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Molecular, cellular, and developmental study of biotin-containing enzymes

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Molecular, cellular, and developmental study of biotin-containing enzymes

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ABBREVIATIONS

ACCase	acetyl-CoA carboxylase
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BCCP	biotin carboxy carrier protein
BSA	bovine serum albumin
bp	base pair
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E-64	L-*trans*-epoxysuccinyl leucylamide(4-guanidino)-butane
EDTA	ethylenediaminetetraacetic acid
GCCase	geranyl-CoA carboxylase
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IPTG	isopropyl-b-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
MCCase	b-methylcrotonyl-CoA carboxylase
MES	2-[N-morpholino]-ethanesulfonic acid
MOPS	3-[N-morpholino]-propanesulfonic acid
NBRF	National Biomedical Research Foundation
PAGE	polyacrylamide gel electrophoresis
PCase	pyruvate carboxylase

PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
PCCase	propionyl-CoA carboxylase
RNAase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylene diamine
Tris-HCl	tris[hydroxymethyl]aminomethane hydrochloride
UV	ultraviolet light

GENERAL INTRODUCTION

Overview

Biotin, a water soluble vitamin also known as vitamin H, is involved in the variety of metabolic and catabolic reactions catalyzed by biotin-containing enzymes in cells. Genetic studies have shown that mutations affecting biotin synthesis and utilization are lethal in bacteria (Eisenberg, 1987), yeast (Mishina et al., 1980), human (Wolf and Heard, 1989), and plants (Shellhammer and Meinke, 1990). These studies confirm the essential biological function of biotin. Due to its importance in biology, the biosynthesis of biotin and the regulation and functions of biotin-containing enzymes have been investigated vigorously in animal and microbial systems in the last 50 years. The central role of biotin in lipogenesis, gluconeogenesis, and the catabolism of branched-chain amino acids in animals and bacteria has been established (Moss and Lane, 1971; Wood and Kumar, 1985; Dakshinamurti and Bhagavan, 1985; Knoles, 1989; Mildvan et al., 1991). By comparison, until recently the knowledge of plant biotin-containing enzymes was meager. Indeed, until 1990 the only biotin-containing enzyme that was known in plants was acetyl-CoA carboxylase (ACCase) (Wurtele and Nikolau, 1990). However, there were indications that additional biotin-containing polypeptides occur in plants (Nikolau et al., 1987; 1984a; 1984b; 1985; Wurtele and Nikolau, 1990; Kannangara and Jensen, 1975; Hoffman et al., 1987). In 1990, 3-methylcrotonyl-CoA carboxylase (MCCase), propionyl-CoA carboxylase (PCCase), and pyruvate carboxylase (PCase) activities were detected in extracts from plant tissues (Wurtele and Nikolau, 1990). Since then, plant MCCase has been purified, and extensively characterized (Chen et al., 1993; Albert et al., 1993; Wang et al., 1994; Song et al., 1994; Diez et al., 1994). The genes and cDNAs for these enzymes have been cloned (Wang et al., 1994; Song et al., 1994). The study presented here has focused on the biological and metabolic functions of biotin-containing

enzymes. In the first section of my thesis, the specific goal is to develop a new approach to study the regulation of the biotin-containing enzymes by depleting the level of biotin in cells to study the regulation of biotinylation and biotin biosynthesis in plant. The second section of my thesis is the characterization of a novel plant biotin enzyme, geranyl-CoA carboxylase (GCCase).

The Biotin Prosthetic Group

Biotin was first isolated from egg yolk in 1936 by Kögl and Tönnis (1936). Its structure was determined in 1942 (Du Vigeaud et al., 1942) and was later verified by X-ray crystallography (Traub, 1956). As shown in Fig. 1, biotin is a bicyclic compound with fused ureido (imidazolidone) and thiophene, and an aliphatic carboxylic acid side chain. The function of the biotin prosthetic group in biotin enzymes is to act as an intermediate-carrier of a carboxyl group. The carboxylation site on biotin is at the N-1 position (Lynen et al., 1961). Biotin is covalently attached to enzymes through an amide linkage between the carboxyl group of the side chain of biotin and the epsilon-amino group of a lysine residue, forming biocytin (biotinyl-lysine).

Biotin-containing Enzymes

Biotin-containing enzymes participate in a number of carboxylation, decarboxylation and transcarboxylation reactions (Moss et al., 1971, Samols et al., 1988). Thus, Biotin-containing enzymes can be classified into three groups: carboxylases, decarboxylases and transcarboxylases (Moss et al., 1971). To date, ten biotin enzymes have been identified (Table I). In the carboxylase category, these are ACCase, MCCase, PCCase, PCase , GCCase, and urea carboxylase. In the decarboxylase category, they are oxaloacetate decarboxylase,

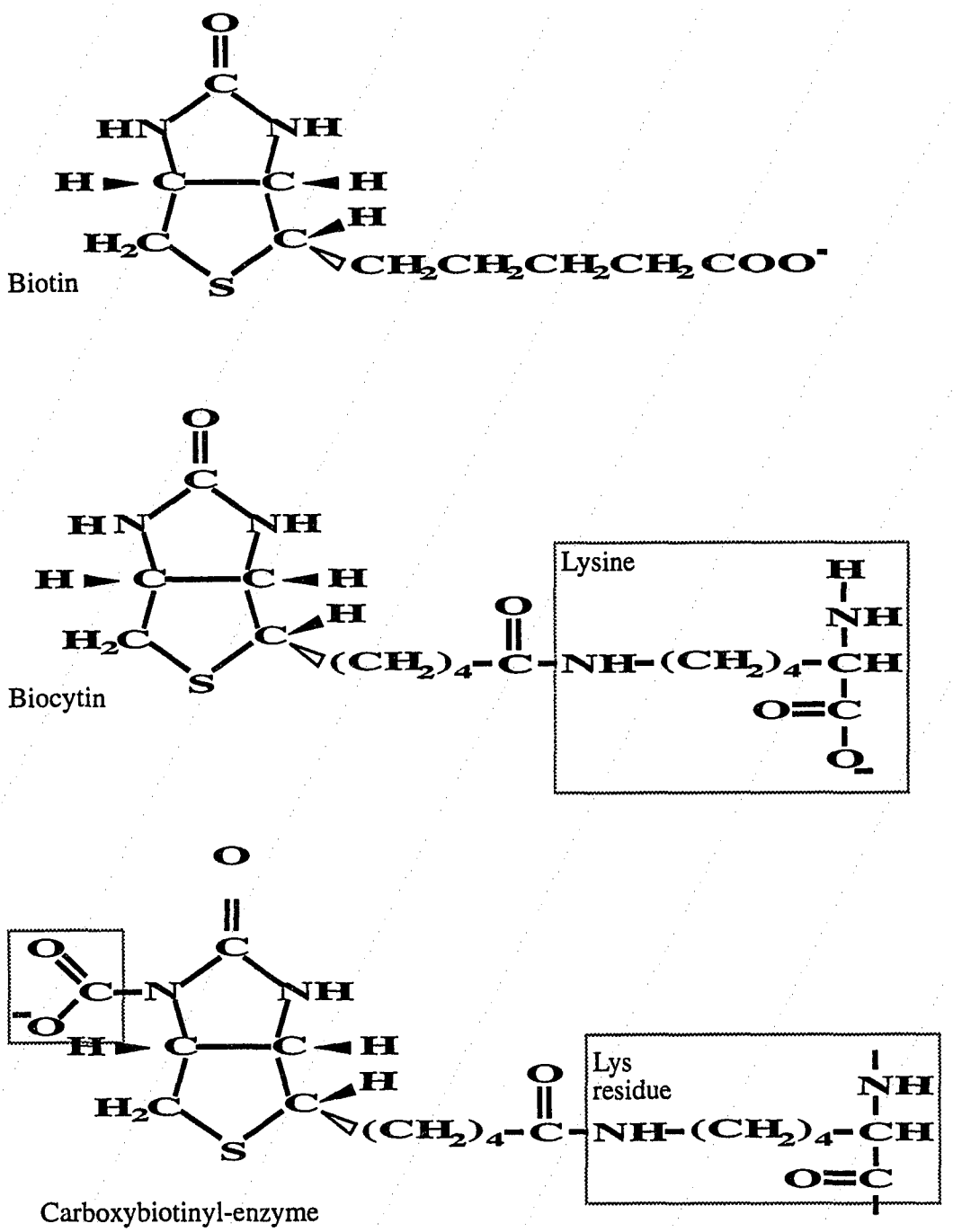

Figure 1. Biotin, biocytin and carboxybiotin-enzyme.
Biotin, Mr = 244.3; biocytin, Mr = 372.5.

Table I. Biotin-containing Enzymes

Enzyme	Substrate	Product
Acetyl-CoA carboxylase	Acetyl-CoA	Malonyl-CoA
Propionyl-CoA carboxylase	Propionyl-CoA	Methylmalonyl-CoA
Pyruvate carboxylase	Pyruvate	Oxaloacetate
3-Methylcrotonyl-CoA carboxylase	3-Methylcrotonyl-CoA	3-Methylglutaconyl-CoA
Geranyl-CoA carboxylase	Z-Geranyl-CoA	Isohexenylglutaryl-CoA
Urea carboxylase	Urea	N-carboxyurea
Methylglutaconyl-CoA decarboxylase	Methylglutaconyl-CoA	Propionyl-CoA
Oxaloacetate decarboxylase	Oxaloacetate	Pyruvate
Glutaconyl-CoA decarboxylase	Glutaconyl-CoA	Crotonyl-CoA
Transcarboxylase	Methylmalonyl-CoA	Pyruvate

methylmalonyl-CoA decarboxylase, and glutaconyl-CoA decarboxylase. The transcarboxylase category has only one member which is transcarboxylase. The reactions catalyzed by these enzymes are involved in diverse metabolic processes. These biochemical pathways are fundamental to life, and include lipogenesis, gluconeogenesis, and the catabolism of branched-chain amino acids. Although each enzyme has distinct metabolic function(s), in every instance the catalytic reaction mechanism can be explained by the role of biotin in these reactions. The role of biotin is to act as an intermediate-carrier of a carboxyl group as it is being transferred from one substrate to another (Moss and Lane, 1971). The overall reactions catalyzed by biotin enzymes are carried out in two steps. The first step involves the carboxylation of biotin on the enzyme. In the second step, carboxyl group is transferred from carboxybiotinyl enzyme intermediate to an appropriate substrate which serves as an acceptor (Knowles, 1989). In my study, I have focused on the enzymes of the carboxylase group.

Biotin-dependent Carboxylases

There are six known biotin-dependent carboxylases. Their substrates and products are shown in Table I. ACCase is found in all species. MCCase is known to be present in animals, fungi, some bacteria, and plants (Moss and Lane, 1971; Knowles, 1989; Albert et al., 1993; Wang et al., 1994; Song et al., 1994). PCase and PCCase have been characterized from animal and microbial sources (Knowles, 1989). Urea carboxylase is found in yeast and unicellular green algae (Moss and Lane, 1971). GCCase has been found only in a few bacteria including *Pseudomaonas citronellolis*, *P. mendocina*, *P. aeruginosa*, and *Acinetobacter* (Samols et al., 1988).

All known biotin-dependent carboxylases catalyze identical first step reactions (Moss and Lane, 1971), as shown in Reaction [1]. The carboxyl group is donated by bicarbonate, and a new carbon-nitrogen bond is formed between biotin and CO_2 at the cost of hydrolysis of

ATP. The carboxyl group is subsequently transferred from the carboxybiotinyl intermediate to an acceptor, as shown in Reaction [2]. The overall reaction is given as Reaction [3]:

$$\text{ENZ-BIOTIN} + HCO_3^- + \text{ATP} \Longleftrightarrow \text{ENZ-BIOTIN-}CO_2^- + \text{ADP} + \text{Pi} \quad [1]$$

$$\text{ENZ-BIOTIN-}CO_2^- + \text{ACCEPTOR} \Longleftrightarrow \text{ENZ-BIOTIN} + \text{ACCEPTOR-}CO_2^- \quad [2]$$

$$HCO_3^- + \text{ATP} + \text{ACCEPTOR} \Longleftrightarrow \text{ADP} + \text{Pi} + \text{ACCEPTOR-}CO_2^- \quad [3]$$

The final acceptor of the carboxyl group is dependent on the specific enzyme involved.

Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA. This reaction is the first and rate-limiting reaction of fatty acid biosynthesis in bacteria, yeast, animals and plants. Mammalian ACCase is allosterically activated by citrate and isocitrate. In additional, long-chain fatty acyl-CoA derivatives are inhibitors of ACCases. Activation is associated with polymerization of the promoter, while inactivation is the result of disaggregation of the active polymer. cDNAs and genes coding for ACCase have been cloned from rat (Lopez-Casillas et al., 1988), chicken (Takai et al., 1987; 1988), yeast (Al-Fell et al., 1992) and *E. coli* (Li and Cronan, 1992a; 1992b; Kondo et al., 1991).

Pyruvate carboxylase (PCase; EC 6.4.1.1) catalyzes the carboxylation of pyruvate to form oxaloacetate. It is involved in gluconeogenesis, lipid biosynthesis, and an anaplerotic reaction for the tricarboxylic acid cycle. PCases from animal and yeast are allosterically activated by acetyl-CoA. The amino acid sequences of PCase from rat (Lim et al., 1988), human (Freytag and Collier, 1984) and mouse (Zhang et al., 1993) have been determined.

Propionyl-CoA carboxylase (PCCase; EC 6.4.1.3) catalyzes the carboxylation of propionyl-CoA to form methylmalonyl-CoA. In animals and bacteria, the methylmalonyl-CoA is then converted to succinyl-CoA, by vitamin B_{12}-dependent isomerase. The succinyl-CoA can be further oxidized through the tricarboxylic acid cycle. PCCase is involved in the catabolism of certain amino acids (isoleucine, valine, methione and threonine) and also the catabolism of odd-chain fatty acids. The amino acid sequences of PCCase from rat (Browner

et al., 1989; Kraus et al., 1986) and human (Lamhonwah et al., 1986; 1987) have been elucidated from their cDNA sequences.

ß-Methylcrotonyl-CoA carboxylase (MCCase; EC 6.4.1.4) catalyzes the carboxylation of ß-methylcrotonyl-CoA to form ß-methylglutaconyl-CoA. A major source of ß-methylcrotonyl-CoA is the degradation of the amino acid leucine. The ß-methylglutaconyl-CoA may be further oxidized to form acetyl-CoA and eventually may be released as CO_2 in the tricarboxylic acid cycle or may be incorporated into other molecules. MCCase is also an enzyme of the mevalonate shunt, a pathway that recycles carbon from mevalonic acid into acetyl-CoA (Edmond and Popjak, 1974). It may also be important for isoprenoid degradation (Cantwell et al., 1978). Genes and cDNAs coding for MCCase have been cloned from plants (Wang et al., 1994, Song et al., 1994).

Geranyl-CoA carboxylase (GCCase; EC 6.4.1.5) catalyzes the carboxylation of geranyl-CoA to form isohexenylglutaryl-CoA. It plays a central role in the degradation of isoprenoid compounds by certain bacteria (Seubert and Remberger, 1963). It has been found in *Pseudomonas citronellolis* (Seubert et al., 1963), *P. aeruginosa, P. mendocina* and *Acinetobacter species* (Cantwell et al., 1978). In *P. citronellolis*, the enzyme can be induced by citronellol (Cantwell et al., 1978). In bacteria, GCCase is composed of biotin-containing subunits of 75 kD and non-biotin containing subunits of 63 kD. The native molecular weight of the holoenzyme is about 550 kD. GCCase activity can also be detected in rat liver extracts (Diez and Nikolau, unpublised data).

