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Development of Acetate and Pyruvate Metabolic Enzyme Activities in Soybean Nodules

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Abstract

Acetate and pyruvate play central roles in the metabolism of many eukaryotic and prokaryotic organisms. Results from several sources indicate that acetate and pyruvate metabolism may be important for the *Bradyrhizobium japonicum*-soybean symbiosis. In determinant symbioses, an assessment of the requirement for a particular enzyme for the support of symbiotic nitrogen fixation can be inferred by the correlation of that enzyme activity relative to the development of nitrogenase activity. The activity of several enzymes of acetate and pyruvate metabolism in the *Bradyrhizobium japonicum*-soybean symbiosis were determined and compared to the development of nitrogen fixation activity as measured by the acetylene reduction method. Plant PEPC, bacteroid PEPC, PK, PEPCK, AK, ACS and PTA appeared to correlate with the development of nitrogen fixation activity. The notable exceptions were the lactate dehydrogenases from both the plant nodule cytosol and the bacteroid which showed no apparent correlation with nitrogen fixation activity. Of those enzymes that correlated with development of nitrogen fixation activity, bacteroid PK and PEPCK rose to their maximal activity prior to that of whole nodule nitrogen fixation activity. These two enzymes may provide a role in the preparation of the bacteroid for the subsequent period of nitrogen fixation. The other enzymes that more closely followed the rise in nitrogen fixation activity may provide support for the actual nitrogen fixation process.

Keywords: soybean nodules, carbon metabolism, *Bradyrhizobium*

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1. Introduction

The carbon metabolism of rhizobia-leguminous plant symbioses has been mostly actively studied in determinant symbioses, particularly that of *Bradyrhizobium japonicum* and soybean. Malate is one of the most abundant organic acids in the soybean nodule (Jackson and Evans, 1966; Stumpf and Burris, 1979; Streeter, 1987). In the plant nodule cytosol, pyruvate may serve as the principal precursor to malate, which is formed by the concerted action of phosphoenolpyruvate carboxylase and cytosolic malate dehydrogenase (King et al., 1986; Day and Mannix, 1988; Schuller et al., 1990). Other metabolic fates of pyruvate are possible in soybean nodules. Tajima and LaRue (1982) showed that soybean nodule cytosol contains pyruvate decarboxylase which forms acetaldehyde and also alcohol dehydrogenase which forms ethanol from acetaldehyde. Under anaerobic or microaerophilic conditions, plant tissues produce lactic acid which originates from pyruvate via lactate dehydrogenase (Davies, 1980). However, Tajima and LaRue (1982) were unable to find appreciable activity of lactate dehydrogenase in either soybean roots or nodule cytosol.

Malate is the principal compound transported from the soybean nodule plant cells to the bacteroid (Price et al., 1987). The citric acid cycle is believed to be the primary pathway for malate metabolism within soybean nodule bacteroids. Operation of the citric acid cycle requires a source of oxaloacetate and acetyl-CoA. Inside the bacteroid, the malate received from the plant cell is converted either to oxaloacetate via malate dehydrogenase or to pyruvate via malic enzyme. Pyruvate dehydrogenase catalyzes the conversion of pyruvate to acetyl-CoA (Karr et al., 1984). Bacteroid malate dehydrogenase (Day and Mannix, 1988; Waters et al., 1985), both NAD-dependent and NADP-dependent malic enzymes (Copeland et al., 1989; Kimura and Tajima, 1989) have been characterized in soybean nodule bacteroids.

Besides malate, acetate is also very abundant in soybean nodules (Jackson and Evans, 1966). Peterson and LaRue (1981, 1982) showed that soybean bacteroids could actively metabolize acetate and support nitrogen fixation as assayed by the acetylene reduction method. Acetyl-CoA is the most common form of acetate, participating in the citric acid cycle, the glyoxylate cycle, amino acid metabolism and the polyhydroxybutyrate cycle. Acetyl-Coa can be generated via several different pathways in bacteria, in addition to pyruvate dehydrogenase. *B. japonicum* bacteroids can activate acetate directly via acetyl-CoA synthetase or the combined activities of acetate kinase and phosphotransacetylase (Preston et al., 1989). In *B. japonicum*, acetyl-CoA synthetase (Preston et al., 1989, 1990), acetate kinase (Preston et al.,

1989), phosphotransacetylase (Preston et al., 1989), two acetoacetyl-CoA thiolases (Suzuki et al., 1987) and two acetaldehyde dehydrogenases (Peterson and LaRue, 1981, 1982; Tajima and LaRue, 1982) have been reported.

