
RELATIONSHIP BETWEEN NITROGEN FIXATION AND CARBON METABOLISM IN LEGUMES: A REVIEW

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ABSTRACT

The process of nitrogen fixation by the legumes imposes carbon (C) energy burden on the plant. The photosynthate translocated from the leaves provides carbon skeleton, reducing power and energy required for the symbiotic nitrogen fixation. Sucrose from the shoot is converted to organic acids, principally dicarboxylates that are supplied to bacteroids to provide reductant for the support of key enzyme nitrogenase. It has been established during the last two decades that an anaplerotic carbon dioxide fixation takes place in nodules, via phosphoenol pyruvate carboxylase (PEPC), a key enzyme for carbon dioxide fixation in plants, algae, cyanobacteria and bacteria, located in the cytoplasm of host cells. Although, it has been shown that nitrogen fixation is fuelled by recently synthesized sucrose translocated to the root nodule, neither sucrose nor hexoses are readily metabolised by isolated bacteroids at rates capable of supporting nitrogenase. In contrast, there is evidence to prove that dicarboxylates play a major and essential role in supporting nitrogen fixation. This review throws light on the relationship between nitrogen fixation and PEPC activity in legume nodules.

The process of nitrogen fixation is energetically very costly requiring huge inputs from the host plant. The nitrogenase reaction alone utilizes as many as 16 molecules of ATP and reductant equivalent to \(8\) \(e^-\) per molecule of nitrogen reduced. Like all synthetic processes, nitrogen fixation imposes a C energy burden on the plant. This C cost approximates \(6\) g C (gN)-1 reduced (Schubert, 1986; Vance and Heichel, 1991). In addition, carbon substrates are required for the assimilation of fixed ammonia, into organic compounds and for nodule growth and maintenance. To meet these demands, nodules must be able to sustain high rates of carbohydrate metabolism in a micro-aerobic environment. However, the metabolic pathways concerned must be strictly regulated to prevent nodules from utilizing photosynthate and carbon reserves to the detriment of other plant functions. The breakdown of carbohydrates in the plant fraction of nodules needs to be geared to the demand for substrates by the bacteroids and for carbon skeletons for the assimilation of fixed ammonia (Day and Copeland, 1991). A number of studies by Lawrie and Wheeler (1975), Kouchi and Nakaji (1985), Streeter (1987) have shown that current photosynthate, translocated into nodules as sucrose, is preferentially used to support nitrogen fixation.

Sucrose is stored in the plant fraction of nodules as starch (Hostak et al., 1987; Walsh et al., 1987). Starch storage in tissues is generally interpreted as excess carbohydrate. Actively nitrogen fixing root nodules of all legume species contain starch in both infected and uninfected cells (Cralle and Heichel, 1985; Gordon et al., 1985; Menegus et al., 1988). Starch accumulates in leaves and roots during the day, although nitrogenase activity occurs unabated throughout both day and night. Shoot carbohydrate pools have been identified as the primary carbon source involved in maintaining nodule activity during darkness (Kouchi et al., 1985). Root starch reserves appear to be unavailable for nitrogen fixation. Finally, nodule starch has a slow turnover rate. Nodule carbohydrate reserves are metabolized
only when external, particularly shoot, reserves are depleted (Kouchi et al., 1985; Walsh et al., 1987). These observations indicate not only that photosynthate in excess of the need of nodules is being produced and transported to the nodule, but also that photosynthate production is excess to the needs of other growing organs. If import and storage of C exceed demand, then what limits nodule function? Rawsthorne and LaRue (1986); Day (1990); Layzell et al. (1990) have suggested there are constraints upon plant C utilization within the nodule or limitations on the conversion of sucrose to metabolites (organic acids) used by bacteroids to reduce nitrogen. Perhaps exchange of appropriate metabolites across cell and organelle (e.g., peribacteroid) membranes limits carbon and nitrogen availability to host plant and bacteroid? (Vance and Heichel, 1991).