Urea carboxylase (UCase) catalyzes the carboxylation of urea to form allophanate. It has been found in certain yeast and unicellular green algae when urea is their sole nitrogen source (Roon and Levenberg, 1969; Domnas, 1962). The gene coding for UCase from yeast was recently isolated (Genbauffe and Cooper, 1991).

E. coli Biotin-containing Enzyme

In *E. coli*, there is only one biotin-containing enzyme, ACCase. ACCase from *E .coli* has three functional components:

1. Biotin carboxyl-carrier protein (BCCP), on which the biotin prosthetic group is covalently bound to a lysine residue.

2. Biotin carboxylase, which catalyzes the ATP-dependent carboxylation of the BCCP-bound biotin in the first half-reaction.

3. Carboxyltransferase, which transfers the carboxyl group from the carboxylated biotin to acetyl-CoA to form malonyl-CoA in the second half-reaction.

All the three components are required for the overall reaction (Alberts et al., 1968; 1969; 1971; Guchhait et al., 1974).

The regulation of fatty acid biosynthesis by ACCase has been studied in *E. coli*. In this organism, fatty acids are mainly utilized for the synthesis of membrane lipids (Cronan and Rock, 1987). Therefore, ACCase activity and consequence fatty acid biosynthesis, are closely coupled to the growth rate of the *E. coli* cells. This regulation is thought to be mediated by the product of the relaxed *(rel)* locus, the nucleotide ppGpp, the concentration of which increases when cell growth decreases (Lazzarini et al., 1971; Golden and Powell, 1972; Polakis et al., 1973; Sokawa et al., 1968; Cashel and Gallant, 1969). One effect of increasing the concentration of ppGpp is to allosterically inhibit the carboxyltransferase component of ACCase and thus decrease fatty acid biosynthesis (Polakis et al., 1973). cDNAs and genes coding for ACCase have been cloned from *E. coli* (Li and Cronan, 1992a; 1992b; Kondo et al., 1991).

Plant Biotin-containing Enzymes

Until recently, the best characterized biotin-containing enzyme in plants was ACCase. One type of plant ACCase is composed of two subunits of 220 kD each (Egin-Buhler and Ebel, 1983; Slabas and Hellyer, 1985; Charles and Cherry, 1986; Hellyer et al., 1986; Slabas et al., 1986; Egli et al., 1992, 1993). This type of ACCase is thought to be cytosolic in dicots, but may be plastidic in monocots. A distinct ACCase with a biotin-containing subunit of 60 kD was reported from maize (Nikolau and Hawke, 1984), soybean (Nikolau et al., 1993) and somatic carrot embryo and is thought to be plastidic (Nikolau et al., In press 1994). Plastidic ACCases with biotin-containing subunits of 35 kD have been reported from pea plant (Sasaki et al., 1993). The reports of these three types of ACCases in plants indicate that these organisms may contain isoenzymes of ACCase. In plants, malonyl-CoA is an intermediate in multiple pathways, including the biosynthesis of fatty acids (Stumpf, 1987), very long-chain fatty acids (Kolattukudy et al., 1976; Bessoule et al., 1989), flavonoids (Hahlbrock, 1981), stilbenoids (Gorham, 1980), anthroquinones (Packter, 1980), malonyl derivatives of D-amino acids (Ogawa et al., 1973; Liu et al., 1983), malonation of 1-aminocyclopropane carboxylic acid (Liu et al., 1983; Su et al., 1985) and malonic acid (Stumpf and Burris, 1981). Thus, ACCase isoenzymes may be located in different compartments in the plant cell or expressed at different developmental stages of the plant's life for generating intermediates for these multiple metabolic processes. The genes coding for some of the ACCases have been cloned (Takai et al., 1987, Kondo et al., 1991; Nikolau et al., In press 1994; Sasaki et al., 1989).

MCCase has been recently purified from carrot somatic embryos (Chen et al., 1993), pea leaves and potato tuber (Alban et al., 1993), maize leaves (Diez et al., 1994), tomato (Wang et al., 1994) and soybean (Song et al., 1994). MCCases from maize, carrot, and soybean have native molecular weights of 850 to 970 kD and appear to be in an $\alpha_6\beta_6$ configuration. Carrot somatic embryo MCCase is composed of two non-identical subunits, a

biotin-containing subunit of 78 kD, and a biotin-free subunit of 65 kD (Chen et al., 1993). The activity of MCCase from tomato root is regulated by the biotinylation of the apoenzyme (Wang et al., 1993). The soybean enzyme appears to have a Bi Bi Uni Uni Ping Pong mechanism (Diez, 1994). The potato and pea enzyme native molecular weights have been reported as 500 kD and 530 kD and the authors suggest a $\alpha_4ß_4$ configuration. The genes coding for MCCase have been cloned from tomato (Wang et al., 1994), soybean (Song et al., 1994), and *Arabidopsis* (Caffrey et al., In press 1994).

GCCase was identified in maize recently (Diez, 1994; Caffrey et al., In press 1994), and has been partially purified and characterized from maize leaves. This enzyme has a biotin-containing subunit of 122 kD. The pH optimum for activity is 8.3. The apparent K_m values for the substrates geranyl-CoA, bicarbonate and ATP are 64 ± 5 µM, 0.58 ± 0.04 mM, and 8.4 ± 0.4 µM, respectively.

Streptavidin

Streptavidin is a protein isolated from the culture supernatant of *Streptomyces avidinii.* It was first found during a screening for secreted antibiotics (Chaiet, 1964). Streptavidin has a native molecular weight of 60 kD and consists of four identical subunits, each with an affinity binding site for D-biotin. Just like avidin, the streptavidin holoprotein binds biotin with an extraordinarily high affinity. The dissociation constant for biotin from streptavidin is approximately 10^{-15} M (Green, 1980). The streptavidin gene has been cloned in 1986 from a genomic library of *Streptomyces avidinii.* (Argarana et al., 1986). The first 24 amino acids represent the signal peptide targeting the protein for secretion and are post-translationaly removed to yield the mature protein (Argarana et al., 1986). The molecular weight of the monomer is 17.5 kD. The previously characterized 60 kD tetrameric protein (with a 15 kD monomer) usually is referred to as core streptavidin, and represents a further processed product

(another 7 amino acids are cleaved from the mature protein). Three tryptophan residues are of particular interest: Trp21, Trp79 and Trp120. They have been implicated as the biotin-binding site of the protein and they are protected by biotin from oxidizing agents. Three Lys residues located next to these three Trp residues might be involved in the formation of the configuration required for the binding of biotin (Green, 1975). Recently, using chemical deletion, it was found that even a fragment which only has the first 50 N-terminal residues has the ability to bind biotin (Gitlin et al., 1990). This fragment includes Tyr33, another AA residue essential for biotin binding. Avidin inactivates biotin-containing enzymes *in vitro* (Nikolau et al., 1985; Wurtele and Nilolau, 1990), it is expected that streptavidin has the same ability.

Dissertation Organization

This dissertation contains two research paper manuscripts preceded by a general introduction to the problem addressed with a critical review of the literature, and followed by a general summary and acknowledgments.

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REDUCTION OF THE ACTIVITY OF ACETYL COENZYME A CARBOXYLASE BY EXPRESSION OF A CLONED STREPTAVIDIN GENE IN *ESCHERICHIA COLI*

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SUMMARY

The streptavidin gene from *Streptomyces avidinii,* was expressed in *E. coli* as a non-fusion protein and as a glutathione S-transferase fusion protein. The expression of streptavidin or the streptavidin fusion proteins caused a slight decrease in the growth of these cells. Non-denaturing gel electrophoresis indicated the assembly of the subunits was only partial. The expressed streptavidin protein was able to bind biotin *in vivo*. The growth rate of biotin auxotrophs of *E. coli* growing in biotin defficient media was decreased by the expression of the glutathione S-transferase fusion streptavidin fusion protein. Decreases in growth rate were correlated with decreases in acetyl CoA carboxylase activity.

ABBREVIATIONS

BSA, bovine serum albumin; IPTG, isopropyl-ß-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris-HCl, tris[hydroxymethyl]aminomethane hydrochloride; GST, glutathione S-transferase

INTRODUCTION

Biotin is a cofactor required for catalysis by a variety of enzymes with diverse metabolic functions. Because of the essential role of biotin to life, a depletion or absence of this molecule has profound consequences. The symptoms of biotin deficiency are well established. In animals and yeast, which are unable to synthesize biotin, insufficient biotin in the diet causes major changes in fatty acid profiles, decreases in growth, and death (Bain and Newbrey, 1988; Watkins et. al., 1990; Proud et. al., 1990; Suchy et. al., 1986; Kramer et al., 1984; Mock et al., 1988). In humans, a variety of disorders of biotin metabolism have been reported and characterized. Such disorders lead to a complex set of metabolic consequences, involving multiple biotin-containing enzymes (Wolf and Heard, 1989). Bacteria are normally biotin heterotrophs, however, several *E. coli* biotin auxotrophs have been isolated (Cleary and Campbell, 1972). Plants also synthesize biotin. Recently, a biotin auxotroph of *Arabidopsis*, *bio1*, has been characterized (Schneider et al., 1989). The embryo of this embryo-lethal mutant develops until the available biotin stored in the seed is depleted, and then dies. In plants which are biotin auxotrophs, such as the *bio1* mutant, just as wildtype plant, contain several biotin enzymes (Wurtele and Nikolau, 1990), it is not known to what extent changes in the activity of the various biotin-containing enzymes are the causes of the symptoms of biotin-deficiency.

We are interested in the regulation and metabolic function of biotin-containing enzymes. One approach to study the regulation and metabolic function of biotin enzymes *in vivo* is to control the level of endogenous biotin. This might be accomplished rapidly and efficiently by introducing a transgene coding for a protein which will bind biotin. *E. coli* has a single biotin-containing enzyme, acetyl coenzyme A (CoA) carboxylase. This biotin-dependent carboxylase catalyzes the first committed step of fatty acid synthesis. In *E. coli*, acetyl-CoA carboxylase

consists of three functionally dissimilar subunits, a biotin carboxyl carrier protein (BCCP), a biotin carboxylase and a transcarboxylase (Guchhait et. al., 1974; Polakis et al., 1974). The biotin cofactor of BCCP plays a central role in the binding of CO_2 in the carboxylation reaction (Fall and Vagelos, 1972). Fatty acids synthesized via acetyl-CoA carboxylase in *E. coli* are mainly utilized for the synthesis of membrane lipids (Cronan and Rock, 1987). Therefore, acetyl-CoA carboxylase activity, and consequently fatty acid biosynthesis, are closely coupled to the growth rate of the *E. coli* cells (Cronan and Rock, 1987; Lazzarini et al., 1971; Golden and Powell, 1972; Polakis et al., 1973). Because acetyl-CoA carboxylase is the only biotin-containing enzyme in *E. coli*, as well as because of the simple genetics of this organism, *E. coli* is a good model system in which to study the effects of expressing a protein that binds biotin on biotin-containing enzymes.

Streptavidin, a protein isolated from *Streptomyces avidinii,* has a molecular mass of 60 kDa and is a holoprotein consisting of four subunits, each with an affinity binding site for the water-soluble vitamin D-biotin (Chaiet et al. 1963, 1964; Buckland, 1986). Streptavidin, like avidin, has a strong and specific binding affinity for biotin ($Kd = 10^{-15}$ M) (Green, 1975). Unlike avidin, it does not interact non-specifically with glycoproteins (Finn et al., 1980; Hofmann et al., 1980). Because of this property, it has been used in a variety of biotin detection systems. Since avidin inactivates biotin enzymes *in vitro*, and since streptavidin that is expressed in *E. coli* shows activity *in vitro* (Sano and Cantor, 1990a, 1990b), we decided to determine whether expressed streptavidin would function *in vivo* to bind biotin. If streptavidin binds biotin *in vivo* and decreases the biotin concentration, then the activity of acetyl-CoA carboxylase might be correspondingly decreased, and this change might affect the growth of *E. coli* cells.

In this publication, we demonstrate that an expressed chimeric streptavidin protein functions *in vivo* to decrease the growth rate and the total growth of *E. coli.* The introduction

of a streptavidin transgene may thus provide a useful method to selectively decrease levels of biotin in living cells.

EXPERIMENTAL PROCEDURES

Strains and materials -- *E. coli* strain JM101 (*supE thi Δ(lac-proAB) F' [traD36 proAB$^+$ lacI^q lacZΔM15])* was used for cloning and expression of pGEX-2T and the streptavidin fusion protein pGEX.SZ. R879 (*F'-*, *BioA24*, *IN(rrnD-rrE)1*, *lac-3350*) (Cleary and Campbell, 1972), a biotin synthetase deficient strain of *E. coli*, was kindly provided by *E. coli* stock center, Yale University, and was used for studies of the *in vivo* effects of expression of streptavidin. *E. coli* strain BL21(DE3) (Novagen) was used for studies of the *in vivo* effects of expression of streptavidin with the pET11 vectors. Restriction enzymes and DNA modifying enzymes were purchased from Bethesda Research Laboratories, United States Biochemicals, or Promega-Biotechnology, and were used as suggested by the suppliers. The radioisotopes were purchased from Amersham or ICN Biomedicals. ^{125}I-protein A was a kind gift of Dr. Basil Nikolau, Iowa State University. Biotin and streptavidin antibody were obtained from Sigma Chemical Company. Other biochemical regents were of analytical grade and obtained from Sigma or Fisher Scientific.

Construction of the expression plasmids -- Construction of the expression vectors was carried out using standard techniques (Sambrook et al., 1989). pGEX-2T (Smith and Johnson, 1988) was used to express the streptavidin gene. The streptavidin gene, in pUC8-SZ (Argarana et al., 1986), was a generous gift from Dr. Takeshi Sano and Dr. Charles Cantor (Columbia University). pUC8-SZ was digested with *Sty* I and cloned into the *Sma* I site of $pGEM_4$ by Dr. Neil Hoffman (Stanford University). The plasmid thus generated is referred to

as $pGEM_4SZ$. Subsequently, we digested $pGEM_4SZ$ with *BamH* I and *EcoR* I, and the fragment containing the streptavidin gene was recovered by using Geneclean and cloned into pGEX-2T which had been previously digested with *BamH* I and *EcoR* I. The generated plasmid, pGEX.SZ, contains 384 bp of the streptavidin coding sequence (from bases 134 to 518 in the sequence reported by Agarana et al., 1986) which has been fused at its 5' end with the glutathione S-transferase (GST) gene and is under the control of a *tac* promoter (Smith and Johnson, 1988). The polypeptide coded by pGEX.SZ contains the GST protein fused at its C-terminus to the streptavidin polypeptide. The portion of the streptavidin polypeptide expressed by using pGEX.SZ contains 11 additional amino acids at the N-terminus and is missing 27 aa from the C-terminal end, compared to the streptavidin polypeptide expressed by Sano and Cantor (1990b).

We constructed a non-fusion expression vector, pET.SZ, by a triple ligation of the following: 1) the streptavidin gene insert of about 500 bp obtained from pUC8-SZ digested by *Bgl* I and *BamH* I; 2) pET11d digested with *Nco* I and *BamH* I; 3) an adapter (5'-C ATG CAA GTT GCC GCC G- 3') which has a half the *Nco* I site at the 5' end and half a *Bgl* I site at the 3' CGG C end.

