Role of Pyruvate Carboxylase, Phosphoenolpyruvate Carboxykinase, and Malic Enzyme during Growth and Sporulation of Bacillus subtilis

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SUMMARY

In extracts of Bacillus subtilis, CO₂ fixation occurs primarily through the apparently constitutive enzyme pyruvate carboxylase, which is strongly activated by acetyl-CoA. This enzyme is necessary for growth on glucose but is not required for sporulation, as was established with a pyruvate carboxylase mutant. The malic enzyme can use either NAD or, less effectively, NADP as cofactor. The ratio of these activities remains constant through enzyme purification and during enzyme induction by L-malate. Enzyme synthesis is not repressed by glucose. Malic enzyme and pyruvate carboxylase form a "pyruvate shunt" to the citric acid cycle, which apparently is necessary during growth on malate for the production of oxalacetate in substrate amounts; malic dehydrogenase functions mainly to provide energy via the citric acid cycle. A specific and sensitive [γ-32P]ATP assay for P-enolpyruvate carboxykinase has been developed. Using this assay, a purified enzyme preparation gave a Kₘ for oxalacetate of about 25 μM. Enzyme synthesis is repressed by glucose. P-enolpyruvate carboxykinase mutants have established that the enzyme is needed for gluconeogenesis and, under normal growth conditions, for sporulation. Sporulation can be restored by the continuous feeding of gluconate.

Microorganisms begin to differentiate into dormant forms when nutritional conditions become growth rate-limiting. This usually occurs when the rapidly metabolizable carbon sources, e.g. carbohydrates, have been exhausted (1, 2). Nevertheless, some carbohydrates are incorporated into polymers during differentiation, indicating the need for gluconeogenesis. We use gluconeogenesis as a general term for the synthesis of any carbohydrate from C₃ and C₄ compounds. To elucidate the control of gluconeogenesis in differentiation, we have investigated the enzymes connecting the citric acid cycle to the Embden-Meyerhof path (Fig. 1).

Since its biochemistry and sporulation have been well studied, we used Bacillus subtilis. In this organism, the roles of glycolytic (2, 3) and citric acid cycle enzymes (4–6) in sporulation have been examined. However, little was known about the enzymes that control CO₂ metabolism, some of which are gluconeogenic. At the branch points of P-enolpyruvate and pyruvate, glycolysis and anaplerosis converge and gluconeogenesis begins. Regulation of this crucial area determines the direction of carbon flow.

We have determined the mechanism controlling the synthesis and activation of the enzymes and analyzed in mutants the effect of enzyme deficiencies on growth and sporulation. We have found that the malic enzyme of B. subtilis uses either NAD or NADP; the activity ratio remains constant during purification and induction by malate. For P-enolpyruvate carboxykinase we have developed a specific sensitive assay and demonstrated glucose repression. This enzyme is required for gluconeogenesis and sporulation, as was shown with a P-enolpyruvate carboxykinase mutant. Sporulation of this mutant could be restored by continuous feeding of gluconate.

MATERIALS AND METHODS

Media—TBAB³ plates, NSMP, and N have been described (7). M medium contained N plus 1 mg per ml of sodium citrate. Both N and M media always contained 25 μg per ml of L-tryptophan and 10 μg per ml of L-methionine, and a carbon source (50 mm, unless stated otherwise).

Bacterial Strains—All strains were derived from the transformable 168 strain of B. subtilis. Our strain, 60015, requires L-methionine and L-tryptophan for growth and sporulates well in NSMP. The two P-enolpyruvate carboxykinase mutants (61101 and 61104) were isolated from ⁶⁰Co-irradiated spores of strain 60015 as colonies that could not grow on plates with N plus L-malate but could grow on N plus glucose. They produced pale colonies on TBAB plates. We obtained from J. Hoch (Scripps Clinic and Research Foundation, La Jolla, Calif.) the pyruvate carboxylase mutant 61437 (C50) and the malic dehydrogenase mutant 61461 (JH421); both require L-tryptophan for growth. The other malic dehydrogenase mutant 61421 (IA21) was obtained from R. Hanson (Department of Bacteriology, University of Wisconsin); it requires L-tryptophan and contains an additional mutation that causes the production of large

³ The abbreviations used are: TBAB, tryptose blood agar base; NSMP, phosphate-buffered nutrient sporulation medium; N, minimal salts medium.
mesosomes. Both malic dehydrogenase mutants produce pale colonies on TBAB or NSMP plates and very few spores. Strains were stored at -60° in N plus 25% glycerol.

