

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

**A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600**

PREVIEW

**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

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

Lincoln, Nebraska

December, 1997

UMI Number: 9815914

UMI Microform 9815914
Copyright 1998, by UMI Company. All rights reserved.

**This microform edition is protected against unauthorized
copying under Title 17, United States Code.**

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

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

SUPERVISORY COMMITTEE:

APPROVED

DATE

Signature	<u><i>[Signature]</i></u>	<u>12/12/97</u>
Typed Name	Raymond Chollet	
Signature	<u><i>[Signature]</i></u>	<u>12/12/97</u>
Typed Name	Robert Klucas	
Signature	<u><i>[Signature]</i></u>	<u>12/12/97</u>
Typed Name	John Marwell	
Signature	<u><i>[Signature]</i></u>	<u>12-12-97</u>
Typed Name	Gautam Sarath	
Signature	<u><i>[Signature]</i></u>	<u>12/12/97</u>
Typed Name	Paul Staswick	
Signature	<u> </u>	<u> </u>
Typed Name	<u> </u>	



GRADUATE COLLEGE
UNIVERSITY OF NEBRASKA

**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***

Xiu-Qing Zhang, Ph.D.

University of Nebraska, 1997

Adviser: Raymond Chollet

Phosphoenolpyruvate 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.

PREVIEW

ACKNOWLEDGMENTS

I am greatly grateful to my adviser, Dr. Raymond Chollet, for his guidance, help and inspiration during the period of my Ph.D. training and research at the University of Nebraska-Lincoln. I would also like to thank Drs. Robert Klucas, John Markwell, Gautam Sarath and Paul Staswick for serving on my Supervisory Committee, their help and many suggestions.

I appreciate Ms. Shirley Condon's help and excellent technical assistance in the lab. Special thanks are due to Drs. Bin Li and Gautam Sarath for their help, suggestions and discussions. I wish to warmly acknowledge Drs. Karen Koch at the University of Florida, Daniel Roberts at the University of Tennessee, Raul Arridondo-Peter (a former postdoc in Dr. Klucas' lab, currently at the Universidad Autonoma de Puebla, Mexico), Desh Pal Verma at the Ohio State University, and Jean Vidal at the Université de Paris-Sud (Orsay, France) for providing me with the anti-maize sucrose synthase antibodies, purified soybean symbiosome membranes, a soybean nodule cDNA library, a partial soybean nodule sucrose synthase sequence, and the antibodies specific to the N-terminal domain of sorghum C₄ PEPC, respectively. Thanks to Drs. Stephen Duff, Gururaj Maralihalli, Shameekumar Patil, Lucy Smith and other colleagues in the lab for discussion. It is always a pleasure to remember friends who shared the moments of frustration and joy in research with me during these years.

To my wife, Jing, my son, David: thank you for a million things, but mostly for your caring and understanding. To my parents, sisters and brother: thank you for your support and patience.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	i
TABLE OF CONTENTS	ii
LIST OF FIGURES	vi
LIST OF TABLES	viii
LIST OF ABBREVIATIONS	ix
CHAPTER 1: Introduction	1
General Outline of Legume Nodule C/N-Metabolism	2
Protein and Gene Structure of Plant PEPC	7
Regulatory Phosphorylation of Plant PEPC	11
Sucrose Synthase: Protein and Gene Structure, and Regulation	14
Literature Cited	18
CHAPTER 2: <i>In Vivo</i> Regulatory Phosphorylation of Soybean-Nodule Phosphoenolpyruvate Carboxylase	28
Abstract	29
Materials and Methods	32
Reagents	32
Plant Material	32
Purification of Soybean Nodule PEPC	33
Antibody Production and Purification	35
<i>In Situ</i> ³² P-Labeling and Immunoprecipitation	36
Phosphoamino Acid Analysis	37
Assay of PEPC Activity and its Inhibitor by L-Malate	37
SDS-PAGE and Immunoblotting	38

Protein Assay	38
Results and Discussion	38
Purification of Soybean Nodule PEPC	38
Effects of Darkness and Stem-Girdling Pretreatments on the Malate Sensitivity and Specific Activity of Nodule PEPC	39
Effects of Extended Darkness, Stem-Girdling and Illumination Pretreatments on the Malate Inhibition, Specific Activity and Amount of Nodule PEPC	48
<i>In Situ</i> ³² P-Labeling of Soybean Nodule PEPC	50
Concluding Remarks	56
Literature Cited	58
CHAPTER 3: Phosphoenolpyruvate Carboxylase Protein Kinase from Soybean Root Nodules: Partial Purification, Characterization, and Up/Down-Regulation by Photosynthate Supply from the Shoots	64
Abstract	65
Materials and Methods	68
Materials	68
Plant Material	68
Preparation of Crude and Enriched PEPC-Kinase Samples	69
Partial Purification of Nodule PEPC-Kinase	70
Purification of Plant PEPCs	71
Enzyme Assays	71
<i>In-Vitro</i> Phosphorylation of Nodule PEPC by the Partially Purified Nodule PEPC-Kinase	72
<i>In-Vitro</i> Phosphorylation of Nodulin-26	73
Protein Determination	73

