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BIOCHEMICAL CHARACTERIZATION OF THE SERYL-PHOSPHORYLATION OF SOYBEAN NODULE PHOSPHO*ENOL*PYRUVATE CARBOXYLASE AND SUCROSE SYNTHASE, AND MOLECULAR CLONING AND OVEREXPRESSION OF A SOYBEAN NODULE SUCROSE SYNTHASE cDNA IN *Escherichia coli*

by

Xiu-Qing Zhang

A DISSERTATION

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Doctor of Philosophy

Major: Biochemistry

Under the Supervision of
Professor Raymond Chollet

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DISSERTATION TITLE

Biochemical characterization of the seryl-phosphorylation of soybean nodule phosphoenolpyruvate carboxylase and sucrose synthase, and molecular cloning and overexpression of a soybean nodule sucrose synthase cDNA in Escherichia coli

BY

Xiu-Qing Zhang

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GRADUATE COLLEGE
UNIVERSITY OF NEBRASKA

BIOCHEMICAL CHARACTERIZATION OF THE SERYL-PHOSPHORYLATION OF SOYBEAN NODULE PHOSPHO*ENOL*PYRUVATE CARBOXYLASE AND SUCROSE SYNTHASE, AND MOLECULAR CLONING AND OVEREXPRESSION OF A SOYBEAN NODULE SUCROSE SYNTHASE cDNA IN *Escherichia coli*

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Phospho*enol*pyruvate carboxylase (PEPC) and sucrose synthase (SS) are critical enzymes in legume nodule C/N-metabolism. In this dissertation it is documented that both of these enzymes in soybean root nodules are phosphorylated *in situ* on a serine residue(s). Stem-girdling or prolonged darkening of the parent plants significantly decreased the apparent phosphorylation state of nodule PEPC. The effect of darkness on PEPC phosphorylation was reversed by illuminating the darkened plants for more than 1 h. This reversal was prevented by concomitant stem girdling, suggesting that the phosphorylation of PEPC in soybean nodules is modulated by photosynthate translocated recently from the shoots. The partially purified PEPC-kinase is Ca^{2+}-independent and has an apparent native molecular mass of about 30 kDa. "In-gel" kinase assays revealed two PEPC-dependent, Ca^{2+}-independent protein kinase polypeptides with molecular masses of about 32 and 37 kDa. Moreover, this protein-Ser/Thr kinase is highly specific to plant PEPC. The Ca^{2+}-independent PEPC-kinase activity in nodules is up- and down-regulated by illumination and stem girdling or prolonged darkness, respectively, suggesting that control of the phosphorylation state of PEPC by current photosynthate in the nodule is mediated largely by this Ca^{2+}-independent protein kinase.

In contrast, SS in soybean nodules (nodulin-100) is phosphorylated by a Ca^{2+}-dependent protein kinase (CDPK). The molecular mass of this SS-kinase is about 55 kDa under both native and denaturing conditions. Unlike nodule PEPC and PEPC-kinase, the phosphorylation state of SS and the total activity of its CDPK are not likely modulated by photosynthate supply from the shoots. A full-length cDNA encoding soybean nodule SS was cloned, sequenced (Accession No. AF030231), and overexpressed in *E. coli* as His-tagged and untagged constructs. This cDNA is 2842 bp long and has an open reading frame of 805 amino acid residues. The soluble recombinant enzymes are catalytically active and phosphorylatable *in vitro* by the partially purified SS-kinase from soybean nodules.

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AAT	aspartate aminotransferase
AS	asparagine synthetase
AI	alkaline invertase
BDA	blue dextran-agarose
BSA	bovine serum albumin
CAM	Crassulacean acid metabolism
CDPK	Ca^{2+}-dependent protein kinase
DTT	dithiothreitol
E-64	*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane
FPLC	fast-protein liquid chromatography
Fru	fructose
Glc	glucose
GOGAT	NADH-glutamate synthase (glutamine 2-oxoglutarate aminotransferase)
GS	glutamine synthetase
I_{50}	50% inhibition constant
IPTG	isopropyl β-D-thiogalactoside
MC-LR	microcystin-LR
MDH	malate dehydrogenase
OAA	oxaloacetate
PEG	polyethylene glycol
PEP	phospho*enol*pyruvate

PEPC	PEP carboxylase
PEPC-kinase	PEPC protein-Ser/Thr kinase
PMSF	phenylmethylsulfonyl fluoride
PVP	polyvinylpolypyrrolidone
SS	sucrose synthase
Suc	sucrose
TLE	thin-layer electrophoresis
W-7	*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