Growth—Bacteria were incubated overnight on TBAB plates and either used directly or, after streaking once more onto plates containing N plus carbon sources, for induction or repression experiments. Media were incubated at an initial $A_{400}$ of 0.1.

Cells for P-enolpyruvate carboxykinase isolation were inoculated from N plus L-malate plates into the same medium in Erlenmeyer flasks of 5 times the liquid volume and were shaken at 120 strokes per min at 37°. When the $A_{400}$ was 1, the cells were inoculated into 12 liters of fresh medium in a fermentor, again grown to $A_{400}$ = 1, harvested, and washed in wash buffer (50 mM Tris-Cl, pH 7.5, 100 mM KCl, 10 mM mercaptoethanol, and 0.1 mM EDTA), and the pellet was stored at -40°.

The malic enzyme was similarly isolated from the malic dehydrogenase strain, 61646, grown in M plus glucose plus 5 mM potassium-L-malate.

Preparation of Extracts and Enzyme Assays—All preparation and purification steps were performed at 0-5°. Cells were suspended in an “extraction buffer” (containing 50 mM Tris-Cl, pH 7.5, 1 mM MgCl2, and 10 mM mercaptoethanol) at a concentration of 0.1 g per ml wet weight, ruptured in a French pressure cell, and centrifuged at 37,000 × g for 30 min. The protein concentration of the supernatant extract was 8 to 10 mg per ml.

CO2 fixation was assayed according to Sundarum et al. (8). P-enolpyruvate carboxykinase activity was determined by a 32P transfer assay described in Fig. 5 or, if purified preparations were used, by the coupled spectrophotometric assay of Shrago et al. (9). Malic dehydrogenase was assayed by oxalacetate reduction according to Yoshida and Freese (10).

For the malic enzyme assay, cells were extracted in extraction buffer plus 1 mM potassium-L-malate and assayed immediately as described under “Results” (see Table III).

Protein was determined by the method of Lowry et al. (11).

Purification of Malic Enzyme—The extract was treated with 20 mg of proteamin sulfate per g of protein. After centrifugation, 131 mg of ammonium sulfate were added per ml of supernatant and the pellet was discarded. Ammonium sulfate, 214 mg per ml, was added and the pellet was dissolved in 1/4 $V_x$ of the original volume of extraction buffer containing 1 mM malate and 50% glycerol and stored at -20°.

Two milliliters of the concentrated enzyme solution containing 12 to 15 mg of protein per ml were dialyzed against a buffer containing 0.01 mM potassium phosphate, pH 6.5, 1 mM MgCl2, 10 mM mercaptoethanol, 1 mM malate, and 20% glycerol. The sample was applied to a hydroxylapatite column (1 × 17 cm2) which had previously been equilibrated in the dialysis buffer. The column was washed with 20 ml of the buffer and was eluted with the following linear phosphate gradients (in the same buffer): 0.01 to 0.05 $M$ (40 ml), 0.05 to 0.05 $M$ (80 ml), and 0.05 to 0.1 $M$ (100 ml). All activity eluted as a single symmetric peak in the final gradient step. This activity was concentrated by ultrafiltration (Diaflo, Amicon Corp.).

Purification of P-enolpyruvate Carboxykinase—Cell extracts were treated with proteamin sulfate as for the malic enzyme. Ammonium sulfate, 351 mg per ml, was added and the pellet was discarded. Additional 179 mg of ammonium sulfate per ml were added and the pellet was stored at -20°.

The pellet was dissolved in 1/4 $V_x$ of the original volume of extract and 277 mg of ammonium sulfate per ml were added. The new pellet was dissolved in extraction buffer containing 10% glycerol and 1 mM ATP and was dialyzed against the same medium. A DEAE-Sephadex A-50 column (1.3 × 15 cm2) was washed with 10 to 15 void volumes of the above dialysis medium and a 1 ml sample containing 20 mg of protein was applied. The column was eluted successively with 20 ml each of dialysis buffer containing 0.05, 0.1, and 0.2 mM Tris-Cl. A linear gradient prepared by mixing 50 ml each of dialysis buffer containing 0.2 and 0.6 mM Tris-Cl was then applied. The activity eluted as a single peak between 0.2 and 0.4 mM Tris-Cl. It was concentrated by ultrafiltration. Activities were determined by the 32P transfer assay prior to the final ammonium sulfate step and by both this assay and the coupled spectrophotometric assay thereafter.