Cell culture conditions -- The growth of the cells was monitored by the changes in optical density at 600 nm (OD 600). For standard culture conditions, JM101 cells harboring pGEX.SZ or pGEX-2T were grown overnight at 37°C in 2 ml LB medium (Sambrook, 1989) with 100 µg/ml ampicillin. Twenty ml of LB medium were innoculated with 100 µl of overnight culture and the expression of fusion protein was induced by adding 0.4 mM IPTG when the cells had grown to mid log phase (OD 600 between 0.6 to 1.0). Cells were harvested by centrifugation at various times after IPTG induction. *E. coli* BL21(DE3) cells, used for the

expression of pET.SZ, and the biotin auxotrophic *E. coli* cell line, R879(*bioA*$^-$), harboring pGEX.SZ or pGEX-2T, also were cultured as described above for JM101 cells.

For cultures of cells to be used for cell growth studies and enzyme assays, cells were grown in 2 ml of LB medium with ampicillin (100 μg/ml) overnight at 37°C. The cells were collected by centrifugation and washed twice with M-9 minimal medium (Sambrook et al., 1989). After the washes, the cells were resuspended in 100 ml M-9 medium and cultured overnight at 37°C. The next morning, the cells were collected by centrifugation and resuspended in 1 ml of M-9 medium. Resuspended cells were inoculated into flasks which contained 100 ml of M-9 medium supplemented with different concentrations of biotin (0 to 10 μM; a 10 mM biotin stock solution was prepared in distilled water with the pH adjusted to 6.5 - 7.0 with KOH). The cells were continually cultured at 37°C. When the OD 600 reached 0.4 - 0.7 (usually between 4 - 6 h after innoculation), 0.4 mM of IPTG was added to induce the expression of the fusion protein. Cell samples were collected at different intervals and harvested by centrifugation. The cells were frozen in liquid nitrogen and stored at -80°C. All experiments involving growth studies and enzyme assays were repeated at least three times with similar results. Data shown are the means ± standard deviations from triplicate samples from individual experiments.

Expression and purification of GST-streptavidin fusion proteins -- The purification procedures were modified from Smith and Johnson (1988). *E. coli* JM101 cells carrying pGEX.SZ were grown at 37°C in 100 ml LB medium supplied with 125 μg/ml ampicillin. When the cells reached mid-log phase (OD 600 between 0.6 and 1.0), IPTG was used to induced the expression of the fusion protein and the cells were grown for an additional 5 h. Cells were collected by centrifugation and the pellet was washed with PBS [150 mM NaCl, 16 mM NaH_2PO_4, 4 mM Na_2HPO_4, pH 7.3]. From this step, all procedures were at 0-4 °C.

Cells were resuspended in 1 ml (1/100 volume) of PBS with 1% Triton X-100 in microfuge tubes, and broken by sonication at 50% strength [a 70-80 setting out of a maximum of 160] for 15 sec on ice, repeated twice. The mixture was centrifuged for 15 min at 10,000g and the supernatant was transfered to another pre-chilled microfuge tube to yield the crude protein extract. A 3 ml glutathione-agarose affinity column was packed and balanced according to the procedures of Pharmacia. One ml of the crude protein extract was loaded on the affinity column. The crude protein extract that came through the column was passed through the affinity column again to ensure complete binding of the GST-streptavidin fusion protein. After three washes of the column with PBS to remove non-specifically bound molecules, the GST-streptavidin fusion protein was eluted from the column with PBS plus 15 mM glutathione. The OD280 was used to estimate the yield of purified protein.

SDS-PAGE, non-denaturing-PAGE and western blot analysis -- After induction of cultures for 4 h, cells from 5 ml of medium were collected by centrifugation at 2,000 g for 10 min and washed once with 50 mM Tris-HCl (pH 7.0). For SDS-PAGE, the cell pellet was resuspended in 600 µl of Tris buffer (50 mM Tris-HCL (pH 7.0), 1 mM EDTA) and 2% (w/v) SDS, and incubated at 100°C in a bath of boiling water for 10 min. The mixture was centrifuged and the supernatant was analyzed by SDS-PAGE (Sambrook et al., 1989) using 12.5% acrylamide gels. For non-denaturing PAGE, washed cell pellets were resuspended in Tris buffer without SDS and sonicated. Cell debris was removed by centrifugation and the supernatants were subjected to non-denaturing-PAGE (Hedrick et al., 1968; Chrambach et al., 1971) using 10% acrylamide gels. Proteins were transferred from the gels to nitrocellulose filters with a semi-dry trans blot apparatus according to the instructions of manufacturer (PolyBlot, American Bionetics, Inc.). Immunological detection of protein was conducted with antiserum diluted between 1:500 to 1:2000 in a solution of 10 mM Tris.HCl (pH 8.0), 100 mM

NaCl, and 3% (w/v) BSA, and the antigen-antibody complexes were detected with ^{125}I-Protein A.

Acetyl-CoA carboxylase activity assay -- The cells from 10 ml of culture were ground to a fine powder in liquid nitrogen with a mortar and pestle. One hundred and fifty µl extraction buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 10 mM ß-mercaptoethanol] (3 to 5 volumes of the pellet) was added and the mixture was further homogenized with the pestle. The mixture was transferred to a prechilled microcentrifuge tube and centrifuged at 10,000g at 4°C for 10 min. The supernatant was used as the source for the enzyme assay. Protein determinations were by the method of Bradford (1976). Acetyl-CoA carboxylase activity was assayed as previously described (Wurtele and Nikolau, 1990) as the rate of incorporation of radioactivity from $NaH^{14}CO_3$ into acid-stable products. For some experiments, the supernatant was adjusted to 20% (v/v) glycerol, frozen in liquid nitrogen and stored at -80°C for future use.

RESULTS

Expression of streptavidin in E. coli -- The streptavidin protein expressed in *E. coli* has been shown to function *in vitro* after it has been solubilized (Sato and Cantor, 1990b). Although the streptavidin protein is predominantly accumulated in inclusion bodies when expressed (Sato and Cantor, 1990b), if some portion of the protein was soluble, or if the protein was exposed to the cellular contents in transit to the inclusion bodies, it might bind biotin *in vivo*. Therefore, to determine whether the expression of the non-fusion streptavidin protein altered growth of *E. coli*, the gene coding the streptavidin protein was ligated into the pET11d vector, which expresses streptavidin as a non-fusion protein. The resultant plasmid,

pET.SZ, was expressed in *E. coli* strain BL21(DE3) (Fig. 1). The protein accumulated to over 50% of the total cell protein. The expressed streptavidin protein had a molecular mass of 17 kDa, as expected. As expected, the majority of this protein was present in inclusion bodies (data not shown).

To test whether the expressed streptavidin decreased cell growth, cells harboring pET.SZ and cells harboring pET11d were grown in minimal medium lacking biotin and induced with IPTG after reaching the log phase. The growth of *E. coli* expressing streptavidin was only slightly less than that of control cells (data not shown). One likely explanation for the failure of the cells expressing streptavidin to show a significant decreased growth compared to control cells was that the expressed streptavidin was not accessible to the biotin.

Expression of GST-streptavidin fusion protein in E. coli -- To solubilize the streptavidin protein, we generated a fusion protein with the soluble GST protein. The streptavidin gene was fused in-frame with the 3' end of the GST (using the expression vector pGEX-2T, which contains the GST gene and is designed to generate a fusion protein). The resulting plasmid (pGEX.SZ) was transformed into *E. coli* strain JM101. Four h after induction of the expression of pGEX.SZ with IPTG, the cells containing pGEX-2T accumulated large amounts of the GST protein (Fig. 2A, lane 3). The induced cells containing pGEX.SZ accumulated large amounts of a 42 kDa protein (Fig.2A, lane 2) which was not apparent in the cells without plasmid (not shown) or the cells containing pGEX-2T (Fig. 2A, lane 3). This protein has the same molecular weight as predicted for the GST-streptavidin fusion protein, 42 kDa (GST is 26 kDa and the streptavidin gene product is about 16 kDa). Protein samples of the identical extracts were analyzed by western blot with an antibody to streptavidin. Only cells harboring pGEX.SZ produced a protein, of 42 kDa, recognized by the streptavidin antibody (Fig. 2B, lane 2), indicating that the 42 kDa protein is the GST-streptavidin fusion protein.

It was determined that the GST-streptavidin fusion protein was a soluble protein, rather than forming inclusion bodies within the cells (data not shown). Therefore, attempts were made to characterize the native protein. The GST-streptavidin fusion protein, as well as the GST protein alone, were purified by using a glutathione affinity column and subjected to non-denaturing PAGE (Fig 3). Coomassie Brilliant Blue staining of the purified fusion protein (Fig. 3, lane 1) shows the predominant band running between the GST protein (26 kDa) and the monomer of BSA (66 kDa). The molecular mass of the GST-streptavidin fusion protein is 42 kDa, indicating the majority of the GST-streptavidin fusion protein may be present in monomeric form. Several proteins were present at much lower levels, running above the GST-streptavidin monomer on the gel (Fig. 3, lane 1); these minor bands may represent small amounts of multimeric assemblies of the GST- streptavidin fusion protein, such as occur for the streptavidin monomer.

Over-expression of functional streptavidin may lower the biotin level inside *E. coli*. One easily detectable consequence of this phenomenum might be a decrease in the growth rate of *E. coli*, brought about by a decreased activity of acetyl-CoA carboxylase. To test whether the rate of growth was decreased in cells expressing the GST-streptavidin fusion protein, we measured the cell density at various intervals after induction of cells growing in minimal medium by using IPTG. Cells harboring pGEX.SZ consistently reached a final density of 5-10% less than that of cells harboring pGEX-2T (Fig. 4). Two possible explanations for the decrease in growth in cells expressing the GST-streptavidin being so small are: 1) the expressed strepavidin fusion protein does not bind biotin, and/or 2) that *E. coli* can compensate for the depletion in free biotin caused by strepavidin by synthesis of additional biotin.

GST-streptavidin fusion protein has the ability to bind biotin in vivo -- The streptavidin tetramer binds tightly to biotin *in vitro*; indeed the K_d of this interaction is the highest known

for a non-covalent protein interaction; the streptavidin monomer also binds biotin, but more weakly (Sano and Cantor, 1990a). Non-denaturing PAGE had indicated that the majority of the GST-streptavidin fusion polypeptide may not assemble *in vivo* into polymeric proteins (Fig. 3). Because the N-terminal GST portion of the fusion protein might prevent the binding of biotin to the fused streptavidin, it was not clear whether either the monomeric or polymeric fusion protein would bind biotin. To test if expressed GST-streptavidin fusion protein has the ability to bind biotin *in vivo*, 10 μM [^{3}H]-biotin (Fall, 1977) was supplied to the cultures of *E. coli* harboring pGEX-2T or pGEX.SZ prior to induction with IPTG. The expressed GST and GST-streptavidin proteins were purified by using a glutathione affinity column and radioactivity in the fractions was monitered by scintillation counting. The GST-streptavidin fusion protein bound about 100-fold more [^{3}H]-biotin than the GST protein alone (Table I). This result indicates that the expressed GST-streptavidin fusion protein has the ability to bind biotin *in vivo*.

To get additional evidence for the binding ability of the GST-streptavidin fusion protein to biotin, we visualized the protein from the above experiments by fluorography (Fig. 5). From the flurograph of crude extracts of *E. coli* containing the pGEX.SZ plasmid, we identified two forms of protein which were associated with radioactivity (Fig. 5, lane 3); these bands were not found in *E. coli* cells with no plasmid or cells harboring the pGEX-2T vector (Fig. 5, lanes 1 and 2). To confirm that the radiolabeled bands were indeed the GST-streptavidin fusion protein, the GST-containing proteins were purified by using a glutathione column, and the eluted protein was subjected to flurography (Fig. 5, lanes 4 and 5). The protein from the cells harboring pGEX.SX and purified by using the glutathione column showed radioactively labeled bands that comigrated with the radioactively labeled bands from the crude extract (Fig. 5, lane 5). Interestingly, neither of the most abundant forms of radiolabeled protein coincided with the predominant GST-streptavidin fusion protein; instead,

they coincided with two bands present at a much lower concentration (see Fig. 4, lane 1). One explanation of this result is that the GST-streptavidin fusion protein associates in the cell to form dimers, trimers or tetramers, and that these polymers have a stronger affinity for biotin than does the monomeric form (as is true for the native streptavidin protein (Sano and Cantor, 1990a). Because expressed GST-streptavidin fusion protein binds biotin *in vivo,* the GST-streptavidin fusion protein is a good candidate for *in vivo* studies of metabolic consequences.

The GST-streptavidin fusion protein decreases the growth rate of E. coli -- To test the effect of expression of the streptavidin gene on bacterial physiology, we expressed streptavidin in a biotin-deficient strain. For these studies, we utilized *E. coli* cell line R879(*bioA*$^-$), which is a biotin auxotroph deficient in biotin synthetase (Cleary and Cambell, 1972). For these studies we wished to use a soluble streptavidin protein. Therefore, the effect of the GST-streptavidin fusion protein in biotin-deficient cells was investigated by transforming pGEX.SZ into R879(*bioA*$^-$) cells. Expression of the GST-streptavidin fusion protein was induced by IPTG in the R879(*bioA*$^-$) cells, whether the cells were cultured in LB medium or in minimal medium supplied with biotin (data not shown).

The growth of *E. coli* R879(*bioA*$^-$) cells expressing the GST-streptavidin fusion protein or expressing the GST protein was quantified in minimal medium containing a range of concentrations of biotin (Fig. 6). In cells grown at the highest biotin concentration tested (10 μM), the growths curves were similar in cells expressing the GST-streptavidin fusion protein and those expressing the GST protein alone (Fig. 6). When cells were grown in 10 nM biotin, the cells expressing the GST-streptavidin fusion protein had a longer lag period than the control cells, although the final concentration attained by both kinds of cells was similar. For cells growing in 2 nM biotin, the growth of cells expressing the GST-streptavidin fusion protein

was significantly lower than the control cells. In 10 pM biotin, cells expressing the GST-streptavidin fusion protein did not grow, whereas the control cells grew to about 30% of their normal density. Cells growing in medium supplied with 1 pM biotin, or in medium with no added biotin, did not grow.

The suppression of the growth rate of E. coli is correlated with a decreased activity of acetyl-CoA carboxylase -- We determined the activity of acetyl-CoA carboxylase, the only biotin containing enzyme in *E. coli* R879 cells, during cell growth. Cells harboring pGEX.SZ, which expresses the GST-streptavidin fusion protein, and cells harboring pGEX-2T, which expresses the GST protein, were cultured in minimal medium supplied with 2 nM biotin in the presence of IPTG. The activity of acetyl-CoA carboxylase was analysed at various stages of growth (Table II). Acetyl-CoA carboxylase activity was positively correlated with the growth rate of *E. coli* in both strains. (Correlation coefficients were $r = 0.991$ for cells harboring pGEX-2T and $r = 0.977$ for cells harboring pGEX.SZ). Interestingly, the coefficient factor (the slope) was different for the cells containing pGEX-2T and pGEX.SZ ($y = 10.9$ versus $y = 17.1$, respectively).

DISCUSSION

The expression of streptavidin has been reported by Sano and Cantor (1990b) as a non-fusion protein, and Nagarajan et al. (1993) as a NPr_{SS}-streptavidin fusion protein. In both cases, the isolated protein functions *in vitro* to bind biotin. The streptavidin gene we used here is somewhat shorter (384 bp) than the core streptavidin used by Sano and Cantor(1990b), as it is missing the C-terminal 15 amino acids, but is long enough to contain the 3 Trp residues

(Trp21, 79, and 120) and Tyr33, which have been implicated in the binding of streptavidin to biotin (Kurzban et al., 1990; Gitlin et al., 1990).

