP, ribose-5-phosphate and glucose-6-phosphate were tested at PEP concentrations below and above the $S_{0.5}$-value. The saturation curves of all four effectors were sigmoidal. In the absence of the inhibitor ortho-phosphate the activators had little effect (Table 3). In contrast to phosphate the inhibitor ATP as the variable component showed hyperbolic saturation curves, the kinetics data of both inhibitors are summarized in Table 4. The inhibitor constant for ATP (1.6 mM) was obtained from a Dixon plot which showed that the inhibition of ATP was competitive with phosphoenolpyruvate.

Table 4. Kinetics data of inhibitors of pyruvate kinase

| Inhibitor | Test conditions | Half maximal saturation constant (mM) | Hill-coefficient | Maximum inhibition (%) |
|---|---|---|---|---|
| Ortho-phosphate | 0.08 mM PEP | 1.62 | 2.1 | 90 |
| Ortho-phosphate | 0.24 mM PEP | 3.66 | 2.6 | 75 |
| ATP | 0.08 mM PEP, 12 mM $MgSO_4$ | 2.9 | — | 100 |
| ATP | 0.24 mM PEP, 12 mM $MgSO_4$ | 4.2 | — | 100 |

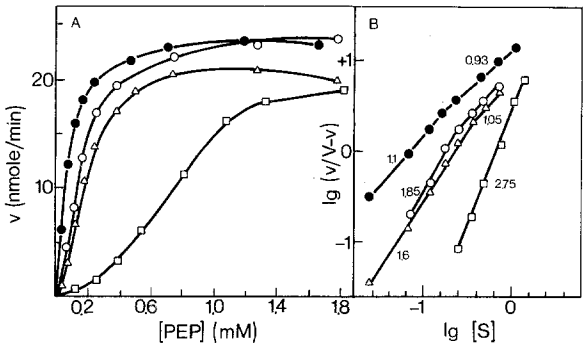


Fig. 2A and B. Effect of ribose-5-phosphate on the PEP saturation of the pyruvate kinase from *A. eutrophus H 16*. The reaction mixture is described in "Materials and Methods"; 3.5 µg of the purified enzyme was used. (A) (●) with 1 mM ribose-5-phosphate, (○) with 1 mM ribose-5-phosphate + 9 mM phosphate, (△) with 2.5 mM ribose-5-phosphate + 9 mM phosphate, (□) with 9 mM phosphate. (B) Hill plot of the data from (A)

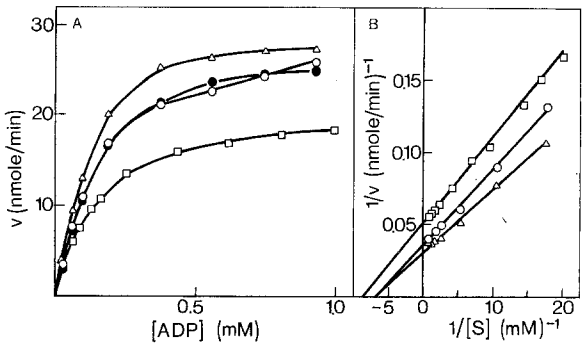


Fig. 4A and B. ADP saturation curves of the pyruvate kinase from *A. eutrophus H 16*. The reaction mixture is described in "Materials and Methods"; 3.5 µg of the purified enzyme was used. (A) (○) without effector, (●) with 9 mM phosphate, (△) with 1 mM AMP, (□) with 5 mM ATP and 12 mM $MgSO_4$. (B) Lineweaver-Burk plot of the data from (A), the curves (○) and (●) are identical