Sporulation The frequency of heat and octanol resistant spores was measured as described by Freese et al. (12).

Chemicals—[$\gamma$-32P]ATP was prepared according to Glynn and Chappell (13) except that the ATP was further purified on a Dowex 1 (chloride form) column (0.3 × 0.6 cm) as recommended by Dr. R. Lazzarini of our laboratory. Five milliliters of the reaction mixture were applied and the column was washed with water and then with 10 ml of 0.01 M sodium formate, pH 3.4, and 0.1 M LiCl. ATP was then eluted with 2 ml of 0.01 M sodium formate, pH 3.4, and 1 M LiCl. It was precipitated by the addition of 8 ml of absolute alcohol. After cold storage overnight, the crystals were obtained by centrifugation, washed in alcohol, dissolved in 0.02 M Tris-Cl, pH 8, plus 0.1 mM EDTA, and stored at -20°.

Nori, acid-washed, was obtained from Pfanzstehl Laboratories, calcium phosphate (hydroxyapatite) from Bio-Rad, Avidin, morpholinopropene sulfonic acid, NAD, NADP, P-enolpyruvate, ADP, ATP, and oxalacetate from Sigma, protamine sulfate from Elanco (Eli Lilly Co.), and NaH14CO3 from New England Nuclear Co.

RESULTS

Properties of Various Mutants—The biochemical block of several mutants employed in this paper is shown in Fig. 1, while their doubling times in different media are summarized in Table I. The two P-enolpyruvate carboxykinase mutants (61101 and 61104) could not grow on malate as sole carbon source but they grew on glycolytic carbon sources. In contrast, the pyruvate carboxylase mutant (61437) grew at a significant rate only on media which supplied citric acid cycle intermediates. Two malic dehydrogenase mutants (61121 and 61161) grew on all carbon sources but at a very low rate on malate alone.

Apart from the enzymes investigated in this paper, several other enzymes had the normal specific activities in the mutants. These include the inducible P-enolpyruvate transferase, phosphofructokinase, and pyruvate kinase in 61437, and isocitrate dehydrogenase, fumarase, and aconitase in 61101 and 61161. Whereas the standard strain (60015) and the pyruvate carboxylase mutant sporulated normally, the malic dehydrogenase and P-enolpyruvate carboxykinase mutants were defective in sporulation. The sporulation deficiency of malic dehydrogenase mutants is typical for mutants of the citric acid cycle (4, 5, 14), while that of the P-enolpyruvate carboxykinase mutant (61104) is analyzed later in this paper.

CO2 Fixation in B. Subtilis Extracts and Control of Pyruvate Carboxylase—The fixation of CO2 was assayed in extracts of our standard strain (60015) in the presence of various additions as summarized in Table II (columns headed by “60015”). With pyruvate and ATP as substrates, the extracts readily fixed CO2 in the presence of acetyl-CoA (Line 1) but not in its absence.

* E. B. Freese, personal communication.
TABLE I
Growth of different mutants on defined media

| Strain | Enzyme deficiency | N + glucose | N + malate | N + glucose + malate | N + glycerol | NSMP |
|--------|-------------------|------------|------------|----------------------|-------------|------|
| 1      | None              | 1.5        | 1.7        | 1.7                  | 0.3         | 0.75 |
| 2      | PEP carboxykinase | 2.0        | NG         | 1.8                  | 2.5         | 0.75 |
| 3      | PEP carboxykinase | 2.0        | NG         | 1.7                  | 2.0         | 0.75 |
| 4      | Pyruvate carboxylase | 4.0 | 2.5        | NG                   | 0.5         |      |
| 5      | Malic dehydrogenase | 2.0        | 5.0        | 1.5                  | ND          | 0.75 |

* PEP, P-enolpyruvate.
* NG, no growth, i.e. doubling times greater than 6 hours.
* ND, not determined.

