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PYRUVATE KINASE OF *BACILLUS SUBTILIS*

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SUMMARY

Conditions are reported which stabilize pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) activity in extracts of *Bacillus subtilis*. These conditions include high protein concentrations and the presence of KCl and phosphoenolpyruvate. A partially purified extract was not activated by fructose diphosphate or adenosine monophosphate and not inhibited by adenosine triphosphate.

Cells harvested during exponential growth on different carbon sources yielded similar specific activities of pyruvate kinase, indicating the constitutive nature of the enzyme. Nevertheless, the specific activity declined at the end of growth on nutrient sporulation medium.

INTRODUCTION

Earlier work on *Bacillus subtilis*[1] indicated the presence of the complete sequence of glycolytic enzymes except for the apparent absence of pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40). The presence of the latter enzyme would be expected, because *B. subtilis* grows well on glucose or other carbohydrates and in their presence produces large amounts of acetoin[2]. A labile pyruvate kinase has been reported for *Bacillus licheniformis*[3] which is closely related to *B. subtilis*. We show here that such a labile activity is present in *B. subtilis* and that it can be stabilized by maintaining high protein concentrations and including both KCl and phosphoenolpyruvate (PEP) in the extraction buffer. This activity is not modified by the adenylates or by fructose diphosphate.

Cells grown on different carbon sources possess almost the same pyruvate kinase activity when the enzyme is extracted and assayed under optimal stability conditions.

Abbreviations: PEP, phosphoenolpyruvate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

MATERIALS AND METHODS

Bacteria

All enzyme preparations were obtained from the sporulating strain of *B. subtilis*, 60015, which requires indole (or L-tryptophan, Trp) and L-methionine (Met) for growth.

Growth and storage of cells

Cells were grown at 37 °C overnight on TBAB or minimal medium plates prepared as previously described[1]. Cultures were started at an initial absorbance (measured in a Gilford spectrophotometer) at 600 nm ($A_{600\ nm}$) of 0.05–0.07 in nutrient sporulation medium (NSMP) or minimal medium (N *plus* carbon source). They were grown to the desired $A_{600\ nm}$, chloramphenicol (100 μg/ml) was added and the cells were centrifuged. For optimal assay conditions, the cells were washed at 0–5 °C in 0.05 M Tris–HCl buffer, pH 7.5, containing 0.1 mM EDTA, 10 mM $MgCl_2$, 100 mM KCl and 10 mM β-mercaptoethanol (wash buffer). The pellet was frozen in liquid nitrogen. The time lapse from taking of sample to freezing was 10–15 min. For sampling at different times of growth, 75–100 $A_{600\ nm}$ units of cells were harvested at each time point. Under these conditions cells stored frozen overnight showed no detectable loss in activity.

Preparation of extracts and enzyme assays

For optimal assay conditions (*i.e.* maximally stabilized preparations), cells were suspended in ice-cold wash buffer *plus* 2 mM PEP at an $A_{600\ nm} = 75$–100 and broken by a single passage through a French pressure cell (Aminco Corp., Silver Spring, Md.). The suspension was centrifuged at 37 000 $\times$ *g* for 20 min in the cold and the supernatant was assayed immediately for pyruvate kinase activity using the method of Bücher and Pfleiderer[4].

The assay mixture contained 50 μmoles of *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) at pH 7.0 (by NaOH), 0.3 μmole NADH, lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) (5–10 I.U. Type VI Sigma Chemical Co.), 4 μmoles PEP, 250 μmoles KCl, 10 μmoles $MgCl_2$, 5 μmoles ADP, and 5–25 μl of extract in a final volume of 1 ml. Controls contained all additions except PEP. Assays were initiated by the addition of extract. Under these conditions reaction rates remained constant for 3–7 min and were linear with enzyme concentration. NADH oxidase activity in the control was always less than 10% of the velocity determined for pyruvate kinase activity. Thin film (< 2 mm thick) dialysis for 3 h against ice-cold wash buffer containing 1 mM PEP produced extracts with 75% or more of the original activity. The extracts could also be stored at 0–5 °C for 3 h with less than 25% loss in activity. Protein was determined by the method of Lowry *et al.*[5] using bovine serum albumin (Fraction IV) as standard. The specific activity was calculated as μmoles of PEP converted to pyruvate per min per mg protein.