CHAPTER 1

Introduction

General Outline of Legume Nodule C/N-Metabolism

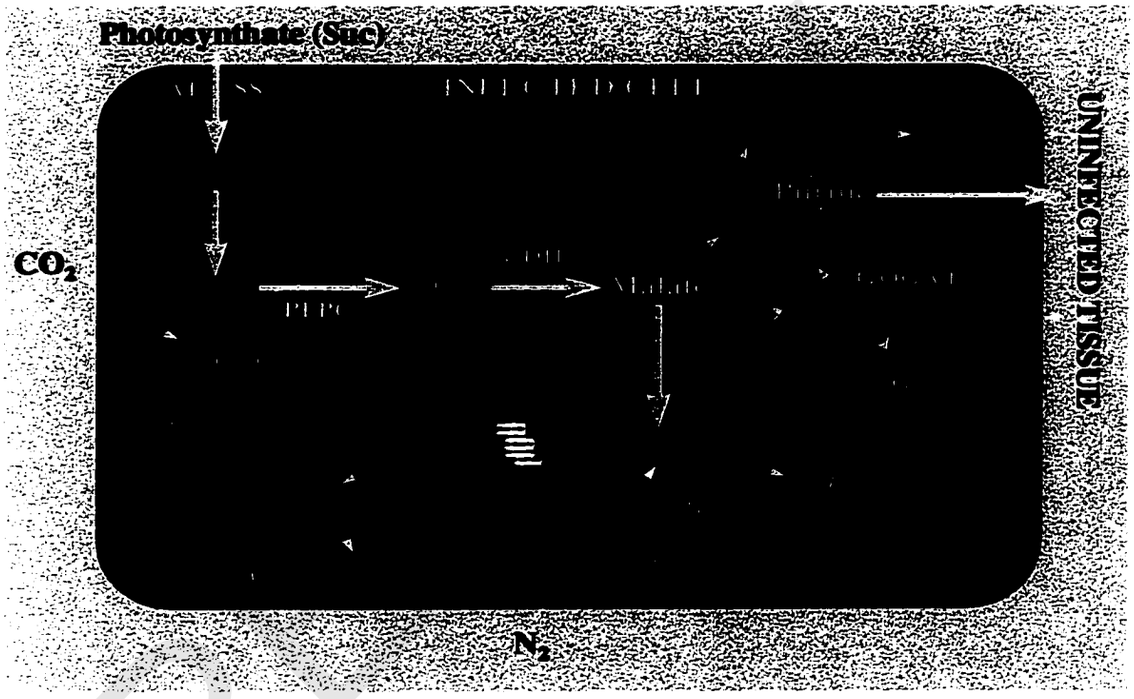
Legumes are able to form a symbiotic association with *Rhizobia* bacteria. The establishment and function of this partnership involve a series of complex biochemical and genetic adaptations by both the host plants and bacteria (Schubert, 1986; Mylona *et al.*, 1995; Geurts and Franssen, 1996). The interaction between the two symbiotic partners starts with recognition and signal exchange during which bacterium-encoded Nod factors play a key role. Legume root hairs deform and curl tightly once the *Rhizobia* have attached. The bacteria subsequently invade the plant by a newly formed infection thread. In the meantime, the nodule primordium is formed by mitosis of reactivated root cortical cells. When the infection thread reaches the primordium, the bacteria are released into the cytosol of the primordial cells, and are subsequently enclosed in the symbiosome membrane (SM) (earlier called the peribacteroid membrane) derived from the host plasma membrane. The bacteria further differentiate into their endosymbiotic form, the bacteroid, and synthesize the nitrogenase complex while the primordium develops into a nodule.

Nodulins, encoded by nodule-specific or nodule-enhanced plant genes, play essential roles in nodule development and function. The early nodulins (*e.g.*, the proline-rich proteins ENOD5 and ENOD12) are expressed before the initiation of dinitrogen fixation and are involved in infection and nodule development. The late nodulins (*e.g.*, the SM-associated water-channel protein nodulin-26 and "cytosolic" nodulin-100) are involved in signaling and metabolite exchange between the bacteroid and infected cortical cell, and in the C/N metabolism of the nodule.