Lines 1-4. The removal of either substrate (Lines 3 and 4) or of MgCl₂ (Line 5) nearly obliterated the activity. The requirements of ATP, magnesium, and acetyl-CoA and the 90% inhibition by the biotin-complexing protein avidin (Line 6) clearly indicated the presence of pyruvate carboxylase (pyruvate:carbon dioxide ligase (ADP) EC 4.4.1.1) whose activity was not dependent on the growth medium. The pyruvate carboxylase-deficient mutant produced 1/4 of the normal activity (Line 1, Columns 61437) whereas the P-enolpyruvate carboxykinase mutant (61104) had normal activity. The pyruvate carboxylase reaction was linear with time and protein concentration in extracts of the standard strain (60015), while the mutant (61437) produced less than 1/2 of this activity (Fig. 2).

Little CO₂ fixation was observed with P-enolpyruvate alone, even in the presence of acetyl-CoA or P₄, regardless of the growth condition (Table II, Line 8), suggesting the absence of P-enolpyruvate carboxykinase (orthophosphate:oxalacetate carboxylase (phosphorylating) EC 4.1.1.31). However, when ADP was also added, good rates of CO₂ fixation were seen in extracts of cells grown on l-malate or NSMP (Line 7). These data indicate the presence of P-enolpyruvate carboxykinase (ATP:oxalacetate carboxy-lyase (transphosphorylating) EC 4.1.1.34) in malate or NSMP grown cells. This activity was absent in the P-enolpyruvate carboxykinase mutant (61104) but present in the pyruvate carboxylase mutant (61437).

Evidence for Malic Enzyme Activity—While the above experiments indicated that the major CO₂ fixing reaction was catalyzed by pyruvate carboxylase, a reaction between malate and NADP suggested the presence of malic enzyme activity (l-malate: NADP oxidoreductase (decarboxylating); EC 1.1.1.40). This reaction could not involve malic dehydrogenase (l-malate:NAD oxidoreductase; EC 1.1.1.37), because this enzyme does not mediate a reaction between NADP and malate or between NADPH and pyruvate (10). Nevertheless, to establish that the observed reaction was caused by malic enzyme we used two mutants (61121, 61161) that displayed 40 times less malic dehydrogenase activity, than the standard strain (Table III). Their
TABLE II

CO₂ fixation in Bacillus subtilis extracts

The B. subtilis strains 60015 (standard), 61437 (lacking pyruvate carboxylase), and 61104 (lacking P-enolpyruvate carboxykinase) were grown in N plus 50 mm potassium-L-malate or glucose or in NSMP to A₆₅₀ = 1.0 to 1.2, washed, ruptured, and the supernatant extract was assayed. The complete reaction mixture (0.2 ml) always contained 100 mm Tris-Cl, pH 7.5, 10 mm MgCl₂, 2 mm NAD, 75 mm NaH₂CO₃ (0.2 μCi), 5 i.u. of malic dehydrogenase, and enzyme. For the pyruvate carboxylase assay, 4 mm sodium ATP, 0.4 mm acetyl-CoA, 2.5 mm sodium pyruvate, and avidin (where indicated) were added, while for the P-enolpyruvate carboxykinase assay 2 mm sodium P-enolpyruvate and 2 mm sodium ADP were added. After mixing in the cold the samples were incubated for 10 min at 30°C. Reactions were stopped with 0.1 ml 2 N HCl and centrifuged; 0.1 ml of the supernatant was dialyzed against a stream of CO₂, counted in a scintillation counter, and corrected for quenching. For strain 60015, the activities of cells grown in NSMP were similar to those grown in N plus malate. The addition of P₇ (5 mm) had no effect on the results of Line 8. The substitution of GDP or IDP (same concentration) for ADP gave about 1/5 of the activity of Line 7.

| Reaction conditions | 60015 | 61437 | 61104 |
|---------------------|-------|-------|-------|
|                     | Glucose | Malate | NSMP | Malate | Glucose | NSMP |
| For pyruvate carboxylase |       |       |       |       |       |       |
| 1. Complete          | 18.0   | 22.0   | 2.0   | 3.0   | 17.0    | 21.0   |
| 2. -Acetyl-CoA       | 0.04   | 0.05   | 0.10  | 0.04  | 0.10    | 0.06   |
| 3. -ATP              | 0.16   | 0.20   | 0.02  | 0.05  | 0.05    | 0.06   |
| 4. -Pyruvate         | 0.17   | 0.22   | 0.18  | 0.40  | 0.30    | 0.50   |
| 5. -MgCl₂            | 0.02   | 0.02   | 0.03  | 0.05  | 0.07    | 0.06   |
| 6. +Avidin           | 2.0    | 2.5    | ND    | ND    | ND      | ND     |
| For PEPc carboxykinase |       |       |       |       |       |       |
| 7. Complete          | 1.0    | 15.0   | 15.0  | 12.0  | 0.75    | 1.0    |
| 8. -ADP              | 0.20   | 0.25   | 0.40  | 0.30  | 0.40    | 0.40   |

a ND, not determined.
b PEP, P-enolpyruvate.

eXtract contained nearly the same specific activity as the standard strains when the relatively labile enzyme activity was stabilized (see "Preparation of Extracts and Enzyme Assays" under "Materials and Methods").