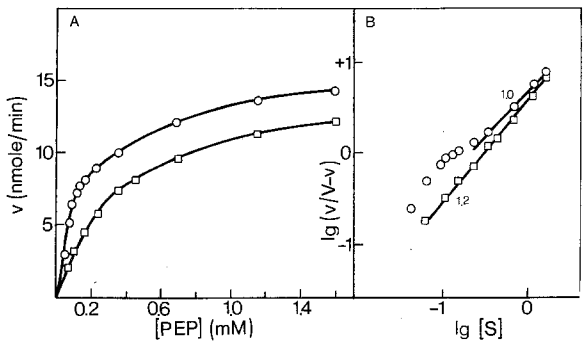


Fig. 3A and B. Effect of ATP on the PEP saturation of the pyruvate kinase from *A. eutrophus H 16*. The reaction mixture is described in "Materials and Methods"; 12 mM $MgSO_4$, 3.5 µg of the purified enzyme was used. (A) (○) with 5 mM ATP, (□) with 8 mM ATP. (B) Hill plot of the data from (A)

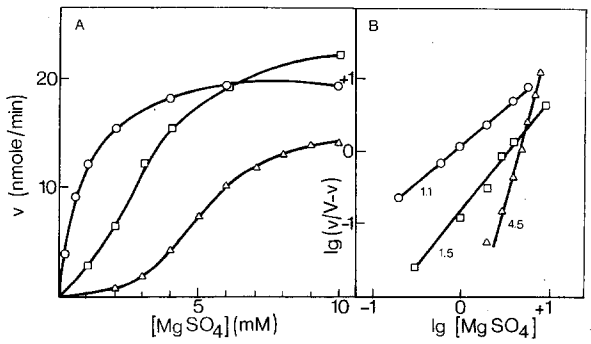


Fig. 5A and B. Magnesium saturation curves of the pyruvate kinase from *A. eutrophus H 16*. The reaction mixture is described in "Materials and Methods"; 3.5 µg of the purified enzyme was used. (A) (○) without effectors, (□) with 18 mM phosphate, (△) with 5 mM ATP. (B) Hill plot of the data from (A)

Phosphate at a concentration of 3 and 9 mM increased the $S_{0.5}$-value for the substrate PEP as well as the Hill coefficient (Fig. 1). This effect was totally relieved by the activators AMP, ribose-5-phosphate and glucose-6-phosphate; PEP-saturation curves in the presence of different concentrations of ribose-5-phosphate are shown in Fig. 2. The PEP-saturation curves in the presence of ATP were hyperbolic, ATP only increased the $S_{0.5}$-value for PEP (Fig. 3).

The substrate(ADP)-saturation curves of the pyruvate kinase were hyperbolic even in the presence of the inhibitors of the enzyme (Fig. 4). The Michaelis-Menten constant $K_m$ in the absence of effectors equalled 0.145 mM ADP. Phosphate and AMP did

not influence this value, ATP (at 12 mM $MgSO_4$) lowered the $K_m$ to 0.111 mM ADP.

The pyruvate kinase of *A. eutrophus H 16* was magnesium-dependent. The magnesium saturation curve was hyperbolic in the absence of enzyme effectors (Fig. 5), the $S_{0.5}$-value equalled 0.85 mM $Mg^{++}$. Phosphate and ATP caused sigmoidal magnesium-saturation curves. 10 mM $MnSO_4$ led to only 37% of the activity at saturating $MgSO_4$ concentrations.

## *Discussion*

### Regulation *in vitro*

The kinetics studies of the partially purified (110-fold) pyruvate kinase from *Alcaligenes eutrophus H 16* indicate that the enzyme is subject to allosteric regulation by its substrate phosphoenolpyruvate (PEP), the inhibitor ortho-phosphate, and the activators AMP, ribose-5-phosphate and glucose-6-phosphate. All these ligands show homotropic cooperative interactions, as is evident from their sigmoidal saturation curves. Furthermore, these ligands cause heterotropic effects and modify the affinity of the enzyme for the other ligands. On the basis of these data the enzyme studied belongs to the K-system of the allosteric model of Monod *et al.* (1965). As could be derived from the Dixon plot ATP, the second inhibitor, is competitive with PEP, although a modified product inhibition cannot be excluded. The inhibition is not relieved by high magnesium concentrations. Saturation curves for ADP, the second substrate of the enzyme, are hyperbolic. With the exception of ATP the effectors only modify the $v_{max}$-value for ADP (V-system according to Monod *et al.*, 1965). The magnesium saturation curves are sigmoidal, however, only in the presence of phosphate and ATP. Because of the complex action of magnesium as a bivalent cation these data are no proof of allosteric interactions.