These data represent the first characterization of the *in vivo* functioning of a streptavidin fusion protein. Expressed streptavidin bound biotin and decreased the growth of *E. coli* cells and the level of acetyl-CoA carboxylase activity in biotin auxotrophs. Furthermore, the streptavidin was expressed as a GST-streptavidin fusion protein. Despite the relatively large size of this soluble fusion protein (GST is 26 kDa compared to the streptavidin protein which is 16 kDa), it appeared to assemble to some extent into dimers or other polymers, similar to native streptavidin. Although the polymeric form only comprised a very small proportion of the total expressed GST-streptavidin fusion protein -- it was just detectable on a Coomassie Brilliant Blue stained gel -- it binds significantly more biotin than does the monomeric GST-streptavidin fusion protein. Furthermore, this protein has a convenient N-terminal tail which would be useful for purification and quantitation of biotin or biotin-containing molecules that are bound to it.

In bacteria, biotin is readily taken up from the medium, and can also be synthesized by the cells. Biotin synthesis is feedback regulated by the internal biotin concentration (Pai and Li chsten, 1965; Barker and and Campbell, 1980). This is accomplished by the product of the birA gene, a bifunctional protein which activates biotin with ATP to form biotinyl-5'-adenylate. The biotinyl-5'-adenylate thus formed may be transfered to the lysine residue of BCCP. Alternately, if the biotin binding lysines are unavailable, the enzyme-biotinyl-5'-adenylate complex acts to corepress the biotin synthetic genes of the *bio* operon (Buoncritiani and Otsuka, 1988; Cronan, 1988, 1989). Consistent with these findings, our results indicate that wildtype *E. coli* cells were able to compensate for the sustained expression of streptavidin, and consequent sustained binding of cellular biotin by the synthesis of additional biotin.

Our results indicate that expressing strepavidin *in vivo* may provide an additional approach to control intercellular levels of biotin, and thus study the function of biotin enzymes

or the regulation of biotinylation. A caveat is that these studies may need to be performed using organisms that do not synthesize biotin, for example, in animals or biotin auxotrophic mutants such as are available in *Arabidopsis* and *E. coli.* In a cell deficient in biotin synthesis, the only source of biotin is from the medium, and this level can be fixed at a known concentration. The expressed streptavidin would then cause the level of free biotin and/or the activity of biotin-containing enzymes in the cell to decrease significantly.

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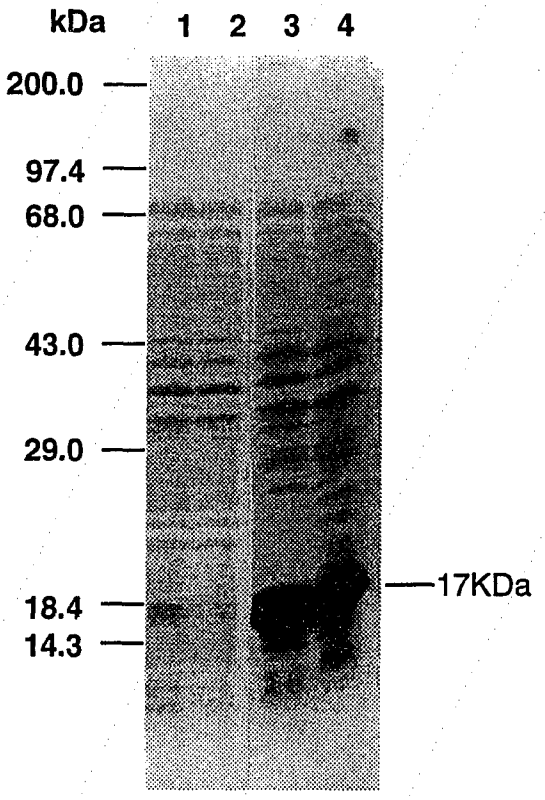

Fig. 1 Expression of the streptavidin gene in *E. coli* as a non-fusion protein. BL21(DE3) cells containing pET11d (lanes 1, 2) or pET.SZ (lanes 3, 4) were cultured in LB medium supplied with 8.2 μM biotin. The cells were induced by IPTG and harvested 6 h after induction (lanes 1, 3) and 24 h after induction (lanes 2, 4). After electrophoresis, the 12.5% SDS gel was stained by Coomassie Brilliant Blue.

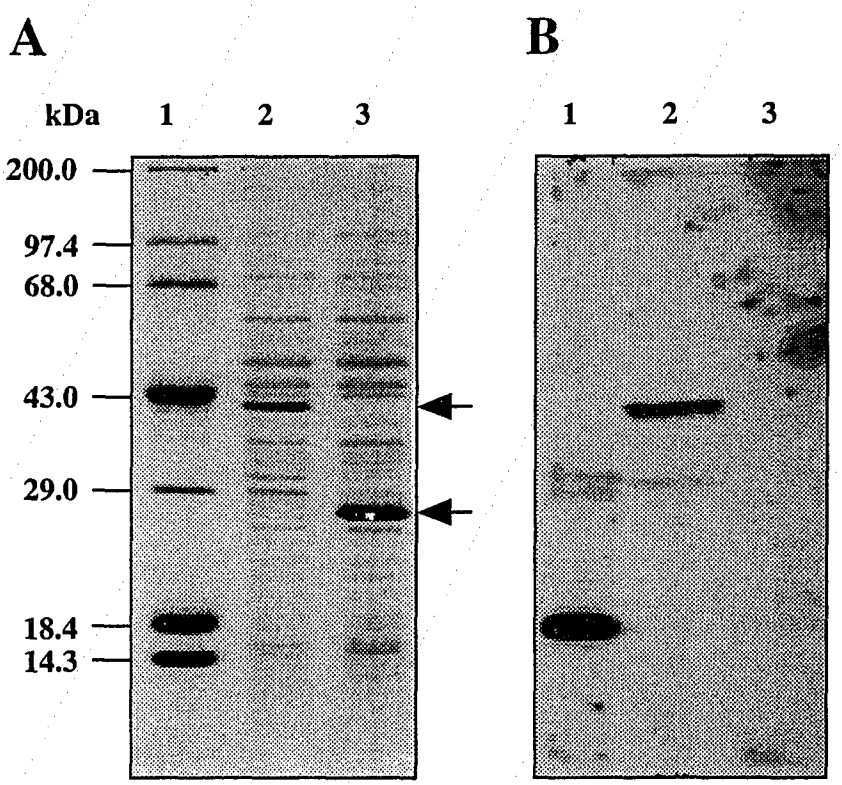

Fig. 2 Expression of the streptavidin gene in *E. coli* as a GST fusion protein. The streptavidin gene was fused, in frame, to the 3'- end of the GST gene in pGEX-2T as described. The resulting plasmid, pGEX.SZ, was introduced into *E. coli*. Expression was induced with IPTG. Six hours after induction, cells from 5 ml of culture were collected by centrifugation and dissolved in 600 µl protein extraction buffer [50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 2% (w/v) SDS]. 25 µl of each sample was subjected to SDS-PAGE. (Panel A) A gel stained with Coomassie Brilliant Blue. Lane 1: molecular weight markers; lane 2: pGEX.SZ; lane 3: pGEX-2T. Upper arrow shows the location of the GST-streptavidin fusion protein; lower arrow shows the location of the GST protein. (Panel B) A gel subjected to western blot analysis, incubated sequentially with antiserum to streptavidin antibody and ^{125}I- protein A. Lane 1: streptavidin standard; lane 2: pGEX.SZ; lane 3: pGEX-2T. The GST-streptavidin fusion protein can be detected in lane 2.

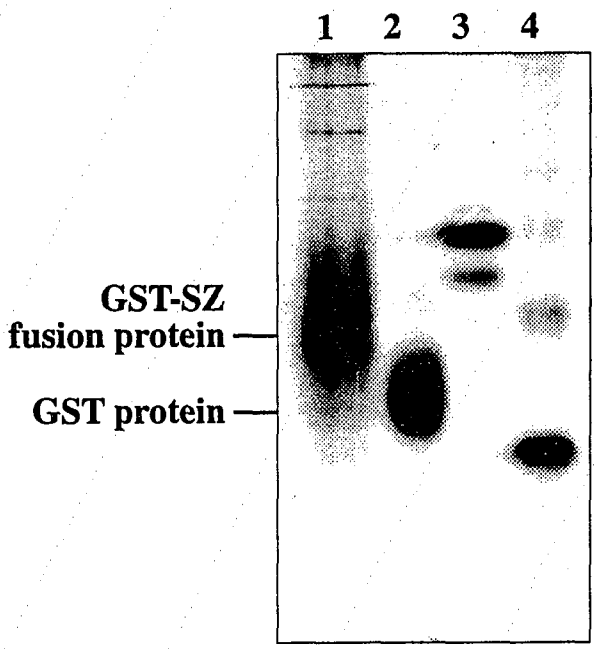

Fig. 3 Purification of the GST-streptavidin fusion protein by affinity chromatography. *E. coli* JM101 cells harboring pGEX.SZ and pGEX-2T were cultured and expression was induced with IPTG. Pellets from 100 ml of culture were washed twice with PBS [150 mM NaCl, 16 mM NaH_2PO_4, 4 mM Na_2HPO_4, pH 7.3], and the cells were resuspended in 1 ml PBS with 1% (v/v) Triton X-100. Cells were broken by sonication, the debris was pelleted by centrifugation, and supernatants were loaded on 3 ml glutathione-agarose affinity columns. After three column washes with PBS, the GST-streptavidin fusion protein was eluted from the column with 15 ml PBS plus 5 mM glutathione. The eluted protein was concentrated to 1.5 ml, and 40 μl of sample was subjected to non-denaturing-PAGE analysis. Protein is from cells harboring pGEX.SZ (lane 1) and pGEX-2T (lane 2). BSA (66 kDa and 133 kDa) (lane 3) and α-lactoabumin (14 kDa) (lane 4) were used as markers.

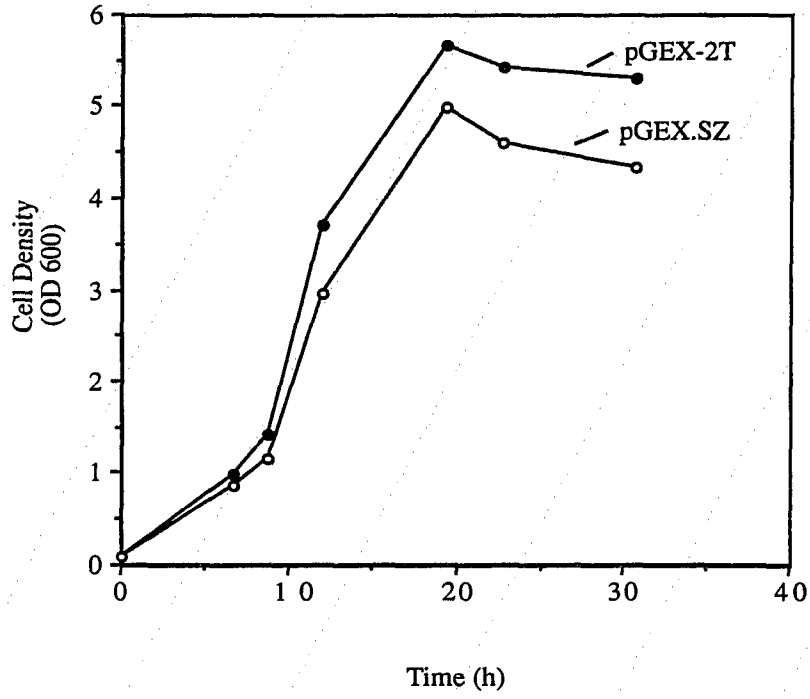

Fig. 4 Growth of cells expressing the GST-streptavidin fusion protein and the GST protein. *E. coli* JM101 cells harboring pGEX.SZ and pGEX-2T were induced to express the GST-streptavidin fusion protein and the GST protein, respectively (as described in methods). Growth of cells was monitered by changes in OD 600.

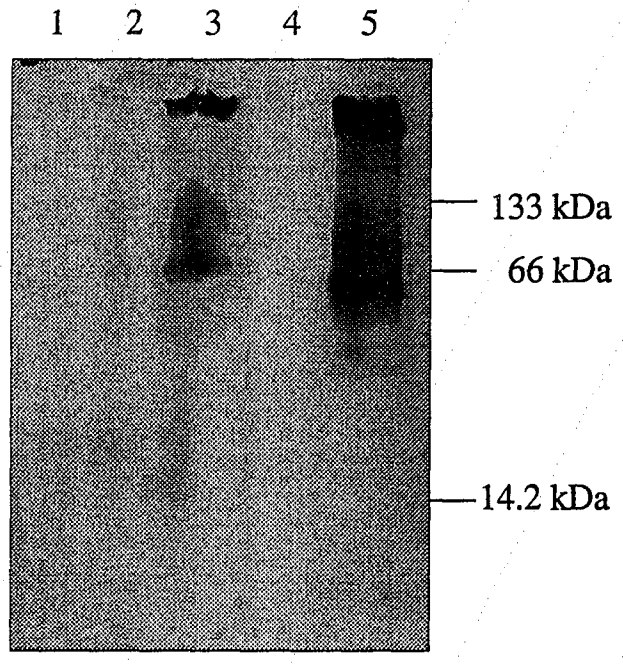

Fig. 5 Flurograph of non-denaturing PAGE of proteins from *E. coli* grown in [^{3}H]-biotin. Protein extracts were from the experiment of Table I. 40 µg protein from extracts of *E. coli* JM101 cells (lane 1) and same strain of cells harboring pGEX-2T (lane 2) and pGEX.SZ (lane 3) were analyzed by 6.5% acrylamide non-denaturing PAGE. 35 µl of the purified GST protein (lane 5) and 60 µl of the purified GST-streptavidin fusion protein (lane 4) were also applied to the gel (purification was as described for Fig. 3).