Acetate and pyruvate are central metabolites in the metabolism of most organisms. Studies on nodule carbon metabolism have focused on the role of dicarboxylic acids because of their obvious importance. However, acetate and pyruvate metabolism also may be integral to nodule development and function. To further access the role of acetate and pyruvate metabolism in nitrogen-fixing soybean nodules, the activity of a number of enzymes that act upon these two metabolites has been determined as a function of plant nodule age. Determinant symbioses permit an assessment of relevance of a particular enzyme by measuring its activity as a function of nodule development (Karr et al., 1984). That is, those enzymes whose activities increase with nitrogenase activity during nodule development may have a necessary role in the symbiotic nitrogen fixation process whereas those enzymes whose activities decrease are probably not required. Here we report that the activities of most of the enzymes of acetate and pyruvate metabolism measured, with the exceptions of plant and bacteroid lactate dehydrogenases, appeared to increase with nodule development.

2. Materials and Methods

Soybean seeds (Cultivar Williams 82) were inoculated with *Bradyrhizobium japonicum* 2143 and grown in a Model GC-15 Environmental Growth Chamber as previously described (Karr et al., 1984). Plants were grown to various ages and nodules at the crown were removed. Plant nodule cytosol extracts were prepared according to Anthon and Emerich (1990). Bacteroid extracts were prepared as described by Karr et al. (1984). The two-step sucrose gradient procedure for the isolation of bacteroids has been shown to be free of mitochondria and cytosolic plant proteins and enzymes (Karr et al., 1984; Waters et al., 1985). The protease inhibitors, leupeptin, PMSF, aminocaproic acid and benzamidine were added to the plant nodule cytosol obtained after nodule maceration and were present in the buffer when the extracts were desalted over a Bio-Gel P6 column (Anthon and Emerich, 1990).

Assay procedures for bacteroid enzymes

The activity of acetate kinase, acetyl-CoA synthetase and phosphotransacetylase was determined as described previously (Preston et al., 1989). Malate dehydrogenase was assayed as described by Waters et al. (1985). The assay

for pyruvate kinase (PK) contained in one ml: 200 mM PIPES, pH 6.8, 5 mM $MgCl_2$, 1 mM EDTA, 150 mM KCl, 2 mM DTT, 1 mM PEP, 2 mM ADP, 160 μM NADH, 20 units lactate dehydrogenase and enzyme extract. The pH optimum for bacteroid PK was between 6.4 and 6.8. Phosphoenolpyruvate carboxylase (PEPC) assays contained in one ml: 100 mM PIPES, pH 6.8, 5 mM $MgCl_2$, 100 mM KCl, 2 mM glutathione, 50 mM $KHCO_3$, 160 μM NADH, 0.005 units of malate dehydrogenase and enzyme extract. The pH optimum of bacteroid PEPC was 6.4 to 7.0 but the broadness of the profile varied with the buffers used; PIPES gave the highest activity. The phosphoenolpyruvate carboxykinase (PEPCK) assays contained in one ml: 200 mM PIPES, pH 6.8, 4 mM $MgCl_2$, 75 mM KCl, 2 mM DTT, 2 mM ADP, 50 mM $KHCO_3$, 160 μM NADH, 0.005 units of malate dehydrogenase and enzyme extract. Lactate dehydrogenase (LDH) was measured as described by Gutmann and Wablefeld (1974).

Assay procedures for plant enzymes

Plant PEPC was measured as described by Schuller et al. (1990). Plant LDH was measured as described by Hoffman et al. (1986). Plant PK was assayed as described by Peterson and Evans (1978).