With NAD instead of NADP, the reaction observed with standard strain extracts (but not malic dehydrogenase mutants) sometimes proceeded nonlinearly for the 1st min, but became linear thereafter. This initial activity apparently resulted from the limited reaction of malic dehydrogenase-forming oxaloacetate. It could be observed separately, by a small increase in the A₆₅₀, if the reaction mixture contained only Tris, malate, and NAD. In contrast, the sustained malic enzyme reaction depended on the complete reaction mixture (Table III) and was linear, after the 1st min, for 5 min for enzyme activities between 0.5 and 10 nmoles per min. We concluded from these observations that malic enzyme activity can be reliably detected in crude extracts of standard strains when the relatively labile enzyme activity was 1 nmoles per min. For PEPc carboxykinase activity in cell extracts. Cells were grown in NSMP and extracts were assayed using the complete reaction mixture described in Table II. Part a, time dependence for the standard strain 60015 (A—A) and the pyruvate carboxylase mutant 61437 (A—A). Each value is the average of two experiments; the same was done for all figures.

TABLE III

Malic dehydrogenase and malic enzyme activities

| Line no. | Strain | Enzyme deficiency | Specific activity |
|----------|--------|-------------------|------------------|
|          |        | Glucose | Glucose + malate | Malate |
|          |        | MDH | ME | MDH | ME | ME |
| 1        | 60015  | None | 400 | 10 | 1200 | 80 | 60 |
| 2        | 61421  | Malic dehydrogenase | 10 | 7.0 | 10 | 60 | NG |
| 3        | 61461  | Malic dehydrogenase | 6.0 | 10 |  |
| 4        | 61437  | Pyruvate carboxylase | NG | NG | 1200 | 30 | 60 |
| 5        | 61104  | PEPc carboxykinase | 200 | 6.0 | 900 | 30 | NG |

Cells were grown in minimal salts medium (N) containing a 50 mm concentration of the indicated carbon sources, harvested, and an extract was prepared.

a Assay conditions for malic dehydrogenase (MDH): 50 mm Tris-Cl, pH 8.0; 1 mm potassium-oxaloacetate; 0.1 mm NADH; and enzyme. With 0.1 mm NADPH, the specific activities of the extracts of cells grown in glucose were less than 1.

b Assay conditions for malic enzyme (ME): 50 mm Tris-Cl, pH 8.0; 50 mm KCl; 10 mm MgCl₂; 10 mm mercuricethoxan; 2.5 mm NADP; and 10 mm potassium-L-malate. With NADP the specific activities of the extracts of cells grown in glucose were 40.0 for 60015, 30 for 61421, and 21 for 61461.

c NG, no growth.
d PEP, P-enolpyruvate.
it decreased much more rapidly when any of the ingredients were omitted.

The specific activity of malic enzyme was always 4 to 6 times higher when the bacteria were grown in the presence of malate than in its absence (Table III). This induction was not prevented by the presence of glucose, whether it was measured in the standard strain or in the malic dehydrogenase mutants (Table IV).

The (sustained) malic enzyme activity measured with NAD was always about 5 times higher than that measured with NADP (Tables III and IV). This constant ratio suggested that the same malic enzyme accepted either cofactor. To establish this more thoroughly, we have partially purified the malic enzyme activity.

Preliminary experiments had shown that malic dehydrogenase would be difficult to separate from malic enzyme. Therefore, we used extracts of the malic dehydrogenase mutant (61161) which had been grown in N plus glucose and malate. During the 3- to 5-hour interval required to purify through the ammonium sulfate steps, more than 90% of the original activity was lost. However, most of this activity was recovered after suspension of the 50 to 80% ammonium sulfate fraction in extraction buffer plus 50% glycerol (Table V). If this suspension was stored at -20°C, no loss in activity was noted over a 6-week period. For further purification, the suspension was dialyzed overnight against elution buffer containing 20% glycerol with no loss of activity and was applied to a hydroxylapatite column. After elution more than 90% of the original activity was recovered.