The pyruvate kinase from *A. eutrophus H 16* is subject to a regulation by the energy charge metabolites AMP and ATP as well as by the key intermediates of carbon metabolism, ribose-5-phosphate and glucose-6-phosphate. The influence of these metabolites deserves special consideration:

1. ATP, an indicator of high energy charge and product of the enzyme reaction, is an inhibitor of pyruvate kinase. In the *in vitro* system the decrease of the ATP concentration as well as the increase of AMP lead to an activation of the enzyme.

2. This effect is intensified by the increase of ribose-5-phosphate concentration. This metabolite could be accumulated by the allosteric inhibition of the phosphoribulokinase by AMP and in the presence of high PEP concentrations (Abdelal and Schlegel, 1974).

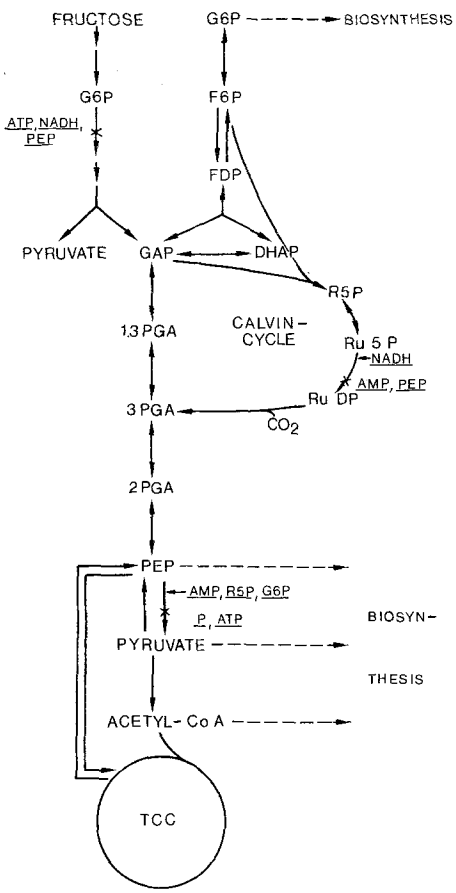


Fig. 6. Metabolic pathways in *Alcaligenes eutrophus H 16* leading to the pyruvate branchpoint. ← activator, × inhibitor. *DHAP* dihydroxyacetone phosphate, *FDP* fructose-1,6-diphosphate, *F6P* fructose-6-phosphate, *GAP* glyceraldehyde-3-phosphate, *G6P* glucose-6-phosphate, *PEP* phosphoenolpyruvate, *PGA* phosphoglycerate, *P* ortho-phosphate, *R5P* ribose-5-phosphate, *RuDP* ribulose-1,5-diphosphate, *Ru5P* ribulose-5-phosphate, *TCC* citrate cycle

3. PEP itself is the substrate of the pyruvate kinase as well as another inhibitor of phosphoribulokinase in strain *H 16*. It may function as an indicator of a saturated $C_3$-pool, inhibit $CO_2$ fixation and promote the function of pyruvate kinase.

4. Glucose-6-phosphate, the key metabolite of sugar degradation via the Entner-Doudoroff pathway, is the weakest activator of the enzyme. A high pool of sugar phosphates, therefore, does not cause a strong stimulation of pyruvate kinase function. In the same way, the glucose-6-phosphate dehydrogenase in *A. eutrophus H 16* is subject only to a negative control by inhibition by ATP, NADH (Blackkolb and Schlegel, 1968) and PEP (Tunail and Schlegel, 1972).

5. Fructose-1,6-diphosphate is not an effector; this observation is in agreement with its purely glucogenetic role. The pathways leading to the pyruvate branchpoint are shown in Fig. 6.