Purification of the enzyme

The enzyme was purified by a modification of the method of Tuominen and Bernlohr[6]. All operations were carried out at 4 °C. 1600 ml of culture, harvested at

$A_{600\ nm} = 1.5$, were centrifuged and the pellet was frozen. The pellet was dissolved in 12 ml of wash buffer and disrupted by passage through the French Press. Debris was removed by centrifugation. 4.7 g of $(NH_4)_2SO_4$ were slowly added to 12 ml of extract (60% saturation) and after 1 h stirring in the cold, the precipitate (which usually contained 40% of the protein and less than 1% of the activity) was discarded. To the supernatant 1.7 g of $(NH_4)_2SO_4$ were added (to bring the saturation to 80%). The pellet (AS I), dissolved in 2 ml wash buffer, usually displayed a gain in total activity. To this solution 0.48 g of $(NH_4)_2SO_4$ was then added (40% saturation) and the suspension was centrifuged. The pellet, which usually contained 50% of the protein and no pyruvate kinase activity, was discarded. To the supernatant 2.6 g $(NH_4)_2SO_4$ were added (60% saturation); all activity was recovered in the pellet (AS II) which contained no detectable ATPase or fructose diphosphatase activity. The pyruvate kinase activity in this pellet was stable to storage at −20 °C for several weeks.

Chemicals

ADP, NADH, PEP, and Tris were obtained from Sigma Chemical Company, St. Louis and *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) from Calbiochem, Los Angeles.

RESULTS

The stability of pyruvate kinase

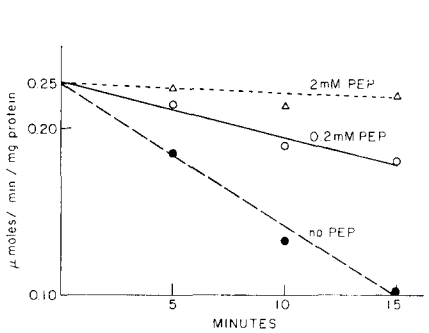
Pyruvate kinase activity was measured by the oxidation of NADH in the presence of PEP, ADP and excess lactic dehydrogenase. When first detected the activity was low, nonlinear with time, and decayed rapidly. Stabilizing conditions were investigated and the results are summarized in Table I. The extraction buffers employed are arranged in the order of increasing specific activity. It is apparent that an increase in the concentrations of KCl, PEP and protein all increased the specific activity of the

TABLE I

EFFECT OF VARIOUS BUFFER ADDITIONS ON THE ACTIVITY OF PYRUVATE KINASE IN EXTRACTS OF *Bacillus subtilis*

The extraction buffer contained 0.05 M Tris, pH 7.3, 10 mM mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EDTA and the compounds stated on a particular line. All cells were harvested from the same culture at $A_{600\ nm} = 1$, suspended in the various buffers to obtain the conditions indicated in the table and ruptured in the French pressure cell. The extract was centrifuged and the supernatant (containing the stated concentrations of protein) was assayed immediately.

Extraction conditions	*Concn of protein (mg)*	*PEP concn (mM)*	*KCl concn (mM)*	*Specific activity (μmoles/min per mg protein)*
1	1.5	—	—	0.005
2	1.5	—	100	0.010
3	2	1	—	0.050
4	3	1	100	0.100
5	8	—	—	0.200
6	10	—	100	0.270
7	9	1	—	0.370
8	10	1	100	0.440