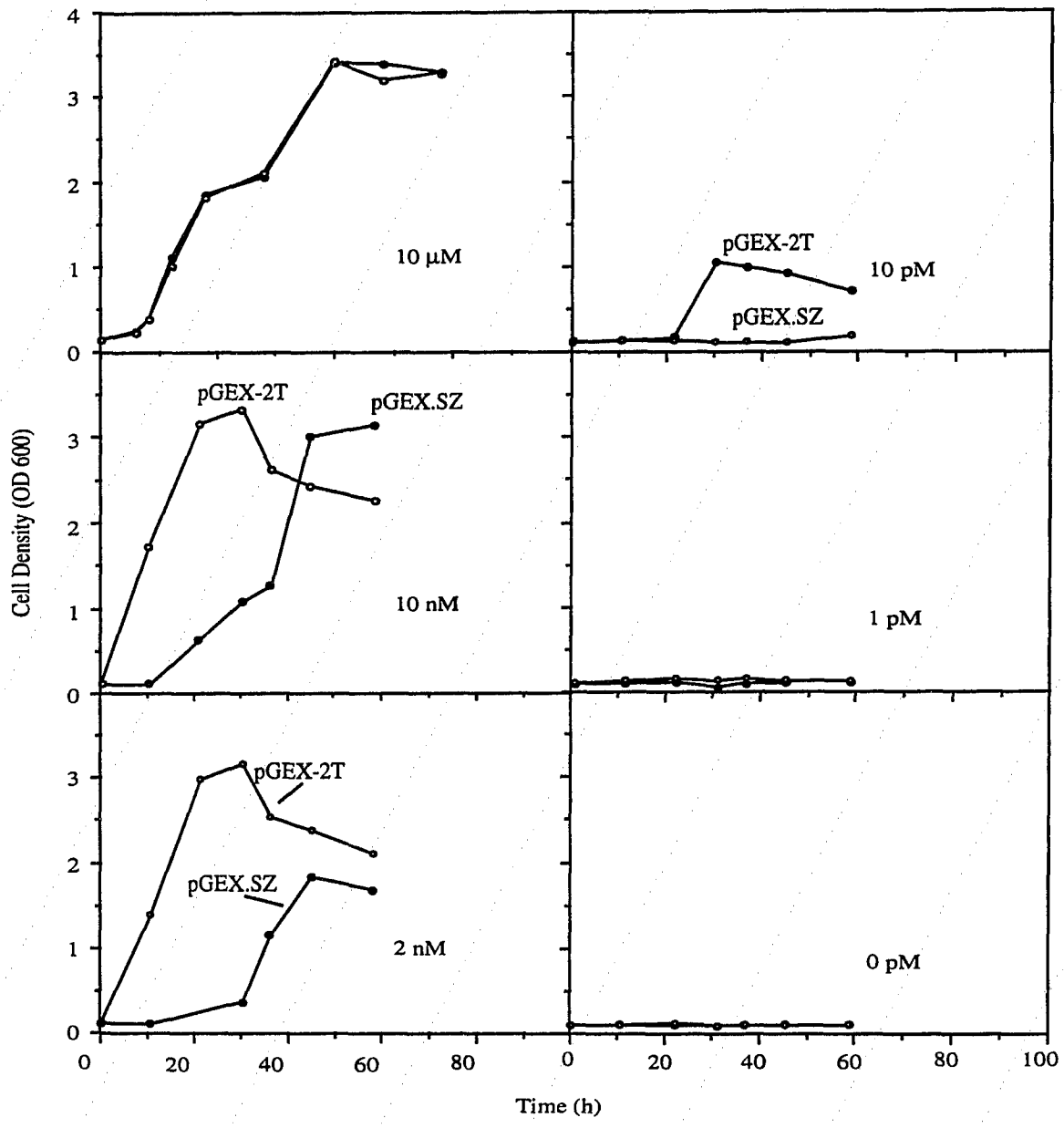

Fig. 6 Time course of the suppression of the growth rate of *E. coli* in cells which express the GST-streptavidin gene. *E. coli* R879 cells expressing pGEX-2T and pGEX.SZ were cultured in M-9 minimal medium in the presence of IPTG with no added biotin and with different biotin concentrations (10 μM; 10nM; 2 nM; 10 pM; and 1 pM biotin) as described in experimental procedures. OD 600s were taken at 0, 4, 12, 25, 30, 48, 60, and 72 h.

Table I. Purification of [^{3}H]-biotin Labeled Streptavidin-GST Fusion Protein by Glutathione-agarose Affinity Column

fraction	dpm	
	pGEX.SZ	pGEX-2T
total ^{3}H-biotin applied	8.1×10^7	8.1×10^7
supernatant	2.7×10^7	ND
pellet	2.9×10^6	2.0×10^6
column wash	2.9×10^5	8.0×10^5
eluted sample	4.2×10^5	5.9×10^3

ND = not determined

Table II. The inhibition of the activity of *E. coli* acetyl-CoA carboxylase by expression of the streptavidin gene*

Time (h)	pGEX.SZ/R879		pGEX-2T/R879	
	growth rate (ΔOD 600/h)	Acetyl-CoA carboxylase (nmole/mg/min)	growth rate (ΔOD 600/h)	Acetyl-CoA carboxylase (nmole/mg/min)
4	0.13	2.3	0.22	3.2
12	0.08	1.2	0.11	1.8
25	0.06	1.2	0.063	1.4
30	0.04	0.7	0.04	1.3

* *E. coli* R879 cells expressing pGEX-2T and pGEX.SZ were cultured in M-9 minimal medium with 2 nM biotin in the presense of IPTG.

CHARACTERIZATION OF GERANYL-COA CARBOXYLASE DURING PLANT DEVELOPMENT

A paper to be submitted to the Journal of Plant Physiology

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and Eve Syrkin Wurtele

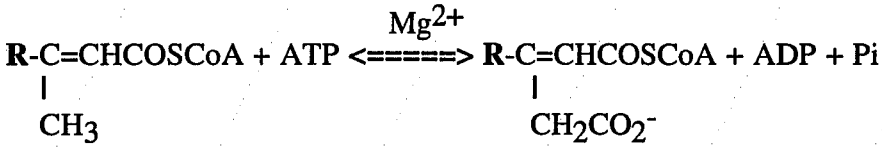
ABSTRACT

Geranyl-CoA carboxylase (GCCase) is an enzyme of monoterpene catabolism previously found in two species of bacteria. Recently, GCCase was also discovered in the plant kingdom (Diez, Guan, Caffrey, Yu, Wurtele, and Nikolau, In preparation). Although the metabolic function of this enzyme in plants is unknown, it may play an important role in isoprenoid catabolism. In the present study, we have characterized this enzyme during plant development in two dicot species (soybean and carrot) and a monocot species (maize). GCCase is present in all of the plant organs examined in both dicot and monocot species. In general, GCCase activity is highest in non-photosynthetic tissues, especially in roots. GCCase activity in maize seedlings is several fold higher than in soybean seedlings. During post-imbibition of soybean cotyledon development, there are two peaks of activity, one during cotyledon expansion and greening and the other during senescence. During carrot embryogenesis, GCCase activity is highest in cells before the induction of embryos. Differential centrifugation indicates that 99% of the GCCase activity in carrot is associated with the organelle fraction (plastids plus mitochondria). Further gradient centrifugation analyses of these organelle fractions of carrot, indicates at least 84% of the activity is localized within the plastids. Organelle fractionation and protease digestion analyses indicate that, in maize, almost

all of the GCCase activity is localized within the plastids. We propose a model describing the possible role of GCCase in isoprenoid catabolism in plastids.

INTRODUCTION

Geranyl coenzyme A carboxylase (EC 6.4.1.4.) (GCCase) is a biotinylated enzyme that catalyzes the ATP-dependent carboxylation of Z-geranyl-CoA to form isohexenylglutaryl-CoA (Seubert and Remberger, 1963; Fall, 1981).



R = $(CH_3)_2C=CHCH_2CH_2$-

This enzyme has been identified in the prokaryotic microorganisms *Pseudomonas citronelilus* (Seubert, 1963), *P. aeruginosa, P. mendocina*, and *Acinetobacter* (Cantwell et al., 1978). In these microorganisms, GCCase was shown to be involved in a pathway that degrades isoprenoids such as citronellol and geraniol to provide energy and a carbon source. GCCase is induced in these bacteria by growing the cells with an isoprenoid as the sole carbon source (Cantwell et al., 1978).

Recently, it was found that GCCase also exists in the plant kingdom (Diez, Guan, Caffrey, Yu, Wurtele, and Nikolau, In preparation). In this study, GCCase activity was identified in maize. The enzyme was purified 180-fold and characterized kinetically. A biotin-containing subunit of 120 kDa was identified. However, the function of this enzyme in plants is unknown. Based on the information from the psuedomonads (Cantwell et al., 1978), we

speculate that it may play an important role in plant isoprenoid catabolism. In plants, the biosynthesis of a variety of isoprenoid compounds has been studied extensively (Gershenzon and Croteau, 1990; Kleinig, 1989; Gray, 1987; Bartley and Scolnick, 1994), but the mechanisms by which these compounds are catabolised are hardly known, except for monoterpenes (Croteau and Sood, 1985; Croteau et al., 1984, 1987; Funk et al., 1992; Falk et al., 1990). To explore the function of GCCase in plants, we have characterized the expression and activity of GCCase during plant development, and the subcellular localization of this enzyme in a monocot (maize) and two dicots (carrot and soybean).

MATERIALS AND METHODS

Reagents

All biochemicals were obtained from Sigma Co.. $NaH^{14}CO_3$ (53.1 Ci/mol) was purchased from Amersham. Geranoic acid was converted from citral by oxidation with Ag_2O (Shamma and Rodrigues, 1968). Geranyl-CoA was synthesized by reacting CoA with geranoic acid in a mixed anhydride reaction (Stadman, 1957; Diez et al., in preparation). The product was purified by extraction with ether and lyophilization. After dissolving in 1 mM MES, the concentration of the geranyl-CoA was determined by the hydroxamate method described by Lipmann and Tuttle (1945).

Plant Materials

Carrot (*Daucus carota L.*) callus cells and carrot embryos were cultured as described previously (Wurtele et al., 1988; Keller et al., 1988). Briefly, cell line JH-4 was initiated from seeds collected from wild carrots growing in Ames, Iowa, and was subcultured every ten days at a density of 1 mg ml^{-1} in 25 ml Murashige and Skoog's medium (MS medium; Murashige and Skoog, 1962) supplemented with 5 μM 2,4-D. Carrot embryos were cultured in 100 ml

MS medium lacking auxin with a density of 0.1 mg ml^{-1}. After 3 to 4 weeks, the total embryo culture was fractionated and collected into 6 fractions according to the size of embryos by a series of screens with different sized meshes (Keller et al., 1988; Wurtele et al., 1993).

To study the effect of isoprenoids on GCCase activity during carrot cell culture, cells were innoculated into a modified MS medium containing specific isoprenoids. In some of these experiments, 8 mM of citronellal, citronellic acid, phytol, octanal, or ß-carotene was added to the MS medium; in other experiments, MS medium was prepared with manitol substituted for sucrose in an equimolar ratio, and 8 mM of citronellal, citronellic acid, phytol, octanal, or ß-carotene was added. For these experiments, cell samples were removed at two day intervals to analyze GCCase activity and cell growth.

Soybean seeds (Glycine max [L.] Merr. cv Corsoy 79), kindly provided by Dr. I. C. Anderson, Department of Agronomy, Iowa State University), were planted in 16 cm high pots containing sterilized soil (a 1:1:1 mixture of soil: peat: perlite) in a greenhouse. The temperature was normally 22 to 24°C. Maximum daily irradiance was no less than 1200 µmol photon m^{-2} s^{-1}. All plants were watered daily. Cotyledons were harvested at 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 days after planting (DAP). Seedlings were collected at the age of 15 DAP and were separated into root, hypocotyl, cotyledon, primary leaf (unifoliate leaf), first trifoliate leaf and shoot tip, as shown in Fig. 6. Immature seeds (0.4 gfw per seed) were collected from 60 DAP plants.

Seeds of maize (*Zea mays L.*) inbred G50 (Pioneer Hi-Bred International Inc., Johnston, IA) were planted in a dark growth chamber at 27°C and grown for 3 days. Maize seedlings used for chloroplast isolations were subsequently transferred to a greenhouse (under the conditions described above) and grown for another 2 to 3 days. All plant samples used for developmental studies were harvested at about 3:30 pm. Immediately upon harvest, all plant samples were frozen in liquid nitrogen.

Preparation of Protoplasts and Subcellular Organelles

Protoplasts were isolated from suspension cultures of carrot embryos at the torpedo stage of development (Wurtele et al., 1993) using procedures similar to those previously described (Nikolau et al., 1984). The intact protoplasts were collected by centrifugation at 250*g* for 10 min and gently ruptured by resuspending in 0.3 M sucrose solution, and passing through a fine nylon mesh (20 μm) attached to the end of a 5 ml disposable syringe. To separate the cytosolic fraction from the mitochondria and plastids, the lysed protoplast suspension was centrifuged to yield a pellet enriched in intact organelles and a supernatant enriched in the cytosolic fraction. The centrifugation was carried out in three steps: 100*g* for 5 min, 300*g* for 5 min, and 12,000*g* for 20 min (Baldet et al., 1993).

The plastids and mitochondria were isolated from the above organelle fraction by differential centrifugation. Typically, the organellar fraction was gently resuspended in an organelle buffer containing 0.3 M sucrose, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 50 mM HEPES, pH 7.8, by using a small paint brush (size 2), and centrifuged at 3,500*g*, for 10 min at 4°C . The pellet was enriched in plastids, and the supernatant was enriched in mitochondria. The mitochondria were recovered by further centrifugation of the supernatant fraction at 10,000*g*, 4°C for 15 min. The isolated plastids and mitochondria were gently resuspended in a small volume of the organelle buffer. In other experiments, the carrot chloroplasts were further purified by percoll density gradient. The chloroplast fractions were loaded on self-generated percoll gradients (Mourioux and Douce, 1981) and centrifuged at 5,000*g* for 20 min at 4°C . One ml fractions were collected at 4°C.

Subcellular organelles from maize tissue were isolated as described by Prasad et al. (1994). Briefly, 15 g of mesocotyls from 3-day-old dark-grown maize seedlings were homogenized in a cold room (10°C) in 25 ml of a grinding buffer (0.4 M sucrose, 165 mM Tricine, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 10 mM EDTA, and 10 mM DTT). The mixture was ground until a smooth paste was obtained. The homogenate was filtered through 4 layers

of cheesecloth and centrifuged at 270*g* for 5 min to remove unbroken cells and debris. The resulting supernatant was centrifuged at 10,800*g* for 15 min. The pellet was resuspended in 4 ml of grinding buffer, and layered onto a sucrose step gradient (from bottom to top: 7 ml each of 57%, 50%, 44%, 33%, and 30% [w/w] sucrose in 10 mM EDTA, pH 7.5, 165 mM Tricine, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, and 10mM DTT. The gradients were centrifuged at 20,000*g* for 1 h using an SW 28 rotor in a Beckman L-4 ultracentrifuge. One ml fractions from the gradient were collected in a cold room.

Protease Digestion

Chloroplasts were isolated from the leaves of light-grown maize seedlings according to the method of Blair and Ellis (1973). Intact or lysed (by repeated freeze-thaw cycles) chloroplasts were incubated with trypsin at 25°C at the ratio of 50:1 (mg chloroplast protein: mg trypsin) in 0.1 M sodium phosphate buffer, pH 7.2 at time intervals of 0, 15, 30, and 45 min. After incubation, the protease activity was inhibited by soybean trypsin inhibitor at the ratio of 3:1 (mg trypsin inhibitor: mg trypsin) and the GCCase, ACCase and PEP carboxylase activities of the digests were determined.

Preparations of Cell-free Extracts

Cell-free extracts were prepared essentially as described by Wurtele and Nikolau (1990). Tissues were homogenized to a fine powder with a mortar and pestle under evaporating liquid nitrogen . After the evaporation of the nitrogen, the sample powder was resuspended in 3 volumes of 100 mM HEPES buffer solution, pH 7.0, containing 1 mM EDTA, 20 mM DTT, 0.1 % (v/v) Triton X-100, 20% (v/v) glycerol, 1 mM E64 and 100 µg/ml of PMSF was added. The mixture was centrifuged at 10,000*g* for 20 min at 4°C. The supernatant, containing the soluble proteins, was collected. Two hundred µl of this protein extract was passed through a 1 ml Sephadex G-25 column preequilibrated in 10 mM HEPES

buffer solution but without PMSF (Wurtele et al., 1984). Fourty µl of the eluate, containing 20-80 µg protein, was used for each enzyme assay.