1. Carbon Supply to the Bacteroid

Nodule homogenates contain a number of carbohydrates and organic acids in sufficient quantities to be considered as potential carbon substrates for bacteroid respiration. However, they must meet two criteria: they must be taken up and metabolized by isolated bacteroids at rates capable of supporting nitrogenase and they must be able to readily cross the peribacteroid membrane (PBM) (Day and Copeland, 1991). Although, it has been shown that nitrogen fixation is fuelled by recently synthesized sucrose translocated to the nodule, neither sucrose nor the hexoses resulting from its hydrolysis are readily accumulated or metabolized by isolated bacteroids (Glenn and Dilworth, 1981; Streeter, 1981; San Francisco and Jacobson, 1986; Copeland et al., 1989). Furthermore, movement of neutral sugars across the PBM of isolated soybean symbiosomes is only by slow, passive diffusion with rates being inadequate to support nitrogenase activity (Day et al., 1989; Udvardi et al., 1990).

Feeding experiments carried out with 13C- or 14C-labeled precursors have shown that label is transferred rapidly from sucrose into the dicarboxylic acids malate and succinate (Reibach and Streeter, 1983; Romanov et al., 1985; Streeter and Salminen, 1985; Kouchi and Yoneyama, 1986). The recovery of label in neutral sugars in the bacteroids following isotope feeding experiments with whole plants has been attributed to passive uptake of neutral sugars and their slow metabolic turnover relative to organic acids (Streeter and Salminen, 1985) and it is generally assumed that organic acids are the most probable substrates supplied to bacteroids in vivo (Appleby, 1984; Dilworth and Glen, 1984; McDermott et al., 1989). Substantial evidence supports the hypothesis that the energy for nitrogen fixation may drive primarily from plant produced dicarboxylic acids, which are taken up by the bacteroids (Arwas et al., 1985; Bolton et al., 1986; Humbeck and Werner, 1987). Malate, malonate and succinate are abundant in legume nodules (Stumpf and Burris, 1979; Streeter, 1987). Of these, malonate is only slowly transported across the PBM (Humbeck and Werner, 1987) and not a potential substrate, whereas malate and succinate are transported rapidly across the PBM of soybean (Udvardi et al., 1988; Day et al., 1989), and results indicate the presence of a distinct PBM dicarboxylate carrier. Isolated bacteroids also possess a dicarboxylate transporter (Dct) capable of rapid rates of malate and succinate transport (Reibach and Streeter, 1984; San Francisco and Jacobson, 1986) and dct mutants of rhizobia form ineffective nodules (Ronson et al., 1981; Finan et al., 1983; Arwas et al., 1985; Garg et al., 2004).

2. Dicarboxylate Transport System

In free-living cells of Rhizobium leguminosarum and Sinorhizobium meliloti, transport of L-malate, fumarate and succinate
occurs via the C₄-dicarboxylic transport (Dct) system (Glenn et al., 1980; Ronson et al., 1981; Engelke et al., 1987). This system consists of three genes: dct A, which codes for the putative transport protein and two divergently transcribed genes, dct B and dct D, which activate transcription of dct A in response to the presence of dicarboxylates (Ronson, 1988; Watson, 1990; Jording et al., 1992; Jording and Puhler, 1993). Dct B and Dct D are well characterized as a two component sensor-regulator pair. Dct B is thought to reside within the inner membrane and function as a sensor of dicarboxylic acids in the surrounding medium (Ronson et al., 1987). The C terminus of Dct B has homology of over 200 amino acids with a large number of sensor proteins (Ronson, 1988). It is capable of both autophosphorylation, presumably at histidine 416, and phosphorylation of Dct D (Giblin et al., 1996).

However, a number of reports have shown that strains with mutations in dct A display constitutive transcription from dct Ap, suggesting that dct A has a role in controlling its own synthesis (Ronson and Astwood, 1985; Yarosh et al., 1989). Dct A would undergo a conformational change, as a result of binding or transporting substrate, which would result in the release and/or activation of dct B (Reid and Poole, 1998). Dct A has two roles in the cell; the first is as a transport protein such that dct A expressed constitutively in strain RU150 with a deletion in dct B or dct D will transport and permit growth on succinate. The second role appears to be the regulation of dct B – dct D. If dct B – dct D becomes more sensitive to phosphorylation in the absence of dct A, then other stimuli, such as osmotic pressure, might cause activation of dct Ap via cross talk. (Reid and Poole, 1998; Poole and Allaway, 2000). Dct B was shown to be the sensor for dicarboxylates itself and does not simply detect the transport state of dct A. However, in the absence of the dct A protein, dct B becomes promiscuous with regard to signaling. Dct A interacts with dct B and alters its signaling ability, shifting the balance between antokinase and phosphatase activity (Reid and Poole, 1998).