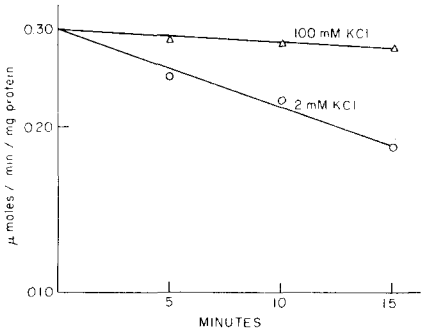
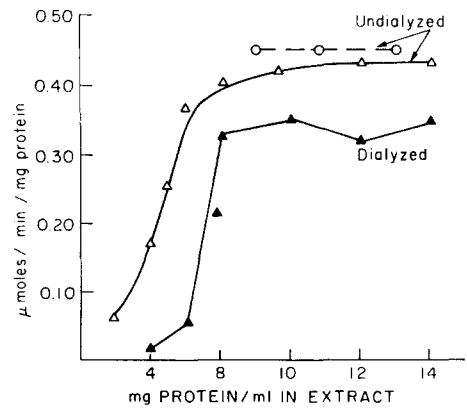


Fig. 1. Effect of PEP on the stability of pyruvate kinase activity in extracts kept on ice. Extraction condition 6 of Table I was used.

Fig. 2. Effect of KCl on the stability of pyruvate kinase activity in extracts kept on ice. Extraction condition 7 of Table I was used.

extracts. Fig. 1 shows the decrease of activity with time at different PEP concentrations when the KCl and protein concentrations were near optimal. Fig. 2 shows a similar decrease for low KCl concentrations when the PEP and protein concentrations were near optimal. Finally, Fig. 3 shows that the specific activities of extracts, obtained at near optimal concentrations of KCl and PEP, was very low when the cells were ruptured at an $A_{600\ nm} < 75$, *i.e.* when the final protein concentration was below 8 mg/ml. The lability of the enzyme at these low protein concentrations was further emphasized by dialysis. At protein concentrations higher than 8 mg/ml the specific activity reached a constant value and dialysis for 3 h against KCl- and PEP-



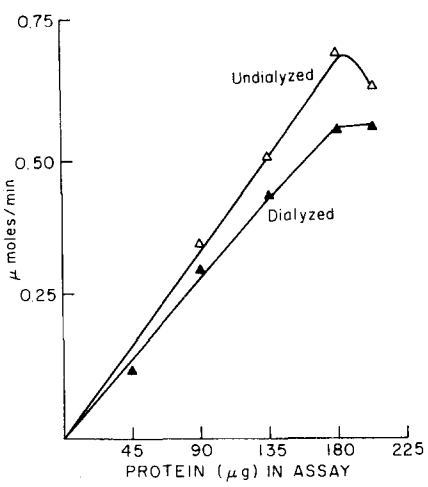


Fig. 3. Effect of extraction at different cell concentrations on the specific activity of pyruvate kinase. The cell concentrations are expressed as protein concentrations of the supernatant. The extraction buffer 8 of Table 1 was used. (a) △—△ and ○---○, different harvests undialyzed; (b) ▲—▲, dialyzed for 3 h against the same buffer.

Fig. 4. Dependence of pyruvate kinase activity on protein concentration. Extraction condition 8 of Table I was used. △—△, undialyzed; ▲—▲, dialyzed.

TABLE II

PURIFICATION OF PYRUVATE KINASE FROM *Bacillus subtilis*

AS I = 60–80% $(NH_4)_2SO_4$ fraction. AS II = a second $(NH_4)_2SO_4$ fractionation, obtained from AS I by discarding the 0–40% fraction and retaining the 40–60%.

Fraction	*Specific activity (units/mg protein)*	*Total activity (units)*	*Overall recovery (%)*
Cell free extract	0.04	1.9	100
AS I	0.06	3.8	200
AS II	0.10	5.6	300

containing buffer did not decrease it by more than 20% (Fig. 3). When the extract was prepared under stabilizing conditions, with or without dialysis, the enzyme activity increased linearly with the protein concentration (Fig. 4).