For each of these enzyme activities, the pH optimum and apparent Km's of each substrate was determined with extracts from nodules of 28 day old plants to ensure optimal measurement of each activity. Quadruplicate measurements were made for each enzyme activity at each nodule age. The maximum variation of all measurements for all enzymes was 13.4%. The complete experiment was repeated twice and several enzyme activities were followed a third time. The figures depict the results from one of the two complete experiments; both experiments yielded similar results.

Acetylene reduction, as an index of nitrogenase activity, was measured as described by Karr et al. (1984). Leghemoglobin was measured as described by Appleby and Bergersen (1980).

3. Results and Discussion

Acetylene reduction activity served as an index of nitrogen fixation activity and when combined with the leghemoglobin content and nodule weight served as measures of nodule development (Fig. 1). These measurements were very similar to those observed previously (Karr et al., 1984; Anthon and Emerich, 1990). Bacteroid malate dehydrogenase activity served as an internal standard by which to compare the efficiency of bacteroid enzyme extraction of each set

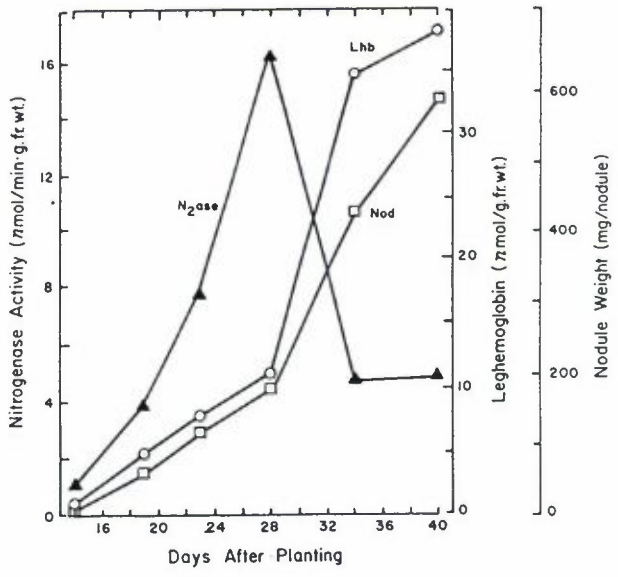

Figure 1. Whole nodule acetylene reduction activity, leghemoglobin content and weight of soybean nodules at various ages formed by inoculation with *Bradyrhizobium japonicum* 2143. Acetylene reduction activity (N_2ase, ▲); leghemoglobin content (Lhb, ○); nodule weight (Nod, □).

of nodules (Karr et al., 1984). The absence of mitochondrial malate dehydrogenase in the bacteroid preparations served as a criterion of purity (Waters et al., 1985). The change in protein content per nodule during development observed here was the same as that reported by Anthon and Emerich (1990), that is, the protein content per nodule was essentially constant during nodule development except in very young nodules.

Soybean root nodule phosphoenolpyruvate carboxylase (PEPC), lactate dehydrogenase (LDH) and pyruvate kinase activities were followed as a function of nodule age (Fig. 2). Soybean root nodule PEPC activity increased markedly, in parallel with acetylene reduction activity and with leghemoglobin content, but the rate of increase was dramatically reduced after the peak of nitrogenase activity was achieved. Day and Mannix (1988) reported that the activity of PEPC in the nodule was more than seven-fold greater than that found in the root indicating enhanced expression in the nodule. The maximal specific activity reported here is about twice the activity Day and Mannix (1988) reported, but their measurements were obtained from plants at a single age and the developmental stage of their plants was not provided. Tajima and LaRue (1982) found that pyruvate decarboxylase activity closely correlated with the