Enzyme Assays and Other Determinations

GCCase, ACCase, and MCCase activities (Wurtele and Nikolau, 1990, Diez, 1994), PEP carboxylase (Nikolau et al., 1984), and Rubisco activity (Wishnick and Lane, 1971) were assayed as the rate of incorporation of radioactivity from $NaH^{14}CO_3$ into the acid-stable products. Cytochrome C oxidase (Douce et al., 1972) and fumarase (Hill and Bradshaw, 1969) were assayed spectrophotometrically. Protein determinations were determined by a dye-binding method (Bradford, 1976). Chlorophyll determinations were perfomed using the method of Arnon (1949). Carotenoid contents were assayed spectrophotometrically (Lichtenthaler, 1987).

SDS-PAGE and Western Blot Analysis

SDS-PAGE was carried out using 5% acrylamide gels as described by Sambrook et al. (1989). Four volumes of 5 X SDS-PAGE loading buffer (250 mM Tris-HCl, pH 7.0, 5 mM EDTA, 5% [w/v] SDS) was added to each volume of sample, and the mixture was incubated at 100°C in a bath of boiling water for 10 min. The mixture was centrifuged and the proteins in the supernatant were resolved by SDS-PAGE. Proteins were transferred from gels to nitrocellulose filters using a semi-dry trans-blot apparatus according to the instructions of the manufacturer (PolyBlot, American Bionetics, Inc.) and the biotin-containing polypeptides were detected with ^{125}I-streptavidin (Nikolau et al., 1985).

RESULTS

Subcellular Localization of GCCase Activity

Protoplasts from carrot embryos were initially used as a source of organelles to determine the subcellular localization of GCCase. Differential centrifugation of lysed protoplast preparations was used to obtain organellar fractions containing predominantly mitochondria and plastids. GCCase activity was associated with this organellar fraction (Table I); while 99% of the GCCase activity was detected in the organellar fraction, only 1% was associated with the cytosolic fraction. Chloroplasts and mitochondria from such organellar fractions were further fractionated by sucrose or Percoll density gradients (see materials and methods). Purity of the fractions was assessed by the activity of marker enzymes such as fumarase and MCCase for mitochondria, and Rubisco and ACCase for chloroplasts. A typical sucrose gradient experiment is shown in Table II. 46% of the GCCase activity recovered from the gradient was found in the chloroplast-enriched fraction and 30% of the GCCase activity was found in the mitochondrial-enriched fraction. 14% and 73% of the Rubisco activity was located in the corresponding fractions, respectively. Despite repeated attempts, we were unable to obtain mitochondrial fractions free of chloroplast contamination, as determined by the distribution of the marker enzymes. If the cross contamination of the fractions, as indicated by the localization of fumarase and Rubisco activity, is taken into account, it is calculated that about 84% of the GCCase activity is associated with the chloroplasts.

As it proved to be difficult to isolate pure chloroplasts and mitochondria from carrot embryo protoplasts, we used maize mesocotyl and leaves as a source of organelles to study GCCase localization. Sucrose gradient analysis indicated that GCCase activity was present in plastids, but not in mitochondria or peroxisomes (Fig. 1).

To localize GCCase activity within the chloroplasts, intact purified chloroplasts were digested with trypsin (Fig. 2). ACCase was used as a marker of an enzyme located in the

chloroplasts. Because some PEP carboxylase activity was found to associate with the chloroplast fraction, we also determined PEP carboxylase activity. Although PEP carboxylase activity was lost following trypsin digestion, the chloroplasts retained most of the GCCase and ACCase activities. In contrast, if chloroplasts were lysed by repeated cycles of freezing and thawing prior to digestion with trypsin, they lost all detectable GCCase and ACCase activities. These results indicate that while PEP carboxylase was bound to the outer chloroplast membrane only as a cytosolic contaminant, the activity of GCCase (as for the activity of ACCase) is localized within the chloroplasts. This conclusion is consistant with our hypothesis that GCCase functions in isoprenoid degradation, since in plants the most abundant isoprenoids, such as the phytol tail of chlorophyll a and chlorophyll b and carotenoids, are synthesized and located inside the plastids (Kleinig, 1989).

GCCase Activity in Cells and Developing Somatic Embryos

We attempted to identify the relative distribution of GCCase activity among plant organs and developmental stages that might signify its importance to growth and development. Initially, we wanted to determine whether GCCase activity was associated with a particular phase in the growth of plant cells. To do this, GCCase activity was determined in cell free extracts from cultured carrot cells at various times after transfer of cells growing at log phase to fresh medium for subculture. GCCase activity was highest at the third day after transfer, just before the onset of the log phase of growth; GCCase activity slowly depleted during the log phase of growth and afterward (Fig. 3). The cell culture reached a maximum fresh weight by 30 days after inoculation, and by this time GCCase activity had decreased to about a sixth of its maximal value.

In some *Psuedomonads*, GCCase activity can be induced by supplying citronellal, nerol, and/or a related isoprenoid to the culture medium as a carbon source; and the ability of a *Psuedomonad* to degrade a particular isoprenoid is dependent on the species and the strain

(Cantwell et al., 1978). To determine whether GCCase activity in carrots was inducible by isoprenoids, carrot cells were grown in the presence of citronellal, citronellic acid, phytol, octonal, or ß-carotene. Phytol and ß-carotene were chosen because they are the most abundant isoprenoids in the chloroplasts of plants. Unlike for pseudomonads, plant GCCase activity is not significantly induced by any of the isoprenoids tested (data not shown).

Cells of carrot suspension cultures develop into somatic embryos through a series of morphologically distinct stages after subculture of the cells into an induction medium, and thus provide an excellent system to study the regulation of GCCase in plant development (Wurtele et al., 1988; Keller et al., 1988). Carrot somatic embryo cultures were fractionated into mature embryos, large torpedo stage embryos, small torpedo embryos, heart shape embryos, globular embryos, and non-embryogenic cells. These developmentally distinct populations were used to assay GCCase activity. As these results indicate, GCCase activity is distributed in all stages of carrot somatic cells and embryos (Fig. 4). The embryogenic cells growing in MS medium containing 2,4-D had the highest GCCase activity. GCCase activity decreased following transfer of the cells to inductive medium, and subsequentally increased during embryo development. The mature embryos had levels of GCCase activity almost as high as those of embryogenic cells. Similar experiments using a different cell line of carrot also gave similar results (data not shown).

GCCase Activity Changes during Post-Imbibition Development of Soybean Cotyledons

Soybean cotyledons progress through physiologically distinct stages: germination, expansion and greening, and senescence (Azcon-Bieto et al., 1989). These stages are associated with major redistribution of carbon via different metabolic pathways. Therefore, assuming that increased GCCase activity may be associated with the isoprenoid degradation in cotyledons at the time of senescence, we determined the GCCase activity in developing

cotyledons (Fig. 5). Until 3 days after planting (DAP), cotyledons remain yellow in color. Once cotyledons emerge from the soil at about 3 to 5 DAP, they begin to green. By 12 DAP, chlorophyll content is maximal and the cotyledons have reached their maximum size and fresh weight. Photosynthetic capabilities are developing during this time. Rubisco activity is maximal at 15 DAP. The cotyledons start senescing and are noticeably yellow by 24 DAP. By about 30 DAP they have lost about 95% of their maximal water content, subsequently they abscise from the plant. From about 5 DAP there is massive hydrolysis of seed storage proteins. The extractable protein content is 11.7, 3.4, and 0.3 mg per cotyledon, at 6, 12 and 30 DAP, respectively.

When the cotyledons are still underneath the soil (3 DAP), GCCase activity is relatively low. As cotyledons begin to expand and green, the GCCase activity increases almost three-fold, and reaching a maximal activity at 12 DAP, which coincides with the maximal fresh weight and chlorophyll content of the cotyledons. GCCase activity decreases between 12 DAP and 18 DAP, and during this time, Rubisco activities is at its maximum. However, the GCCase activity then increases, reaching a second, somewhat lower, peak at about 30 DAP, just before abscission.

Distribution of GCCase Activity in Plant Organs

The distribution of the GCCase activity was examined in the organs of 15 DAP soybean seedlings (Fig. 6). The highest GCCase activity was in the root. GCCase activity of the root was about 20-fold higher than GCCase activity of the unifoliate leaf, and 6-fold higher than GCCase activity of the cotyledon. The activities of the shoot apex and hypocotyl were about 7 times higher than that of the primary leaf.

Because the root is a developmental gradient of dividing, elongating, differentiating and mature cells, we wished to determine which part of the root had the highest GCCase activity. However, roots of 15 DAP soybean seedlings have numerous secondary roots emanating from

the primary root, thus it is difficult to dissect roots of plants of this age into different developmental sections. Therefore, we examined root tips and mature roots, as well as other organs, of younger (4 DAP) soybean seedlings, which had not yet begun lateral root formation. The root tips contained the root meristem and differentiating cells, the remainder of the root included the mature cells. Compared to other organs, GCCase activity was the highest in the roots of these younger seedlings (Fig. 7). Activity was similar in the root tip (the first 1.5 cm of the root), and the more mature regions of the root.

To compare the activity of GCCase in a monocot with that of soybean, GCCase activity also was examined in organs of young dark grown maize seedlings (3 DAP) (Fig. 8). In maize, however, GCCase activity was higher in the mesocotyl than in other organs. The lowest GCCase activity was in the mature root. GCCase activity of maize was consistently higher than that of soybean. Similar experiments were repeated with organs of 5 DAP dark-grown and 5 DAP light-grown maize seedlings, with similar results (data not shown).

DISCUSSION

Little is known about catabolism in plants, in part because of the importance to the biosphere of the net anabolic capabilities of members of this kingdom. However, studies have shown that biosynthesis of secondary products may occur simultaneously with catabolism (Barz and Koster, 1981). For example, flavonoids are degraded extensively at times of rapid growth (Barz and Koster, 1981). We propose that GCCase in plants is required for the catabolism of some, possibly most, isoprenoids (Fig. 9). Isoprenoids might be oxidized and converted to 10 carbon acyclic forms and eventually to geranyl-CoA by enzymes in part specific for each isoprenoid. The geranyl-CoA would then be catabolized to isohexenylglutaryl-CoA, which in turn would be converted by a hydratase and lyase, analogous to the *Pseudomonad* metabolism (Cantwell, 1978), to the resulting branched chain

acyl-CoA. Further catabolism might proceed via ß-oxidation and methylcrotonyl-CoA carboxylase, also analogous to the pathway in *Psuedomonads* (Cantwell et al., 1978).

The presence of geranyl-CoA carboxylase in plastids indicates that at least a portion of isoprenoid catabolism may occur in this organelle. Because carotenoids and the phytol tail of chlorophyll, typically the most abundant plant isoprenoids, are localized in the plastids, this organelle is a logical site for the initial degradation of these isoprenoids. Plastids are typically thought of as a major site of synthetic reactions in plants, however, several enzymes associated with catabolic pathways also are localized in plastids (Barz and Koster, 1981, Butt and Lamb, 1981; Poulton, 1981). At some step subsequent to conversion of geranyl-CoA to isohexenylglutaryl-CoA, plastid isoprenoid catabolites might be translocated to the mitochondria, where possibly ß-oxidation (Thomas et al., 1988; Masterson et al., 1990) and methylcrotonyl-CoA carboxylase are localized (Baldet et al., 1992; Nikolau et al., 1993), and further degraded in the mitochondria into acetyl-CoA and acetoacetate.

In carrots, in six independent experiments, although the majority of GCCase activity was associated with the plastids, mitochondrial fractions always retained a small amount of GCCase activity. Therefore, we can not exclude the possibility that GCCase activity exists in mitochondria, as well as in plastids. Because so little is known about isoprenoid catabolism and its localization, it is not yet possible to predict whether isoprenoids from other cellular compartments might be translocated to the plastid for degradation via GCCase, whether GCCase is also located in other compartments (such as the mitochondria), or whether the pathways for isoprenoid degradation differ for different isoprenoids.

Unlike the several species of bacteria which can use GCCase to degrade particular isoprenoids as a major carbon source, plants do not rely on the catabolism of isoprenoids as a major source of carbon. Furthermore, we do not know whether the exogenously applied isoprenoids such as phytol and carotenoids actually get into the plastids of the cells, where the

early catabolic pathway may be compartmentalized. This may explain why plant GCCase was not induced by addition of isoprenoids to the medium of cultured cells.

One stage in which degradation of isoprenoids would occur in the plant is during senescence. As plant organs senescence, constituent organic molecules are degraded into forms that can be exported into the plant body, so that the carbon can be retained before abscission of the organ. Therefore, if the hypothesis that GCCase is involved in degradation of carotenoids and the phytol tail of chlorophyll is correct, cotyledons might have a peak of GCCase activity as they entered senescence. This is indeed what was observed.

Another time of high degradation of some isoprenoids might be associated with rapid cellular metabolism, as has been found for other phytochemicals (Barz and Koster, 1981). A possible interpretation of the first peak of GCCase activity during cotyledon development is that this occurs during a stage of rapid isoprenoid synthesis, and this process proceeds concomitantly with rapid degradation of particular isoprenoids.

To try to begin to understand when GCCase activity might be the most important to the plant, we analysed GCCase activity in a variety of maize and soybean organs at different stages of development. GCCase activity was present in all plant organs and all stages of development examined. In general, GCCase activity was elevated in organs and cells undergoing rapid growth, such as the root tip, mesocotyl and shoot of young maize seedlings. However, no clear pattern is discernable in the levels of activity of GCCase in different organs. Roots of both 15 DAP and 4 DAP soybean had the highest GCCase activity of the organs examined; in the 15 DAP soybean, GCCase activity in the root was up to 20-fold greater than in the unifoliate leaf. An increased understanding of the role of GCCase in plant development and physiology will require detailed studies of plastidic isoprenoid catabolism, such as those that are being done currently for monoterpenes (Croteau and Sood, 1985; Croteau et al., 1984, 1987; Funk et al., 1992; Falk et al., 1990), and sophisticated studies as to the

compartmentalization and networking of isoprenoid catabolism between different subcellular compartments.

