Nitrogen fixation and carbon metabolism in legume nodules

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A large amount of energy is utilized by legume nodules for the fixation of nitrogen and assimilation of fixed nitrogen (ammonia) into organic compounds. The source of energy is provided in the form of photosynthates by the host plant. Phosphoenol pyruvate carboxylase (PEPC) enzyme, which is responsible for carbon dioxide fixation in C4 and crassulacean acid metabolism plants, has also been found to play an important role in carbon metabolism in legume root nodules. PEPC-mediated CO2 fixation in nodules results in the synthesis of C4 dicarboxylic acids, viz. aspartate, malate, fumarate etc. which can be transported into bacteroids with the intervention of dicarboxylate transporter (DCT) protein. PEPC has been purified from the root nodules of few legume species. Information on the relationship between nitrogen fixation and carbon metabolism through PEPC in leguminous plants is scanty and incoherent. This review summarizes the various aspects of carbon and nitrogen metabolism in legume root nodules.

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Nitrogen fixed symbiotically in the nodules of legumes accounts for over half of nitrogen fixed in nature annually. The bacteria are released into newly formed nodule initials and become bacteroids surrounded by plant host tissue. The legume nodules use large amounts of carbohydrates for the fixation of nitrogen and assimilation of fixed ammonia into organic compounds as well as for nodule growth and maintenance.

The photosynthates translocated from the leaves provide carbon skeleton, reducing power and energy required for symbiotic nitrogen fixation. Sucrose from the shoot is converted to organic acids, principally dicarboxylates, which 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.

Nitrogen fixation and metabolism

The process of symbiotic nitrogen fixation involves the reduction of dinitrogen to ammonia by nitrogenase enzyme complex which is composed of Fe and MoFe proteins.

\[ 8H^+ + 8e^- + N_2 + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \]

Ammonia produced by nitrogen fixation is converted to organic nitrogen before it is exported from the nodule for utilization by the host plant.

The initial organic product is the amino acid glutamine; the assimilation of ammonia into glutamine is catalysed by two enzymes-glutamine synthetase (GS) and glutamate synthase (GOGAT). In higher plants, GOGAT occurs in two distinct isoforms, NADH-GOGAT and ferredoxin-dependent GOGAT (Fd-GOGAT). These differ in molecular mass, subunit composition, enzyme kinetics, antigenic and reductant specificity, and metabolic function. Fd- and NADH-GOGAT are encoded by distinct genes.

One of the most critical problems faced by a nitrogen-fixing organism is the sensitivity of nitrogenase to molecular oxygen. Both the Fe protein and the MoFe protein are rapidly and irreversibly inactivated by molecular oxygen. This extreme sensitivity of nitrogenase to oxygen raises a problem for nitrogen fixing organisms since the large amounts of energy required (in form of ATP and reductant) are produced through cellular respiratory pathway that can only operate efficiently when molecular oxygen is present.

In legume nodules, the oxygen supply is regulated to a large extent by an oxygen-binding protein called leghemoglobin which is produced by host plant and located within the bacteroid-infected host cell. It constitutes as much as 30% of the host cell protein. Its function is to bind oxygen and control release of oxygen in the region of the bacteroid. The equilibrium concentration of oxygen in the bacteroid zone is thus kept at a level (about 10nM) sufficient to support bacteroid respiration and the production of ATP and reducing potential, while preventing excess oxygen from inactivating dinitrogenase. Oxygen levels must

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be carefully balanced because a too low oxygen concentration can also limit dinitrogenase activity in nodules. This is made possible by the existence of a variable nodule oxygen permeability (Po), which acts as an oxygen diffusion barrier. The large carbon requirement imposed by symbiosis may be a consequence of limited ATP production by the plant resulting from low oxygen induced fermentative pathways leading to C4-dicarboxylic acid synthesis.

Carbon supply to bacteroids

The energy for N2 fixation is derived primarily from plant-produced dicarboxylic acids which are taken up by the bacteroids. The dicarboxylic acids are most likely derived from host cytosol, as low oxygen tensions in infected zone of the nodule may limit mitochondrial respiration.

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 activity. Also the movement of neutral sugars across the peribacteroid membrane (PBM) of isolated soybean symbiosomes is only by slow passive diffusion with rates being inadequate to support nitrogenase activity. In contrast, there is evidence to prove that dicarboxylates play a major and essential role in supporting nitrogen fixation.

Tracer studies on the metabolism of 14CO2 in alfalfa nodules have been done. The incorporated radioactivity was found to be higher when the nodules were attached to roots than when they were isolated and on fresh weight basis the incorporated radioactivity was 8-fold higher in the nodules than the roots. 14CO2 metabolism studies do not indicate radioactivity associated with glucose and fructose, most of the radioactivity being in malate and some in glutamate, aspartate and asparagine.