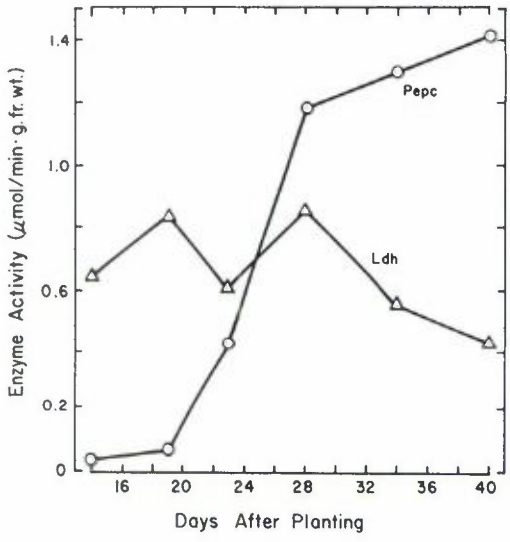

Figure 2. Activity of phosphoenolpyruvate carboxylase (Pepc) and lactate dehydrogenase (Ldh) in the plant portion of soybean nodules at various ages formed by inoculation with *Bradyrhizobium japonicum* 2143. Phosphoenolpyruvate carboxylase (Pepc, ○); lactate dehydrogenase (Ldh, △).

rise of acetylene reduction activity. In fact, pyruvate decarboxylase activity appears to be more closely correlated with nodule acetylene reduction activity than PEPC.

Soybean nodule lactate dehydrogenase activity was variable during the development of nitrogen fixation activity, but appeared to decline somewhat during senescence. Tajima and LaRue (1982) found much lower lactate dehydrogenase activity in the nodule cytosol than reported here. The discrepancy between their data and ours is not known, but may be due to the difference in assay conditions.

The relatively high activity of lactate dehydrogenase was surprising since the activity of pyruvate kinase was low by comparison: 30–40 nmoles/min·g.fr.wt. and did not change appreciably during nodule development (data not shown). Soybean root nodule pyruvate kinase is a highly regulated enzyme (Peterson and Evans, 1978) and its *in vitro* activity may not reflect its *in vivo* activity. The apparent difference in activities between PEPC and pyruvate kinase indicates the metabolic flux from PEP is directed towards the formation of malate and not the formation of pyruvate. The malate produced is then transported into the bacteroids. The high lactate dehydrogenase activity may simply be in response to the low oxygen condition of the nodule tissue (Davies, 1980;

Hoffman et al., 1986) and not a nodule specific response. Alternatively, it may provide a mechanism to reduce the elevated NADH/NAD ratio caused by the low oxygen conditions within the nodule (Salminen and Streeter, 1990).

The activities of several acetate and pyruvate metabolic enzymes were measured in desalted bacteroid extracts. The bacteroid pyruvate metabolic enzyme activities showed several developmental patterns (Fig. 3). Bacteroid PEPCK

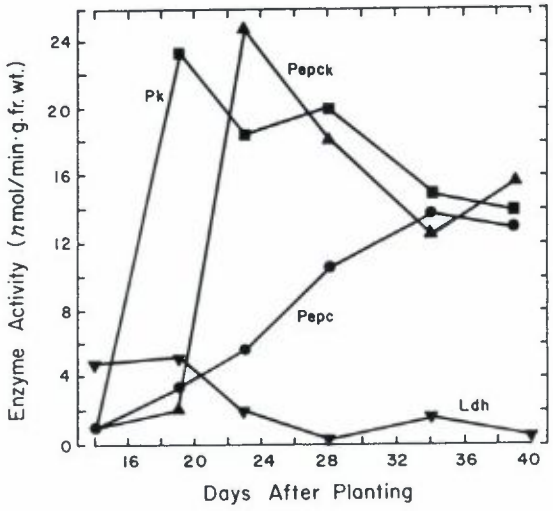

Figure 3. Activities of several bacteroid pyruvate metabolic enzymes from soybean nodules at various ages formed by inoculation with *Bradyrhizobium japonicum* 2143. Pyruvate kinase (Pk, ■); Phosphoenolpyruvate carboxykinase (Pekck, ▲); Phosphoenolpyruvate carboxylase (Pepc, ●); Lactate dehydrogenase (Ldh, ▼).

and PK activities increased rapidly during the early phase of nodule development, reached their maximal activity prior to the peak of maximal nitrogenase activity and then declined. Bacteroid PEPC activity increased in parallel with nitrogenase activity but continued to increase after the acetylene reduction activity declined, until 40 days, and then appeared to decrease slightly. We have previously shown that bacteroid pyruvate dehydrogenase also increases in parallel with acetylene reduction activity (Karr et al., 1984). Bacteroid lactate dehydrogenase activity did not increase during nodule development but rather declined during the rapid rise in acetylene reduction activity and remained low as nodule development continued.