All of these studies showed that blocking the uptake of dicarboxylates by bacteroids prevent nitrogen fixation, establishing firmly that the C₄-dicarboxylates are the primary carbon source for bacteroid respiration.

3. Pathways for Organic Acid Metabolism in Bacteroids

Both direct and indirect lines of evidence indicate that dicarboxylic acids, especially malate and succinate are the most likely forms in which carbon is supplied to the bacteroid to support nitrogenase. Since succinate can readily be converted to malate via the TCA cycle, these two substrates are considered as equivalent. Malic enzyme (ME) activity has been demonstrated in R. leguminosarum (McKay et al., 1988) and B. japonicum (Kimura and Tajima, 1989) bacteroids. This enzyme, which oxidatively decarboxylates malate to pyruvate, could, together with malate dehydrogenase (MDH), provide an effective means of generating both acetyl-CoA and oxaloacetate for TCA cycle operation. An NADP and NAD dependent form of ME has been detected in soybean bacteroids by Copeland et al. (1989); Driscoll and Finan (1993; 1996); Mitsch et al. (1998). It has been shown that NAD⁺ but not NADP⁺ malic enzyme is essential for nitrogen fixation (Driscoll and Finan, 1993). The role of ME has always been assumed to be the provision of pyruvate, and hence acetyl-CoA, as an anaplerotic function to enable the TCA cycle to operate (Driscoll and Finan, 1996).

4. Anaplerotic CO₂ Fixation by PEPC

Strong evidences support the theory that PEP carboxylase in legume nodules
provide carbon skeletons for NH₃ assimilation and export (Coker and Schubert, 1981). The fixation of carbon dioxide by PEPC has been shown to be important in the synthesis of the substrates taken up by the bacteroids and for the provision of carbon skeletons for the assimilation of fixed nitrogen (Vance et al., 1985; King et al., 1986; Rosendahl et al., 1990). This anaplerotic reaction could also be significant in reducing the energy cost of nitrogen fixation by providing a mechanism for the reabsorption of some of the carbon lost in respiratory reaction in the nodules (Deroche and Carayol, 1988).

PEPC (Orthophosphate oxaloacetate carboxylase, PEPC) a Mg²⁺ requiring enzyme, was first discovered by Bandurski and Grenier in 1953. It is a cytosolic enzyme present in all living plant cells (Utter and Kolenbrander, 1972) and is also widely distributed in bacteria, cyanobacteria, and green algae (Toh et al., 1994; Lepiniec et al., 1994). It catalyzes the irreversible 'b-carboxylation of phosphoenol pyruvate (PEP) in the presence of HCO₃⁻ and Me²⁺ to yield oxaloacetate (OAA) and Pi and thus is involved intimately in C₄-dicarboxylic acid metabolism in plants. Besides its cardinal roles in the initial fixation of atmospheric CO₂ during C₄ photosynthesis and Crassulacean acid metabolism (CAM), PEPC functions anaplerotically in a variety of non-photosynthetic systems such as C/N partitioning in C₃ leaves, seed formation and germination, and fruit ripening (Leegood and Osmond, 1990). PEPC has also been partially purified from frenchbean, soybean, lupin, alfalfa root nodules (Christeller et al., 1977; Peterson and Evans, 1979; Merczewski, 1989) and in other C₃, C₄ and CAM plants (Vance and Heichel, 1991).

The anaplerotic fixation of CO₂ by PEPC is believed to play a variety of roles in support of symbiotic nitrogen fixation by legume root nodules. These include: (a) The synthesis of dicarboxylic acids (malate, succinate) used as respiratory substrates by the bacteroids (King et al., 1986; Rosendahl et al., 1990); (b) Provision of carbon skeletons for NH₄⁺ assimilation (Christeller et al., 1977; Rosendahl et al., 1990); and (c) Synthesis of organic acids to maintain charge balance and neutral pH intracellularly and in the xylem (Israel and Jackson, 1982). Rosendahl et al. (1990) examined the distribution of metabolites of dark CO₂ fixation in pea root nodules inoculated with effective and ineffective strains of Rhizobium leguminosarum and demonstrated that organic acids produced following nodule CO₂ fixation are taken up by the bacteroids.