Malate, malonate and succinate are abundant in legume nodules and are transported rapidly across the PBM of soybean and French bean. In vitro and in vivo radioactive studies of 14CO2 metabolism showed high PEPC activity in the nodules of genetically diverse cultivars of chickpea; this activity increased further in the tolerant cultivars when the plants were subjected to salt stress. Similar genetic variability associated with host plant in their tolerance to salinity and drought have been reported among different legume species and cultivars. These results indicate the presence of a distinct PBM dicarboxylate carrier. Isolated bacteroids also possess a dicarboxylate transporter (Det) capable of rapid rates of malate and succinate transport and dct mutants of rhizobia form ineffective nodules. Recently the dct operon has been implicated in the nifA regulatory system of rhizobia further emphasizing the importance of dicarboxylates in symbiosis. Thus, malate and succinate are the most effective substrates for supporting the nitrogenase activity of isolated bacteroids.

Nodule phosphoenol pyruvate carboxylase

Nodule PEPC constitutes up to 1-2% of total soluble proteins of efficient nodules. This enzyme is a tetramer of identical monomers. PEPC is ubiquitous and especially known as a carbon dioxide harvesting enzyme. It is located in the mesophyll cells of C3 leaves and also occurs in an inactive state in C4 plants; PEPC works exclusively during night in CAM plants. The PEPC activity of nodule expressed on fresh weight basis is as high as the activity generally found in leaves of C4 plants. Unlike the bacterial enzyme which is allosteric in nature, PEPC responsible for carbon dioxide fixation in root nodules of legumes is non-allosteric plant enzyme.

Dicarboxylic acids resulting from PEPC activity have been suggested to support bacteroid metabolism in support of nitrogenase activity. Based on oxidation of dark carbon dioxide fixation products in soybean root nodules, it has been estimated that the dicarboxylic acids produced by PEPC activity would be capable of providing 48% of energy for nitrogenase activity.

The first product of carbon dioxide fixation via PEPC is oxaloacetic acid which is highly unstable and is rapidly reduced into malate by malate dehydrogenase (MDH) or transaminated into aspartate by aspartate aminotransferase. The malate formed can be utilized in three different ways in nodule metabolism. A part of malate formed can be used as a carbon and energy source for the nitrogen fixing bacteroids. Malate can enter tricarboxylic acid (TCA) cycle to produce energy (ATP), reducing power and carbon skeletons used in the functioning of nitrogenase and biosynthesis of amino acids from ammonium. Malate could also play a role in adjustment of charge balance in vacuoles and in xylem fluid.

PEPC-gene regulation

Many plants possess multiple isoforms of PEPC, perhaps each being associated with a different
metabolic pathway. Although PEPC clearly plays a central role in symbiotic nitrogen fixation, little is known about the mechanisms underlying PEPC gene expression during establishment and maintenance of symbiosis.

PEPC from photosynthetic and non-photosynthetic tissues of a number of C₃, C₄ and CAM species were analysed and based on kinetics and ion-exchange chromatography at least four distinct isoforms of PEPC were proposed. These isoforms are the C₄ photosynthetic PEPC present in leaves of C₄ plants, C₃ PEPC in leaves of C₃, C₄ and CAM plants, CAM PEPC in leaves of CAM plants and non-autotrophic PEPC present in the roots of all plants. The functionally active form of all these PEPCs consists of a heterotetramer with a monomeric molecular mass of 100 kDa.

Studies on PEPC gene expression in nitrogen fixing (effective) and non-nitrogen fixing (ineffective) alfalfa nodules suggest that full gene expression is associated first with nodule initiation and development and then with initiation and maintenance of effective symbiosis. Several workers have isolated PEPC cDNAs and genes from a wide variety of plants. They isolated and characterized an alfalfa PEPC gene (PEPC-7) whose transcripts were found at elevated levels in nodules, relative to either roots or leaves. The intron/exon structure of this gene was identical to that of most other plant PEPC genes except for the presence of an additional intron in 5' untranslated region. The analysis of promoter deletions suggests that the region between -634 and -536 is of particular importance in directing transcriptional activity to the infected zone of nodules. Thus, PEPC-7 has a central role in nitrogen fixing nodules and the regulation of transcription is an important determinant of its activity.

Conclusion
The limited need of oxygen for proper functioning of nitrogenase results in energy burden on symbiotic nitrogen fixation. This need for energy is compensated by carbon dioxide fixation via PEPC in plant part of nodules. The dicarboxylic acids supplied to bacteroids provide reductants for support of nitrogenase. However, detailed information on the structure, function and genetic control of PEPC activity in a wide spectrum of legume species is lacking. Such information is expected to be helpful in the breeding programmes to improve symbiotic nitrogen fixing efficiency of legumes.

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