Table 5. Average intracellular metabolite concentrations[a] and the respective kinetics data of the pyruvate kinase from autotrophically grown cells of *A. eutrophus H 16*

| Metabolite | Metabolite concentrations (mM) of cells kept under | | | Constants of half maximal effect (mM) |
|---|---|---|---|---|
| | $H_2 + O_2$, C plus N deficiency | $H_2 + O_2 + CO_2$, N deficiency | $H_2 + O_2 + CO_2 + NH_4^+$ growth condition | |
| PEP | 0.15 | 0.6 | 1.2 | 0.120 without effectors |
| AMP | 0.4 | 0.5 | 0.6 | 0.166 at 0.24 mM PEP + 9 mM phosphate |
| ADP (free + $Mg^{++}$ bound) | 0.7 | 1.0 | 1.3 | 0.145 at saturat. $Mg^{++}$ and PEP concentr. |
| ATP (free + $Mg^{++}$ bound) | 4.2 | 2.8 | 3.6 | 4.2 at 0.24 mM PEP + 12 mM $MgSO_4$ |
| Glucose-6-phosphate | 0.2 | 1.75 | 1.1 | 3.2 at 0.24 mM PEP + 9 mM phosphate |
| % activity[b] of the pyruvate kinase | < 35 | ~ 60 | ~ 70 | |

[a] These are unpublished data from Cook, using an improved version of the method described in Bowien *et al.* (1974) (Manuscript in preparation).
[b] % of $v_{max}$, *i.e.* at saturating PEP concentration in absence of ATP.

### Regulation *in vivo*

Conclusions about the activity of an enzyme in the cell can only be drawn when the intracellular metabolite concentrations are known. The concentration in the cell of PEP, ADP, ATP, AMP and glucose-6-phosphate have been measured (Table 5). The intracellular concentration of ortho-phosphate is not known but assumed to be below 10 mM. The concentration of ribose-5-phosphate is also unknown. The known values represent the total concentration of each metabolite within the cell. Only the "free" metabolite concentration can be assumed to be active in regulation. This concentration may be considered as the total intracellular concentration less the amount of metabolite bound to enzymes as ligands. Up to this point the free metabolite concentration in question cannot be calculated, since the enzyme interactions of the adenylates for instance are too complex. Therefore, conclusions with respect to the *in vivo* regulation can be drawn with certainty only if the known intracellular concentrations are far above the corresponding half maximal saturation constants.

From the intracellular pool sizes one can assume that the AMP concentration is sufficient for full allosteric activation under all physiological conditions except carbon plus nitrogen deficiency. Glucose-6-phosphate should be without additional influence, since its concentration is far below the half maximal activation value. PEP and possibly ADP are also limiting only under the conditions of C plus N deficiency. The concentration of ATP is sufficiently high to assume a significant regulation.

Using the intracellular concentrations of PEP and ATP the activity of the pyruvate kinase was calculated and expressed as per cent of maximum activity, *i.e.* at saturating PEP concentration in the absence of ATP, the other effectors were not considered, since their influence on $v_{max}$ is negligibly small (Table 5). Although the ratio of activity under different conditions should be constant, the absolute values are influenced by either ATP and PEP binding to other enzymes or by magnesium concentrations far below the value of 12 mM. Since the intracellular AMP concentration is very high an intensification of the ATP inhibition by allosteric effects can be supposed only for conditions of C plus N deficiency with energy excess.

The investigations on the kinetics data of pyruvate kinase, on the enzyme control by various metabolites and the comparison of these data to the intracellular concentrations of metabolites in strain *H 16* justify at least one conclusion: The economic use of the catabolic and energy providing function of pyruvate kinase is guaranteed by its inhibition by ATP. Although this enzyme is characterized by a physiologically plausible pattern of allosteric interactions, the meaning of these interactions *in vivo* seems to be small compared to the regulation of the pyruvate kinase by ATP which occurs in a non-allosteric manner.

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