Throughout all purification steps and in all active fractions, the NAD:NADP ratio of activity remained constant (Table V). In the pooled column fractions the NAD-associated activity had a pH optimum of 8.0 whereas the NADP-associated activity had a broad pH profile with a midpoint of about pH 8.0 (Fig. 3). Therefore, we assayed the NADP-associated activity in the foregoing experiments (Tables IV and V) at pH 6.5 to minimize the NAD-associated activity, which in turn was measured at pH 8.

When the pooled activity of enzyme was passed through a Sephadex G-200 column, the NAD- and NADP-dependent activities had a pH optimum of 8.0 whereas the NADP-associated activity had a broad pH profile with a midpoint of about pH 8.0 (Fig. 3). Therefore, we assayed the NADP-associated activity in the foregoing experiments (Tables IV and V) at pH 6.5 to minimize the NAD-associated activity, which in turn was measured at pH 8.

When the pooled activity of enzyme was passed through a Sephadex G-200 column, the NADP- and NADP-dependent activities eluted in the same peak distinctly after the void volume. All of our results support the assumption that the NAD and NADP activities are associated with the same enzyme.

**Purification and Control of P-enolpyruvate Carboxykinase—**Initially, we used the CO2 fixation assay shown in Table I to determine the linearity of P-enolpyruvate carboxykinase activity with time and protein concentration in crude extracts (Fig. 4, Curve 60015). Although MnCl2 (2 mM) in the assay mixture produced twice as much activity as MgCl2 (10 mM) we avoided manganese because it often produced a precipitate.

To enable accurate comparisons of the enzyme activities at different times of growth in different media and in mutants a reliable, specific, and sensitive assay was needed. Previously, P-enolpyruvate carboxykinase had been detected by a variety of methods. Simplest are the direct spectrophotometric assays coupled either to malic dehydrogenase (9) or to pyruvate kinase and lactate dehydrogenase (15). Since NADH and the other substrates can react in other ways, these assays are unreliable in crude extracts. Other methods, including the one used here, measure the fixation of 14CO2 into acid-stable counts (8); they are more specific, but less sensitive. Assays in the direction of P-enolpyruvate formation have the advantage that enzyme turnover is 7 to 10 times higher than in the reverse. But methods employing the Warburg apparatus for determining CO2 liberation or measuring the production of P-enolpyruvate colorimetrically (16) are also insensitive. These difficulties can be overcome by measuring the transfer of 3P from ATP to P-enolpyruvate. 3P can be specifically and quantitatively cleaved from the produced 3P-labeled P-enolpyruvate by hydrolysis with HgCl2 (17). We employed the CO2 fixation assay shown in Table I to determine the linearity of P-enolpyruvate carboxykinase activity with time and protein concentration in crude extracts (Fig. 4, Curve 60015). Although MnCl2 (2 mM) in the assay mixture produced twice as much activity as MgCl2 (10 mM) we avoided manganese because it often produced a precipitate.

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### Table V

| Step | Total | NAD | NADP | Total | NAD | NADP |
|------|-------|-----|------|-------|-----|------|
|      | mg    | uMoles/min | uMoles/min | mg    | uMoles/min | uMoles/min |
| 1. Crude | 3500 | 0.170 | 505 | 0.026 | 91 | 6.5 |
| 2. Protamine sulfate | 1800 | 0.340 | 612 | 0.050 | 90 | 6.8 |
| 3. Ammonium sulfate | 450 | 1.04 | 488 | 0.160 | 72 | 6.5 |
| 4. Hydroxylapatite | 65 | 3.80 | 247 | 0.631 | 41 | 6.0 |

*Activities were determined using the assay conditions of Table IV.*

**Fig. 3.** pH dependence of NAD and NADP activities of malic enzyme. Purified enzyme collected after the hydroxylapatite column step was assayed according to the conditions of Table V. For pH 6.5 to 7.3 and 7.5 to 9.0, morpholinopropane sulfonic acid and Tris buffers were used, respectively.
TABLE VI
P-enolpyruvate carboxykinase activities in standard and mutant strains

|               | Standard strain | 60015 | 61104 | 61101 |
|---------------|-----------------|-------|-------|-------|
| Growth media  | PEP mutant       |       |       |       |
| N + glucose   |                 | 20    | 16    | 15    |
| N + glucose + l-malate |     | 18    | 11    | 10    |
| N + l-malate  |                 | 150   | NGb   | NG    |
| NSMP          |                 | 200   | NGb   | NGb   |