This symbiosis depends on the exchange of C- and N-containing compounds

between the host plant and the bacteroid (Schubert, 1986; Mellor and Werner, 1990; Day and Copeland, 1991; Streeter, 1991; Vance and Heichel, 1991; Vance and Gantt, 1992; Werner, 1992; Vance et al., 1994; Udvardi and Day, 1997) (Fig. 1). The host plant supplies the symbiotic bacteroid with the requisite carbon sources, derived mainly from shoot photosynthesis, to fuel respiration and, thus, to produce ATP and reductant which are required for the overall nitrogenase reaction. Photosynthate, mostly as sucrose (Suc) (see Mellor and Werner, 1990 for review), is transported from the source leaves and stems to the root nodules through the phloem. In the cytosol of nodule cells, two plant-encoded enzymes, sucrose synthase (SS or nodulin-100) and alkaline invertase (AI), are capable of hydrolyzing Suc. Physiologically, it is believed that SS plays the most critical role in Suc cleavage in mature soybean nodules given that its activity is well correlated with nitrogenase activity, whereas that of AI is not (Gordon *et al.*, 1997). The UDP-Glc from the SS-catalyzed cleavage reaction (Suc + UDP $\rightleftharpoons$ Fru + UDP-Glc) is converted to Glc-1-P by UDP-Glc pyrophosphorylase. The resulting Fru and Glc-1-P enter glycolysis to form phospho*enol*pyruvate (PEP). Most, if not all, of this glycolytic PEP is carboxylated to form oxaloacetate (OAA), and a small fraction, if any, is converted to pyruvate by pyruvate kinase for consumption by mitochondria. At this metabolic branch-point, PEP carboxylase (PEPC) plays a key role in catalyzing the carboxylation of PEP. The resulting OAA is readily converted to malate by NADH-malate dehydrogenase (MDH). Several lines of evidence indicate that malate is critical for nodule C-metabolism. Firstly, C_4-dicarboxylic acids (*e.g.*, malate, succinate) are the major carbon compounds

Figure 1. A simplified scheme for primary C/N metabolism in legume root nodules



transported across the SM from the plant cytosol and utilized by the enclosed bacteroids to obtain C, ATP and reducing power (see Udvardi and Day, 1997). Secondly, malate can be transported into nodule mitochondria via the malate/OAA carrier and there enter the citric acid (TCA) cycle to produce ATP and other carboxylic acids (*e.g.*, OAA, succinate, 2-oxoglutarate). ATP is needed for NH_3 assimilation via cytosolic glutamine synthetase (GS). The OAA and 2-oxoglutarate can provide carbon skeletons for NH_3 assimilation. Finally, malate can also enter the plastid directly (see Day and Copeland, 1991) and provide both OAA and NADPH via NADP-malate dehydrogenase for purine (i.e., ureide) synthesis (Fig. 1).

In the bacteroids, malic enzyme (ME), together with MDH, provide both pyruvate and OAA for operation of the citric acid cycle and, thus, supply reducing power and ATP for nitrogenase. Ammonium, the initial product of dinitrogen fixation catalyzed by nitrogenase, is exported to the host cytosol and assimilated initially into Gln by the reaction catalyzed by GS. Gln is then transported into the plastids for net incorporation into Glu via NADH-glutamate synthase (GOGAT). Some Glu is recycled back to the cytosol to support the continued operation of GS. The remaining Glu transfers its amino-N to OAA to form Asp via aspartate aminotransferase (AAT). The resulting 2-oxoglutarate is recycled to maintain the continued operation of GOGAT, and Asp is used for Asn synthesis via the Gln-dependent amidation of Asp catalyzed by asparagine synthetase (AS).

In indeterminate nodules, such as those from alfalfa and peas, amides, principally Asn, are exported to the shoots. In determinate nodules, such as those from soybean and

Phaseolus beans, Asp and Glu are further used for purine synthesis in the plastids (and in mitochondria, see Atkins *et al.*, 1997). Purines are exported from the plastid of the infected cell to the uninfected cell and there used for the synthesis of ureides (allantoin and allantoic acid). It is believed that xanthine dehydrogenase, uricase (nodulin-35) and allantoinase are key enzymes for purine oxidation in the uninfected cells.

Protein and Gene Structure of Plant PEPC

PEPC (EC 4.1.1.31) is a ubiquitous cytosolic enzyme in plants, bacteria, cyanobacteria and green algae (Chollet *et al.*, 1996). In higher plants, in addition to its aforementioned critical functions in legume nodule C-metabolism, the enzyme plays various other important roles in a variety of physiological contexts. Of these, the best-known and most extensively studied is its critical role in photosynthetic CO_2 fixation by C_4 and CAM plants (Jiao and Chollet, 1991; Nimmo, 1993; Chollet *et al.*, 1996).

PEPC is a homotetramer with a monomeric molecular mass of about 110 kD in most cases (O'Leary, 1982; Andreo *et al.*, 1987; Jiao and Chollet, 1991; Chollet et al., 1996). However, a recent report indicated that PEPC in banana fruits is perhaps an $\alpha_2\beta_2$ heterotetramer (Law and Plaxton, 1995). The α subunit has a molecular mass of about 103 kD while that of the β subunit is about 100 kD.

PEPC cDNAs have been isolated from numerous microbial and plant tissues (Lepiniec *et al.*, 1994; Rajagopalan *et al.*, 1994). There are several highly conserved motifs and residues in all the deduced amino acid sequences (see Chollet *et al.*, 1996). These domains/residues are likely involved in the active and/or regulatory sites of the enzyme. It is believed that most of the active-site determinants are located within the C-