The rise of bacteroid PEPCK and PK prior to the rise in both nodule acetylene reduction activity, and in plant cytosol PEPC, may indicate the

dependence of the bacteroid on glycolytic metabolism to provide pyruvate and oxaloacetate before the plant can provide sufficient malate as a source of these two metabolites. The increase in bacteroid PEPC may function to conserve energy by recycling respiratory carbon dioxide.

The activities of the bacteroid acetate metabolic enzymes as a group appeared to correlate more closely with the acetylene reduction activity than did the pyruvate metabolic enzymes. Acetate kinase and phosphotransacetylase activities closely followed the development of acetylene reduction activity and its subsequent decline, whereas acetyl-CoA synthetase activity continued to increase to 38 days before it then declined (Fig. 4).

The development of the acetate metabolic enzymes also correlates with that of the accumulation of polyhydroxybutryate, a polymer of acetate (Karr et al., 1983). Bergersen and Turner (1990) have indicated that an active turnover of polyhydroxybutyrate may support symbiotic nitrogen fixation in soybean. Thus, the increase in acetate metabolic enzymes may reflect the turnover of this polymer.

In summary, most of the enzymes of acetate and pyruvate metabolism measured increased in activity as the plant age increased. Only the plant and

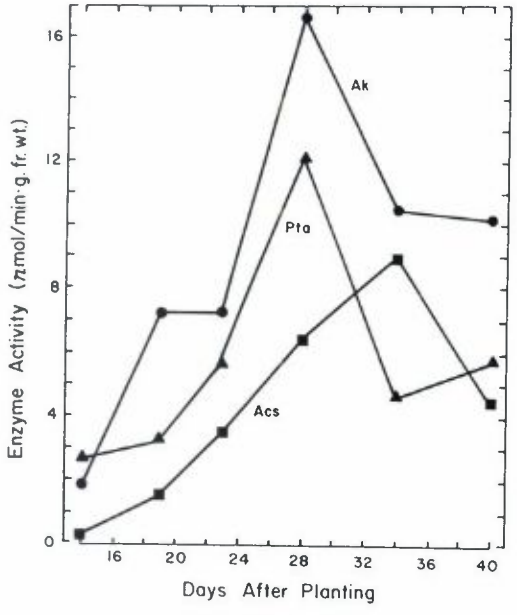

Figure 4. Activities of several bacteroid acetate metabolic enzymes from soybean nodules at various ages formed by inoculation with *Bradyrhizobium japonicum* 2143. Acetate kinase (Ak, ●); Phosphotransacetylase (Pta, ▲); Acetyl-CoA synthetase (Acs, ■).

bacteroid lactate dehydrogenase activities did not increase during nodule development. This was a rather unexpected result as lactate dehydrogenases are frequently expressed under anaerobic conditions (Davies, 1980; Hoffman et al., 1986). However, the activity of the plant lactate dehydrogenase was considerable and may have been expressed to this level at an earlier time in nodule development than measured in this study.

Of those enzymes that increased with plant nodule age, bacteroid PK and PEPCK were the only two enzymes which rose to maximal activity prior to the rise of acetylene reduction activity. This may indicate a need for these enzymes in early nodule development prior to the initiation of nitrogen fixation activity. Those enzymes whose increases in activity correlated with acetylene reduction activity during nodule development imply that elevated expression of these enzymes is required for the symbiotic nitrogen fixation process. However, a definite role for these enzymes in providing energy and/or reducing equivalents to nitrogenase or other support functions remains to be demonstrated.

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