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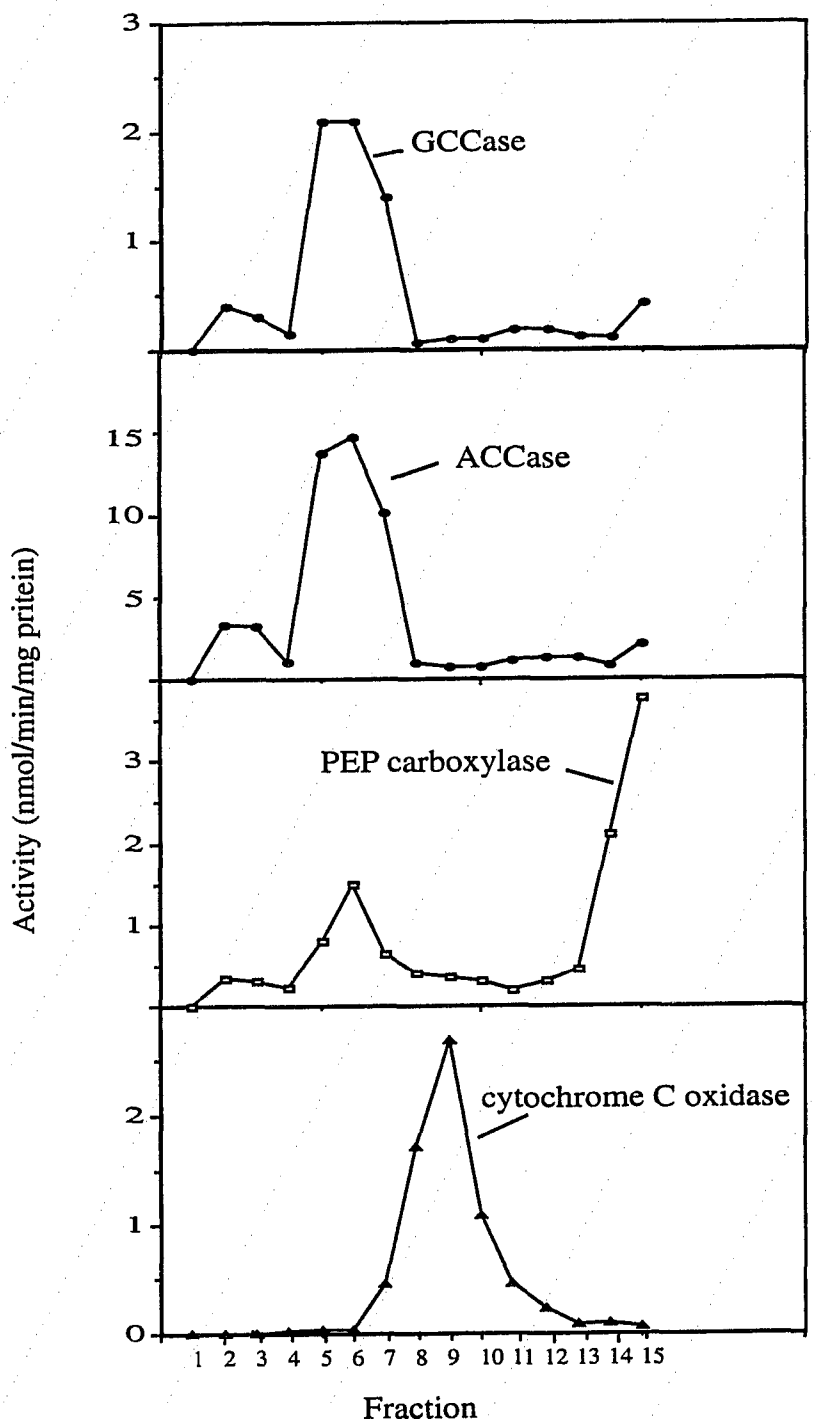

Fig. 1 Subcellular localization of GCCase activity in sucrose density gradient (30% to 57%) from mesocotyl of dark-grown 3 DAP maize seedlings. One ml fractions were collected.

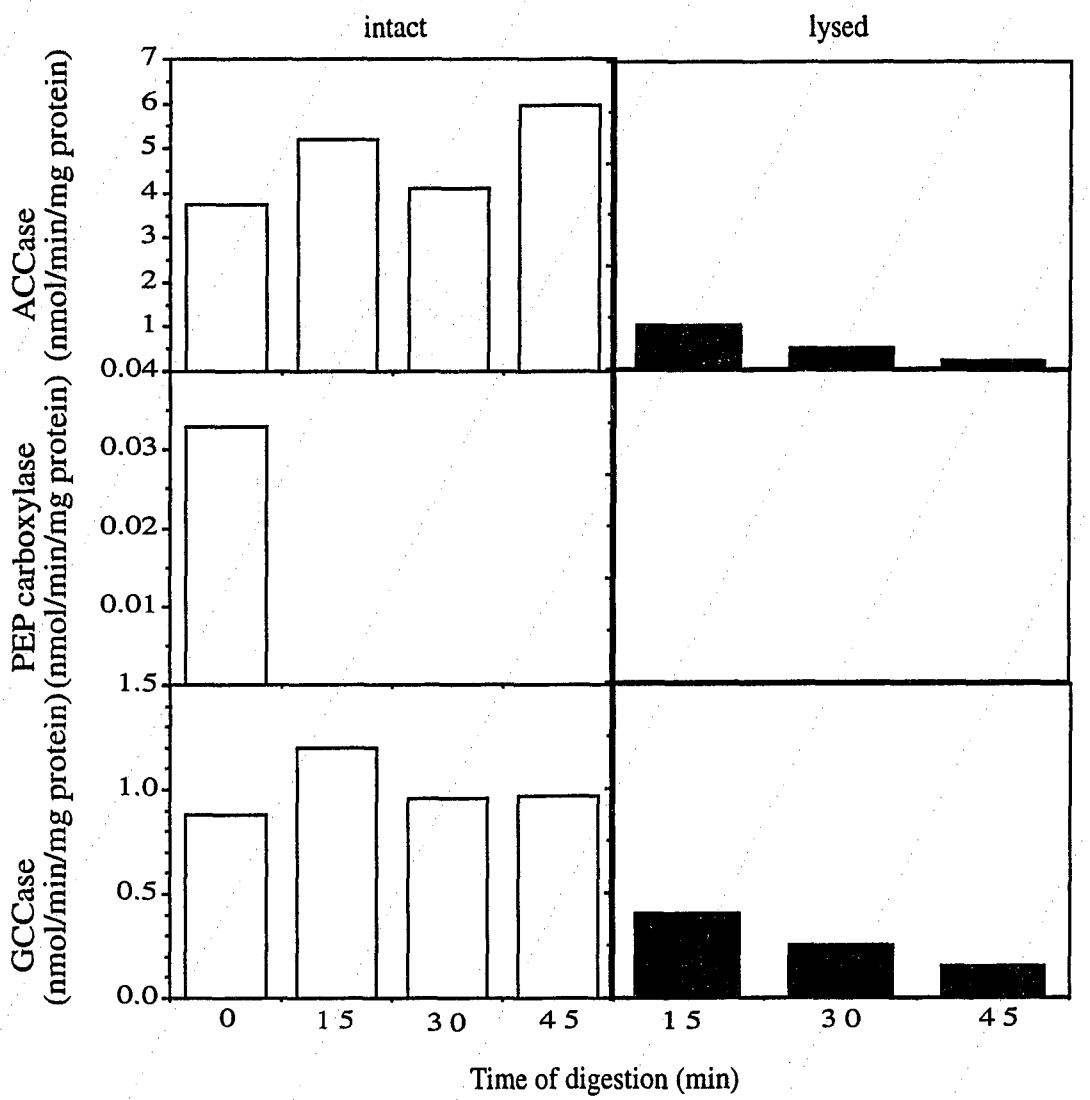

Fig. 2 Effect of trypsin digestion on GCCase activity of isolated chloroplasts of maize. Intact chloroplasts from maize were isolated and digested with trypsin for 0, 15, and 45 min at RT. The GCCase activity was measured before and after the digestion. ACCase assay was used as a chloroplastic marker and PEP carboxylase was cytosolic marker. PEP carboxylase activity was eliminated after the trypsin digestion whereas ACCase and GCCaseactivities were not effected. All activity were eliminated (45 min digestion) if chloriplasts were lysed by freezing-throw procedure before digestion with trypsin.

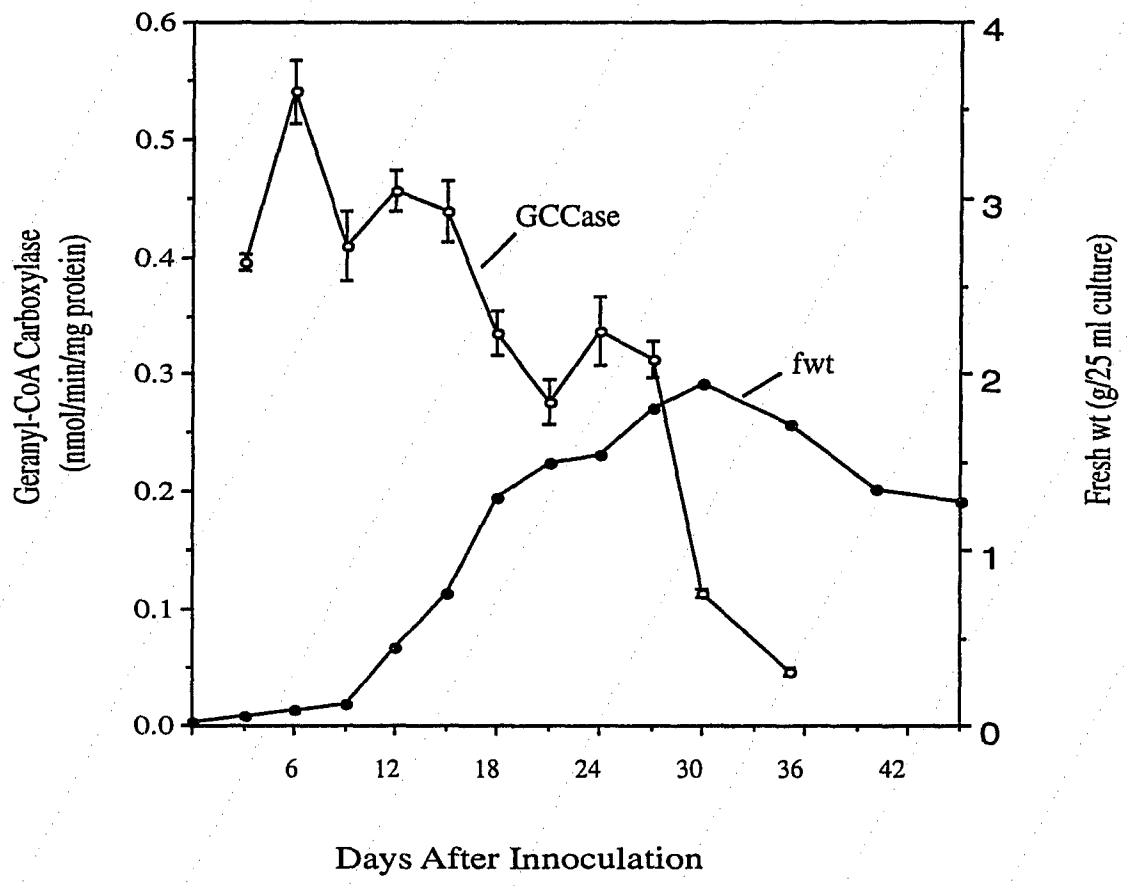

Fig. 3 GCCase activity during the culture of carrot callus cells. Data are the means ± SD of three determinations.

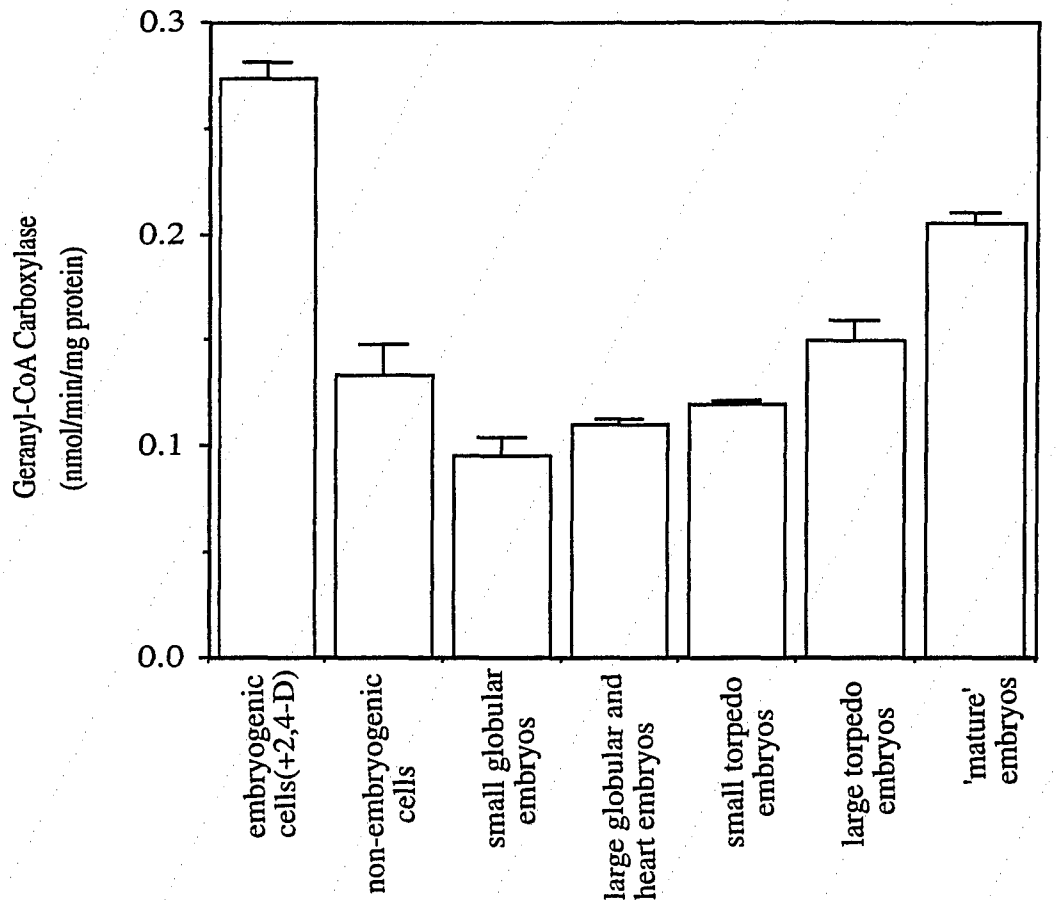

Fig. 4 Geranyl-CoA carboxylase activity during carrot embryogenesis. Fractions of different developmental stages were collected from cultures of somatic cells and embryos as described in "Materials and Methods" section. Data are the means ± SD of three determinations.

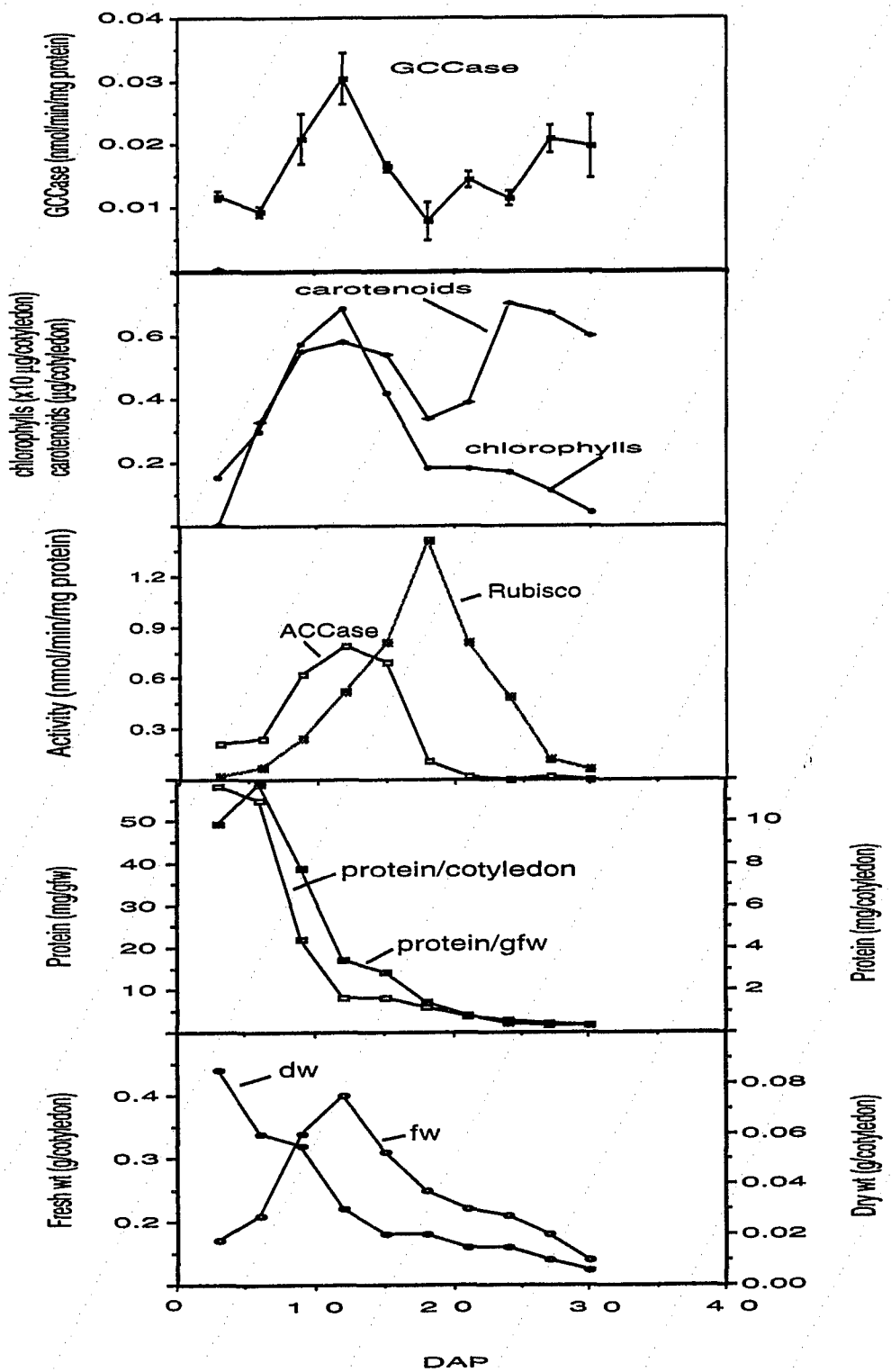

Fig. 5 GCCase activity during soybean cotyledon development Data for GCCase activity are the means ± SD of three determinations.