* PEP, P-enolpyruvate.

b NG, no growth.

of the original P-enolpyruvate carboxykinase activity which could now be measured reliably by the coupled spectrophotometric assay. Dialysis against extraction buffer containing substrates and 10% glycerol and elution from a DEAE-column produced a single symmetrical activity peak with 85 to 90% recovery. The activity of the pooled peak, concentrated by ultrafiltration, was unstable, making further purification difficult.

The enzyme eluted (recovery about 50%) from a Sephadex G-100 column with the exclusion volume, indicating a molecular...
weight above 100,000. Thus the *B. subtilis* enzyme appears to resemble the cytoplasmic enzymes of Tetrahymena (18) and yeast (19) rather than the rat liver enzyme (20).

Kinetic measurements of the P-enolpyruvate carboxykinase purified through the DEAE-step showed the greater reliability of the $^{32}$P transfer assay (Fig. 6). Oxalacetate saturated the enzyme at 75 $\mu$M concentration with half-saturating concentrations of 20 to 30 $\mu$M. The saturation curve was a normal hyperbola with no suggestion of cooperativity. The $K_m$ for oxalacetate was an order of magnitude lower than that of other reports (21, 22). No activation or inhibition by 1 to 5 mM amounts of fructose-P$_2$, glucose-6-P, or AMP was detected either in the coupled spectrophotometric or in the $^{32}$P transfer assay. Similarly, 0.1 $\mu$M acetyl-CoA was without effect.

Enzyme Changes during Growth and Sporulation in NSMP—Since some enzyme activities change during sporulation (3, 5, 12, 23), the activities of the above enzymes were measured during growth and sporulation in NSMP. Pyruvate carboxylase and P-enolpyruvate carboxykinase activities remained essentially constant while malic enzyme increased toward the end of exponential growth (Fig. 7). If NSMP was supplemented with an amount of glucose (25 mM) which did not prevent eventual sporulation (2), P-enolpyruvate carboxykinase activity remained repressed to the end of growth and increased subsequently when the cells entered the sporulation process. Glucose did not influence the activities of pyruvate carboxylase or malic enzyme.

**Sporulation of Pyruvate Carboxylase and P-enolpyruvate Carboxykinase Mutants**—Although the carboxylase mutant (61437) grew slower, in NSMP, than the standard strain (60015) (Table I), it sporulated almost as well (Table VIII).

The P-enolpyruvate carboxykinase mutant (61104) grew but lysed after growth had ceased (Fig. 8). Even among the surviving cells very few heat-resistant spores (Table VIII, Fig. 8) or octanol-resistant spores (Fig. 8) were produced. The few sporulating cells may have salvaged the necessary carbohydrate from the debris of the lysed cells. The lysis could be prevented by the addition of carbohydrates. The restoration of sporulation was more difficult, because the onset of spore development is suppressed by carbohydrates (3). When we added small amounts of glucose (a slowly metabolizable carbohydrate) at different times after the end of exponential growth, the mutant

![Fig. 6. Dependence of the P-enolpyruvate carboxykinase reaction rate on the oxalacetate concentration, measured by the $^{32}$P transfer assay. Partially purified enzyme from the DEAE-column step (Table VII) was assayed as in Fig. 5. Inset: plot of 1/velocity against 1/oxalacetate concentration.](http://www.jbc.org/)

**Table VII**

**Purification of P-enolpyruvate carboxykinase**

Activities were determined by the $^{32}$P transfer assay (see Fig. 5).

| Step                  | Asw/Asa | Total protein | Total activity | Specific activity | Recovery % |
|-----------------------|---------|--------------|----------------|------------------|------------|
| Crude                 | 0.62    | 4000         | 1600           | 0.4              | 100        |
| Protamine sulfate     | 0.91    | 3200         | 1600           | 0.5              | 100        |
| Ammonium sulfate I    | 0.87    | 800          | 1600           | 1.3              | 65         |
| Ammonium sulfate II   | 0.91    | 400          | 810            | 2.0              | 50         |
| DEAE-Sephadex         | 1.00    | 40           | 640            | 16.0             | 40         |