When the stabilized enzyme (in wash buffer, which contains 100 mM KCl, *plus* 2 mM PEP) was 200-fold diluted in a reaction mixture that contained 100 mM NaCl instead of KCl, the observed activity was 7–10-fold less than with KCl. However, 100 mM NH_4Cl gave about the same activity as KCl. Stabilized extracts could be subjected to $(NH_4)_2SO_4$ fractionation with full recovery of activity. Even when extracts had been prepared in wash buffer without PEP, and were therefore less active, $(NH_4)_2SO_4$ precipitation recovered much of the activity (see Table II).

The regulation of pyruvate kinase

The reaction rates of pyruvate kinase, partially purified by ammonium sulfate precipitation as described in Materials and Methods and in Table II, were measured at different PEP concentrations (using 5 mM ADP). A sigmoidal curve was obtained which showed half maximal velocity at a PEP concentration of 0.25 mM. At this concentration of PEP or at 1 mM PEP, addition of 1 mM Mg:ATP, Mg:AMP, or FDP did not alter the reaction velocity.

TABLE III

SPECIFIC ACTIVITY OF PYRUVATE KINASE IN CELLS GROWN IN DIFFERENT MEDIA

B. subtilis strain 60015 was grown in the stated media and cells were harvested in mid exponential phase at an $A_{600\ nm}$ = 0.5–1.5. They were ruptured in wash buffer containing 2 mM of PEP, to give protein concentration of about 10 mg/ml. The reported specific activities are averages of 3 or more separate cultures. For composition of media, see Materials and Methods. N, minimal medium; NSMP, nutrient sporulation medium.

Media	*Concn (mM)*	*Specific activity (μmoles/min per mg protein)*
N *plus* citrate	15	0.35 ± 0.05
N *plus* malate	50	0.50 ± 0.05
N *plus* pyruvate	50	0.45 ± 0.05
N *plus* glucose	50	0.29 ± 0.04
NSMP	—	0.50 ± 0.05
NSMP *plus* glucose	50	0.35 ± 0.03
N *plus* glucose *plus* malate	50	0.40 ± 0.05
	25	
N *plus* glucose *plus* citrate	50	0.53 ± 0.05
	15	