Many plants possess multiple isoforms of PEPCs, perhaps each being associated with a different metabolic pathway. Ting and Osmond (1973), analyzed PEPCs from photosynthetic and non-photosynthetic tissues of a number of C₃, C₄ and CAM species and proposed that at least four distinct isoforms exist based on kinetics and ion exchange chromatography. These include the C₄ photosynthetic PEPC present in the leaves of C₄ plants, C₃ PEPC in the leaves of C₃, C₄ and CAM plants, CAM PEPC in the leaves of CAM plants, and a non-autotrophic PEPC present in the roots of all plants. The functionally active form of all these PEPCs consists of a homotetramer with a monomeric molecular mass of about 100 kDa. The molecular mass of the subunit of PEP carboxylase determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was Ca 130 kDa in leaves of spinach (Miziorko et al., 1974), 90 kDa alfalfa nodule (Vance and Stade, 1984) and 101 kDa (Miller et al., 1987). Sequence analysis of PEPC cDNAs and genes suggests that most plant PEPCs can be loosely placed into three related groups (Toh et al., 1994). Inspite of their high level of expression, the characterized alfalfa and soybean nodule
PEPCs are most related by their sequences to PEPCs of dicots that are constitutively expressed at low levels (Pathirana et al., 1997).

Although PEPC clearly plays a central role in symbiotic nitrogen fixation, little is known about the mechanisms underlying PEPC gene expression during the establishment and maintenance of symbiosis. In developing nodules, PEPC activity increases to a level that is 10- to 15-fold greater than that found in roots and leaves. This increase is correlated with comparable increases in PEPC protein and transcript. Full gene expression is associated first with nodule initiation and development and then with the initiation and maintenance of effective symbiosis (Pathirana et al., 1992; 1997). Immunogold cytochemistry studies on alfalfa nodule PEPC antibodies showed that PEPC is a cytosolic enzyme and is distributed relatively equally in infected and uninfected cells of the nodule symbiotic zone but reduced by 60 percent for ineffective nodule suggesting a direct role of the enzyme in supporting nitrogen fixation (Robinson et al., 1996).

5. Isoenzymes of PEPC

Two isoenzymes of PEPC in alfalfa nodules have been resolved (Vance and Slade, 1984; Deroche et al., 1984). Peterson and Evans (1979) reported five isoenzymes in soybean nodule using imidazole – PAGE system. PEPC-I and PEPC-II isoforms of lupin root nodules differed in their kinetic properties. PEPC-I and PEPC-II had Km (PEP) of 0.09 and 0.18 mM respectively at pH 8.0 (Merczewski, 1989). Higher activity of PEPC I in the nodule infection region (Christellar et al., 1977; Merczewski, 1989) and its high affinity for PEP and HCO₃⁻, suggest a possible involvement of PEPC-I in reassimilation of respired carbon.

6. Carbon Metabolism in the Host

Comparative studies have shown that the specific activities of most of the enzymes involved in the conversion of sucrose to oxaloacetate and to pyruvate are substantially higher in the plant fraction of nodules than those in the roots (Kouchi et al., 1988; Copeland et al., 1989). This indicates that the expression of enzymes concerned with the utilization of sucrose is enhanced in nodules compared to roots.

6.1. Conversion of Sucrose to Hexose Monophosphates

In soybean nodules, sucrose may be cleaved by the action of an alkaline invertase or by sucrose synthase. Alkaline invertase appears to be the main enzyme of sucrose cleavage in young nodules and the specific activity of this enzyme remains essentially, unchanged during nodule development; in contrast, the specific activity of sucrose synthase is low in immature nodules but increases in parallel with the increase in nitrogenase activity as the nodules develop (Morell and Copeland, 1984; Anthon and Emerich, 1990). This enhancement of sucrose synthase activity may be due to the synthesis of an enzyme form that is specific to nodules (Thummler and Verma, 1987). Sucrose synthase is abundant in mature soybean nodules, making up 3-4 per cent of the total soluble protein in the plant fraction (Morell and Copeland, 1985). Neither sucrose synthase nor alkaline invertase of soybean nodules is subject to fine metabolic control. Sucrose synthase has a high affinity for uridine diphosphate (UDP) (km 5 mM) and its activity, as well as that of alkaline invertase, is likely to be regulated mainly by the availability of sucrose in the plant cytosol. The Km values for sucrose of soybean nodule alkaline invertase and sucrose synthase are 10 mM and 30 mM, respectively (Morell and Copeland, 1984, 1985). Nodules contain only a small amount of acid invertase activity, which is unlikely to be quantitatively important in the cleavage of sucrose (Robertson and Taylor, 1973; Streeter, 1982). Further metabolism of the glucose and
fructose produced in the above reactions will require phosphorylation of the hexoses to form corresponding hexose-6-phosphates. In soybean nodules, as in other plant tissues, glucose and fructose are likely to be phosphorylated by separate kinases which have specificity for the respective hexoses (Copeland and Turner, 1987). The UDP-glucose produced by sucrose synthase may act as a precursor of other nucleotide sugars and certain polysaccharides. Alternatively, it may be converted to glucose-1-phosphate by UDP-glucose pyrophosphorylase. The catalytic potential for the readily reversible reaction is high in the plant fraction of soybean nodules (Salminen and Streeter, 1986). Glucose-1-phosphate is converted to glucose-6-phosphate and glucose-6-phosphate to fructose-6-phosphate by phosphoglucomutase and phospho hexose isomerase, respectively. These enzymes have been found to be highly active in extracts from the plant fraction of soybean nodules (Copeland et al., 1989; 1991).