Results and Discussion	73
Partial Purification and Characterization of PEPC-Kinase from Soybean Root Nodules	73
Effects of <i>In-Vitro</i> Phosphorylation of Nodule PEPC by the Partially Purified Nodule PEPC-Kinase	85
Effects of Prolonged Darkness, Stem Girdling, and Illumination Pretreatments of Soybean Plants on Nodule PEPC-Kinase Activity	90
Concluding Remarks	99
Literature Cited	102
CHAPTER 4: Seryl-Phosphorylation of Soybean Nodule Sucrose Synthase (Nodulin-100) by a Ca²⁺-Dependent Protein Kinase	107
Abstract	108
Materials and Methods	110
Plant Material	110
Purification of Soybean Nodule SS and PEPC	110
<i>In-Situ</i> ³² P-Labeling, Immunoprecipitation, and Phosphoamino Acid Analysis of Nodule SS	111
Partial Purification of Nodule SS-Kinase and PEPC-Kinase	111
Enzyme Assays	113
Results and Discussion	114
Soybean Nodule SS is Phosphorylated <i>In Situ</i> on a Serine Residue(s).....	114
Characterization of Nodule SS-Kinase	114
Concluding Remarks	125
Literature Cited	127

CHAPTER 5: Cloning and Overexpression of a cDNA Encoding Sucrose Synthase (Nodulin-100) from Soybean Root Nodules in <i>Escherichia coli</i>	131
Abstract	132
Experimental Procedures	134
Isolation of cDNA Clones	134
Construction of the Plasmid for Expression of SS cDNA in <i>E. coli</i>	135
DNA Sequencing	136
Overexpression of the SS cDNA in <i>E. coli</i>	139
Purification of Soybean Nodule and Recombinant SS	139
SS Assay and Protein Determination	141
SDS-PAGE and Western Blotting	142
<i>In Vitro</i> Phosphorylation of SS	142
Results and Discussion	142
Isolation and Characterization of SS cDNA Clones	142
Expression of the SS cDNA Clone in <i>E. coli</i>	146
Recombinant SS is Phosphorylated by Nodule CDPK <i>In Vitro</i>	156
Concluding Remarks	159
Literature Cited	160
CHAPTER 6: Concluding Remarks	163
Literature Cited	168

LIST OF FIGURES

Chapter 1

1. A simplified scheme for primary C/N metabolism in legume root nodules. 4
2. Limited comparison of the plant-specific, N-terminal phosphorylation domain of PEPC 9

Chapter 2

1. FPLC-based purification of soybean nodule PEPC 41
2. Immunoblot analysis of soybean nodule PEPC from plants subjected to various pretreatments 43
3. L-malate sensitivity and suboptimal activity of soybean nodule PEPC extracted from plants subjected to various pretreatments 46
4. Immunoprecipitation of PEPC from extracts of soybean nodules labeled with ^{32}Pi *in situ* 51
5. Two-dimensional thin-layer electrophoretic analysis of phosphoamino acids 54

Chapter 3

1. Partial purification of soybean nodule PEPC-kinase by sequential FPLC on phenyl Sepharose, BDA, and Superdex 75 columns 75
2. Ca^{2+} -independence of the activity of soybean nodule PEPC-kinase. 78
3. Phosphorylation of various plant PEPCs by the partially purified soybean nodule PEPC-kinase 80
4. Inability of soybean nodule PEPC-kinase to phosphorylate nodulin-26 83
5. In-gel assay of soybean nodule PEPC-kinase activity 86
6. Time course of changes in specific activity and I_{50} (L-malate) of purified soybean nodule PEPC phosphorylated *in vitro* by the partially purified nodule PEPC-kinase preparation 88
7. Effects of stem girdling, extended darkness, and illumination pretreatments of soybean plants on nodule PEPC-kinase activity 91
8. Reversible effects of light and dark pretreatments of soybean plants on nodule PEPC-

kinase activity	95
9. Comparison of renaturable PEPC-kinase activity in nodules from illuminated and stem-girdled soybean plants	97

Chapter 4

1. <i>In-situ</i> ³² P-labeling and phosphoamino acid analysis of soybean nodule SS	115
2. A soluble, Ca ²⁺ -dependent protein kinase is involved in the phosphorylation of nodule SS, and this kinase activity is not affected by stem-girdling pretreatment of soybean plants	117
3. <i>In-vitro</i> phosphorylation of nodule SS by the partially purified SS-kinase, and detection of autophosphorylation by this purified nodule CDPK by an in-gel assay	120
4. Ability of the partially purified nodule SS-kinase to phosphorylate nodule PEPC, and inability of partially purified nodule PEPC-kinase to phosphorylate nodule SS	123