**Table VIII**

**Sporulation properties of pyruvate carboxylase and P-enolpyruvate carboxykinase mutants**

Cells were inoculated into NSMP at an $A_{600}$ of 0.1 and sporulation was determined after 24 hours by platings for heat-resistant particles (S) and total viable cells (V).

| Strain   | Enzyme deficiency | Viable cells (V) per ml | Heat resistant particles (S) per ml | S/V |
|----------|-------------------|-------------------------|-----------------------------------|-----|
| 60015    | None              | $9 \times 10^9$         | $9 \times 10^6$                   | 0.6 |
| 61437    | Pyruvate carboxylase | $8 \times 10^9$      | $4 \times 10^8$                   | 0.5 |
| 61104    | PEP* carboxykinase | $2 \times 10^6$        | $2 \times 10^3$                   | 0.01|

* PEP, P-enolpyruvate.
The major CO₂-fixing enzyme of B. subtilis is pyruvate carboxylase which is strongly activated by acetyl-CoA and apparently constitutive. Its activity is required for growth on carbohydrates, since a pyruvate carboxylase mutant grows in glucose only if the medium also contains a citric acid cycle compound. While P-enolpyruvate is converted to oxalacetate by P-enolpyruvate carboxykinase in enteric bacteria (24, 25), two constitutive enzymes, pyruvate kinase (26) and pyruvate carboxylase, perform this function in B. subtilis (Fig. 1).

The major enzyme controlling the reverse path from oxalacetate to P-enolpyruvate is P-enolpyruvate carboxykinase, whose activity is repressible by glucose. Mutants lacking this activity cannot grow on citric acid cycle compounds, although they grow normally on glucose. Similar results have been reported for Escherichia coli (27, 28). The repression of P-enolpyruvate carboxykinase by glucose is necessary to prevent a loss of ATP by the cycle of enzymes which convert P-enolpyruvate to pyruvate, pyruvate to oxalacetate, and oxalacetate back to P-enolpyruvate, because the other two enzymes of this cycle are constitutive. In media (e.g. N plus malate or NSMP) in which P-enolpyruvate carboxykinase is not repressed and the above cycle could therefore function, P-enolpyruvate presumably is used for gluconeogenesis sufficiently fast that little of it re enters the cycle. The cycle is also controlled by the acetyl-CoA activation of pyruvate carboxylase.

The malic enzyme also is involved in CO₂ metabolism. In B. subtilis this enzyme reacts with either NAD or NADP, with an activity ratio of about 6:1. In excess malate, this reaction, in contrast to that of malic dehydrogenase, causes a rather complete reduction of the nucleotides. The major physiological direction of the enzyme apparently is from malate to pyruvate, since the activity is induced by malate and the pyruvate carboxylase mutant cannot grow on glucose. This enzyme together with pyruvate carboxylase thus form an inducible "pyruvate shunt" which can be used to supply oxalacetate for gluconeogenesis, aspartate production, and metabolism via the citric acid cycle. However, this shunt apparently consumes too much energy to be the only way of converting malate to oxalacetate; malic dehydrogenase is a necessary alternative, as mutants deficient in its activity grow only extremely slowly on malate. In the shunt one ATP is used and thus more energy is required than in the direct conversion of malate to oxalacetate. Nevertheless, malic dehydrogenase alone does also not produce oxalacetate at a rate necessary for optimal growth, as is shown by the slow growth of a pyruvate carboxylase mutant on malate. The equilibrium constant of malic dehydrogenase and the stabilizing effect of NADH (10) favor the production of malate. We conclude that during growth on malate the inducible pyruvate shunt is used mainly for the production of substrate amounts of oxalacetate, while malic dehydrogenase functions primarily to provide energy via the citric acid cycle.

At the end of growth in NSMP all rapidly metabolizable carbon sources have been exhausted and the massive development of spores begins. Energy demands are then satisfied by the oxidation of direct acetyl-CoA precursors (acetate, reetin, etc.) via the citric acid cycle (5). In addition, gluconeogenesis is required since mutants lacking P-enolpyruvate carboxykinase activity cannot sporulate, unless they are slowly and continuously supplied with a carbohydrate. In fact glucosamine is required for the synthesis of murepptides (12), components of the spore cortex (29, 30).

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