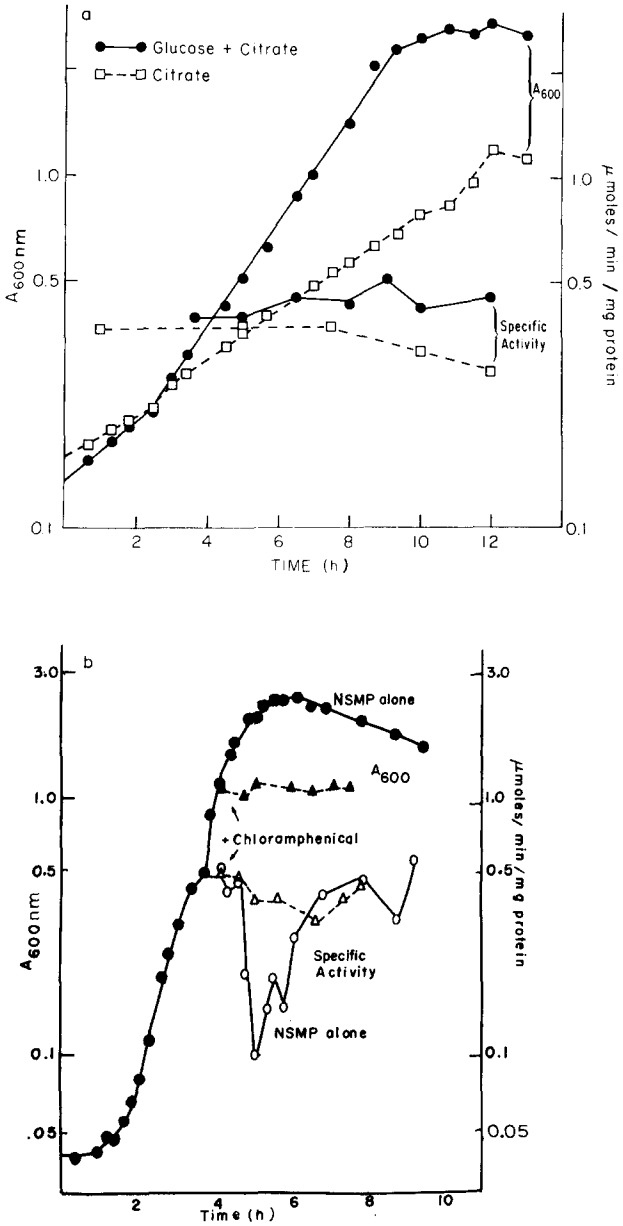


Fig. 5. (a) Pyruvate kinase activity of cells grown in minimal medium (N) *plus* 55 mM glucose and 4.4 mM citrate and in N *plus* 15 mM citrate. $A_{600\ nm}$: ●—●, in glucose and citrate; □---□ in citrate. Specific activity: ●—●, glucose and citrate; □---□ in citrate. (b) Pyruvate kinase activity of cells grown in nutrient sporulation medium (NSMP). $A_{600\ nm}$: ●—●, NSMP alone; ▲---▲, NSMP *plus* 100 μg/ml chloramphenicol added at time of arrow. Specific activity: ○—○, NSMP alone; △---△, NSMP *plus* chloramphenicol.

When cells were grown in synthetic media, containing different carbon compounds, about the same specific activity of pyruvate kinase was observed (Table III). The constitutive nature of pyruvate kinase was also demonstrated when the specific activity was assayed at different times of growth, for cells grown on glucose *plus* citrate or on citrate alone as carbon sources (Fig. 5a). The values remained almost constant and did not differ significantly under the two growth conditions.

When cells were grown in nutrient sporulation medium (NSMP) the activity of pyruvate kinase decreased at the end of exponential growth (Fig. 5b). This decrease could not be prevented by the administration of chloramphenicol at the time of harvesting, but it was avoided when chloramphenicol was added during exponential growth, stopping any further development (Fig. 5b).

DISCUSSION

Conditions have been determined which stabilize the activity of pyruvate kinase in *B. subtilis* extracts. These stabilizing conditions, which include PEP, KCl and high protein values in the extraction buffer, are similar to those used to stabilize this enzyme in *B. licheniformis*[3], *Azotobacter vinelandii*[7], and *Bacillus liquefaciens*[8]. The physiological significance of the enzyme's lability is not known. Addition of PEP and KCl does not reactivate inactive preparations of the enzyme. However, $(NH_4)_2SO_4$ fractionation of somewhat less active preparations results in a partial recovery of activity. The reason for the gain in activity upon $(NH_4)_2SO_4$ fractionation is not known. It could be an activation of the enzyme either by NH_4^+ or by high protein values. It could also be due to the loss of an inhibitor; but that possibility appears unlikely, as addition of crude extract (before $(NH_4)_2SO_4$ fractionation) to the partially-purified enzyme did not significantly decrease activity.

In contrast to the inducible pyruvate kinase of *Escherichia coli*[9], the *B. subtilis* enzyme is apparently constitutive. However, at the end of growth in nutrient sporulation medium the activity declines; this phenomenon could result from the enzyme instability during the deficiency of PEP or from the action of a protease at that time. Whether the decline is important for sporulation is not known.

We have found no evidence for modification of enzyme activity by millimolar amounts of ATP, AMP, or fructose 1,6-diphosphate. Only the substrate (PEP) activated the enzyme. These results are at variance with the findings in *B. licheniformis*, in which the enzyme was activated by AMP and inhibited by ATP, but not affected by FDP[6]. In *E. coli* K_{12} a fructose 1,6-diphosphate-activated form of the enzyme has been found which is not affected by AMP or ATP[9]. However, in *E. coli* B, the enzyme is activated by both AMP and fructose 1,6-diphosphate[10]. It thus appears that the metabolite sensitivity of the enzyme activity differs in different bacterial species. Detection of enzyme modifiers depends to a large extent on the activity state of the enzyme. Consequently, we have maintained maximal activity conditions in preparations used to test for enzyme induction and modification.

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