Chapter 5

1. Subcloning of the full-length soybean nodule SS cDNA into <i>E. coli</i> expression vectors, pET 11a and pET 28a	137
2. Nucleotide and deduced amino acid sequences of soybean nodule SS (accession no. AF030231)	143
3. Comparison of the N-terminal amino acid sequences of various plant SSs	147
4. SDS-PAGE analysis of recombinant soybean nodule SS in <i>E. coli</i>	150
5. Western blot analysis of soybean nodule SS expressed in <i>E. coli</i>	152
6. SDS-PAGE analysis of purified soybean nodule, recombinant His-tagged and untagged SS	154
7. <i>In-vitro</i> phosphorylation of purified soybean nodule and recombinant SS by the partially purified nodule CDPK (SS-kinase)	157

Chapter 6

1. Proposed organization of the signaling elements involved in the PEPC phosphorylation cascade in C ₄ , C ₃ and CAM leaves, and legume root nodules	165
--	-----

LIST OF TABLES**Chapter 2**

1. Purification of PEPC from soybean root nodules 40
2. *In vivo* effects of light, extended darkness and stem girdling on the L-malate sensitivity and activity of soybean nodule PEPC 49

Chapter 3

1. Effects of stem girdling, prolonged darkness, and light on the L-malate sensitivity and suboptimal activity of soybean nodule PEPC 94

PREVIEW

LIST OF ABBREVIATIONS

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	<i>trans</i> -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 <i>enol</i> pyruvate

PEPC	PEP carboxylase
PEPC-kinase	PEPC protein-Ser/Thr kinase
PMSF	phenylmethanesulfonyl fluoride
PVP	polyvinylpyrrolidone
SS	sucrose synthase
Suc	sucrose
TLE	thin-layer electrophoresis
W-7	<i>N</i>-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

CHAPTER 1

Introduction

PREVIEW

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.

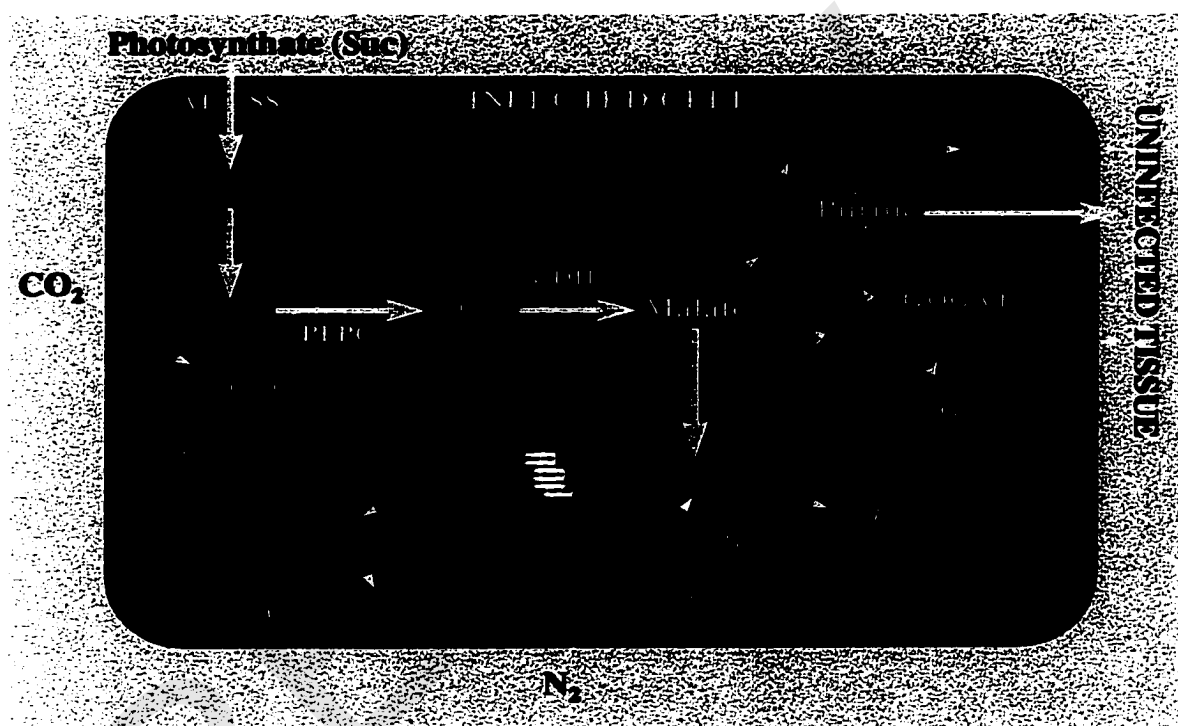
Nodulins, encoded by nodule-specific or nodule-enhanced plant genes, play essential roles in nodule development and function. The early nodulins (*e.g.*, the proline-rich proteins ENOD5 and ENOD12) are expressed before the initiation of dinitrogen fixation and are involved in infection and nodule development. 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 ($\text{Suc} + \text{UDP} \rightleftharpoons \text{Fru} + \text{UDP-Glc}$) is converted to Glc-1-P by UDP-Glc pyrophosphorylase. The resulting Fru and Glc-1-P enter glycolysis to form phosphoenolpyruvate (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, C₄-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

PREVIEW



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 CO₂ fixation by C₄ 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-