6.2. The Glycolytic Pathway

The first step in the incorporation of fructose-6-phosphate into the glycolytic pathway is phosphorylation to form fructose-1,6-bisphosphate. Plant tissues contain two enzymes capable of catalyzing this step, namely phosphofructokinase (PFK) and phosphofructotransferase (PFP). PFK catalyzes an essential irreversible reaction and occurs in the cytoplasm and plastids. The enzyme from plant, animal and microbial sources has complex regulatory properties, which are likely to be important in the control of glycolysis (Dennis and Greyson, 1987). In soybean nodules, PFK may have a more important role than PFP in the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate. The catalytic potential of PFK in the plant fraction of soybean nodules is considerably higher than that of PFP (Salminen and Streeter, 1987). Moreover, the specific activity of PFK, but not that of PFP, is increased in the nodules in comparison to roots (Copeland et al., 1989).

The nature of the end product of the glycolytic reactions will be determined by the relative activities of PEP carboxylase and pyruvate kinase. The catalytic potential of PEP carboxylase in the plant fraction of nodules exceeds that of pyruvate kinase and is greatly enhanced over that in roots (Copeland et al., 1989). PEP carboxylase and pyruvate kinase of nodules have roughly comparable Km values for PEP (Vance and Stade, 1984) whereas in other plant tissues the affinity of PEP carboxylase for PEP is usually an order of magnitude lower than that of pyruvate kinase (O'Leary, 1982). The fixation of CO₂ by PEP carboxylase has been shown to be important in the synthesis of the substrates taken up by the bacteroids and for the provision of carbon skeletons for the assimilation of fixed nitrogen (Rosendahl et al., 1990). This anaplerotic reaction could also be significant in reducing the energy cost of nitrogen fixation, by providing a mechanism for the reabsorption of some of the carbon lost in the respiratory reactions in nodules (Deroche and Carrayol, 1988).

CONCLUSIONS

It can therefore, be concluded that sucrose from the shoot is converted to organic acids, principally dicarboxylates, which are supplied to the bacteroid to provide reductant for the support of nitrogenase. Much of the organic acid production occurs in the uninfected cells of the nodule. Recent work has shown that isolated infected cells of soybean can accumulate malate but are impermeable to sucrose and poorly permeable to hexoses. The infected zone lacks malic enzyme but contains large quantities of malate dehydrogenase and efficient malate and oxaloacetate carrier (Rawsthorne and La Rue, 1986; Day and Mannix, 1988). These properties, together with low activity of some
TCA cycle enzymes, lead to rapid malate-in/oxaloacetate-out exchange in isolated nodule mitochondria (Bryce and Day, 1990). Thus, malate oxidation by nodule mitochondria produces both ATP (needed for NH₃ assimilation via glutamine synthetase) and oxaloacetate in the cytosol. The oxaloacetate could provide some of the carbon skeletons needed for the GOGAT reaction and purine synthesis in the plastids. Malate would also provide carbon and reducing power to the bacteroid (Day and Copeland, 1991).

For an effective symbiosis, carbon and nitrogen sources must cross both the bacteroid and the peribacteroid membranes. Transport across the bacteroid membrane usually, but not always, resembles transport across the membrane in free-living cells (Brewin, 1991; Verma and Hong, 1996). Although, major changes in our understanding have occurred recently, there are still significant unanswered questions.

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REFERENCES