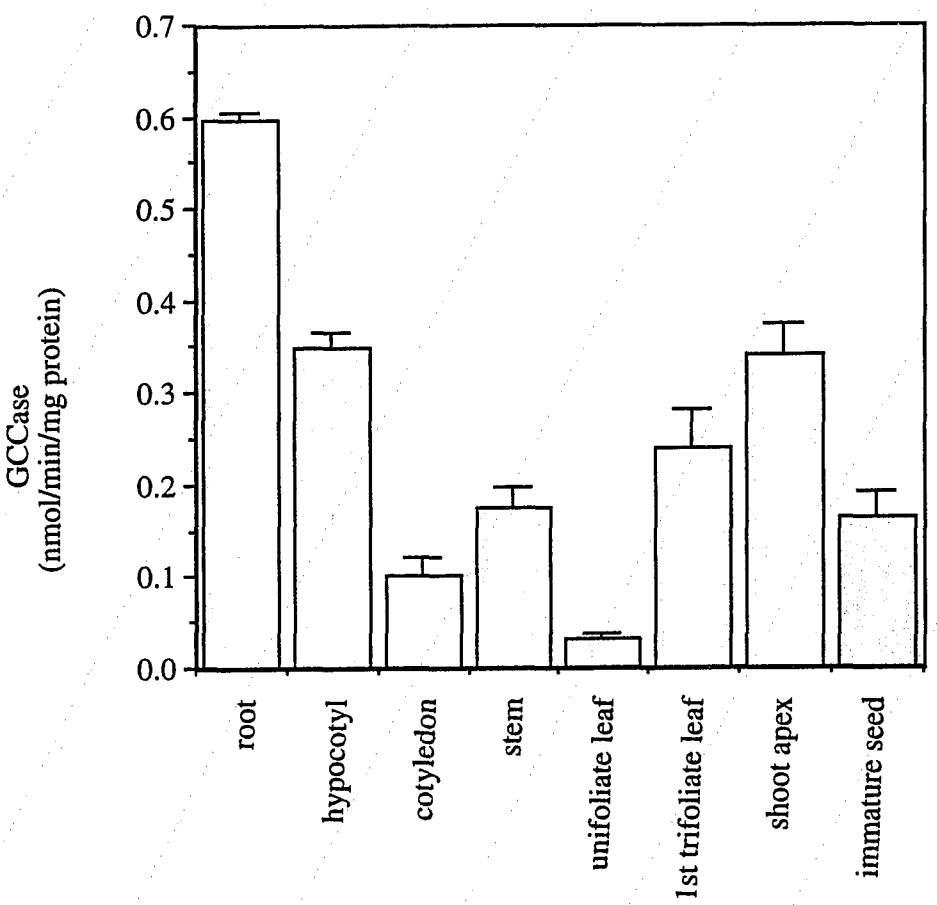

Fig. 6 The distribution of GCCase activity in organs of 15 DAP soybean plants. Immature seeds (0.4 gfw/seed) from 60 DAP old plants are also included. Data are the means ± SD of three determinations.

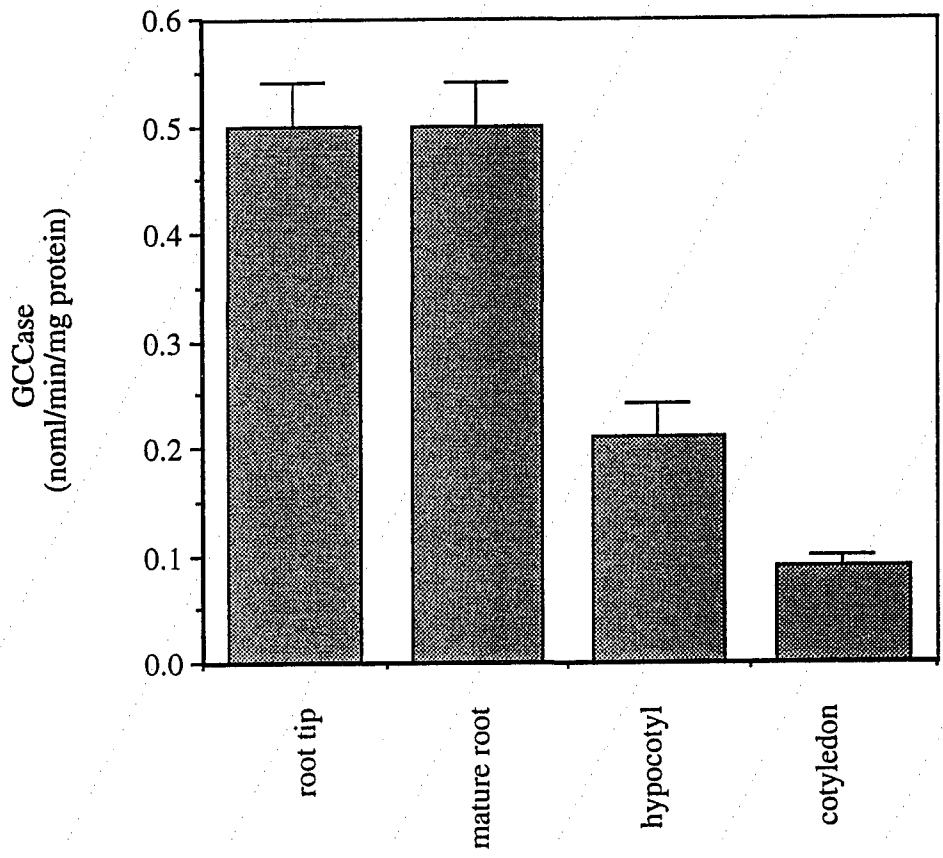

Fig. 7 The distribution of GCCase activity in organs of 4 DAP soybean seedlings. Data are the means ± SD of three determinations.

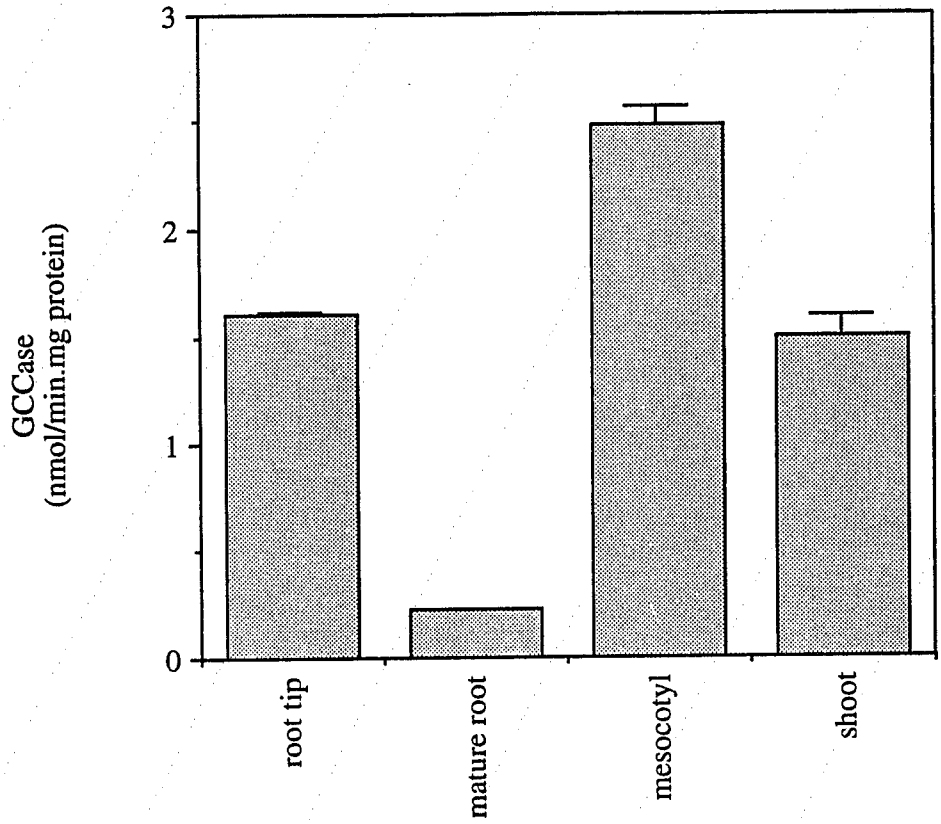

Fig. 8 The distribution of GCCase activity in organs of 3 DAP maize seedlings. "Shoot" refers to the portion of the seedling shoot including and above the coleoptilar node. Data are the means ± SD of three determinations.

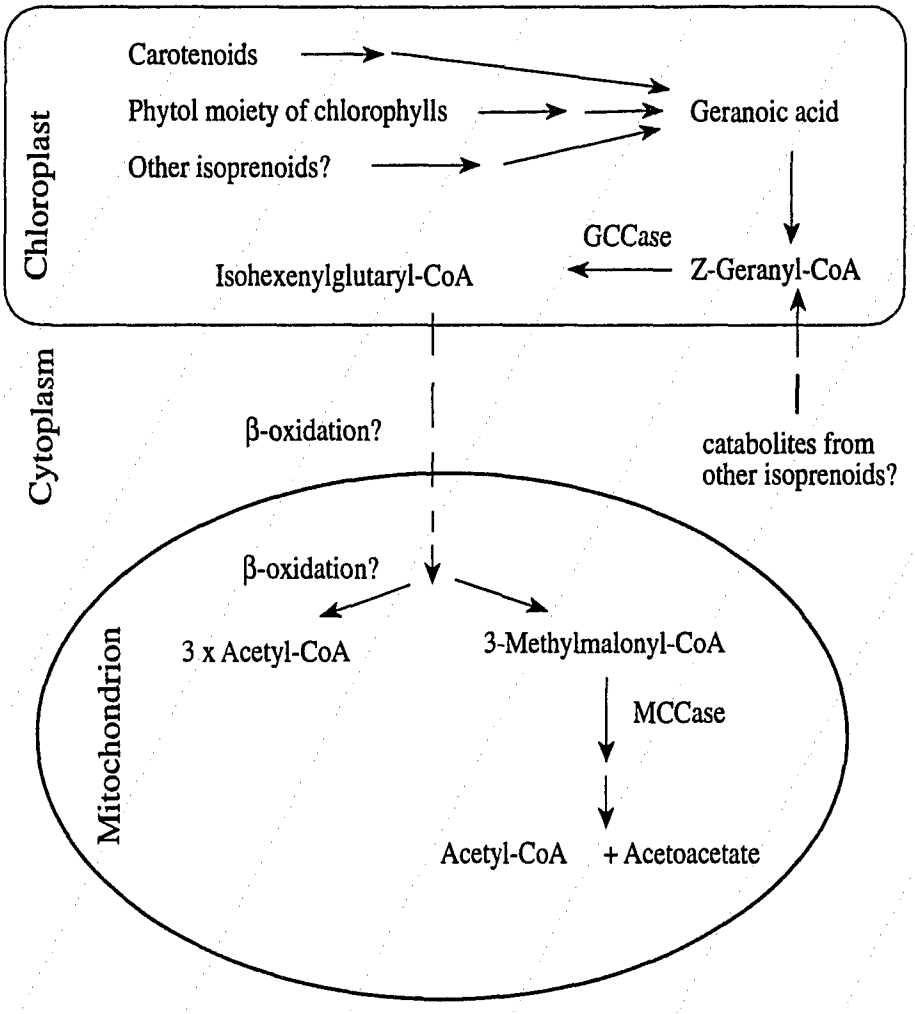

Fig. 9 Postulated role of GCCase in degradation of isoprenoids

Table I DISTRIBUTION OF GERANYL-CoA CARBOXYLASE ACTIVITY IN ORGANELLES FROM CARROT EMBRYO PROTOPLASTS

Enzyme or marker	Total activity (nmol/min)			% of recovered activity in each fraction	
	protoplast	cytosol	organelles	cytosol	organelles
MCCase	59.6	3.7	41.4	8	92
GCCase	14.2	0.07	7.2	1	99

Table II DISTRIBUTION OF GERANYL-CoA CARBOXYLASE ACTIVITY IN ORGANELLES FROM CARROT EMBRYO PROTOPLASTS*

Enzyme or marker	Total activity (nmol/min) (% recovered activity)		
	cytosol (fractions 1-3)	mitochondria (fractions 16-20)	chloroplast (fractions 26-29)
Fumarase	9 (28)	21.8 (67)	1.6 (5)
Rubisco	12.2 (12)	15.7 (14)	76.3 (73)
GCCase	15.9 (24)	20.2 (30)	30.8 (46)

* 84% of the GCCase is associated with chloroplasts if cross contamination (as indicated by marker enzyme distribution) is taken into account.

GENERAL SUMMARY

This thesis presents a study of the metabolic function of biotin-containing carboxylases, with special focus on ACCase and GCCase.

A novel approach was used to regulate the activity of biotin-containing enzymes through the binding of streptavidin to biotin and depletion of the level of biotin in the cells. The streptavidin gene was expressed in *E. coli* as a non-fusion protein and as a glutathione S-transferase fusion protein. The majority of the non-fusion protein was insoluble; in contrast, the glutathione S-transferase streptavidin fusion protein was soluble. Non-denaturing gel electrophoresis indicated the assembly of the subunits of the fusion protein was only partial. However, the expressed glutathione S-transferase streptavidin fusion protein was able to bind biotin *in vivo.* The growth rate of biotin auxotrophs of *E coli* in biotin deficient media was decreased by the expression of the glutathione S-transferase streptavidin fusion protein. Decreases in growth rate were correlated with decreases in ACCase activity. These experiments have provided a valuable approach that can be used to study the metabolic function of biotin-containing enzymes, the regulation of biotin biosynthesis, and the biotinylation of biotin-containing enzymes.

GCCase is the most recently identified biotin-containing enzyme in plants. The metabolic function of this enzyme in plants is unknown. However it may play an important role in isoprenoid catabolism. We have characterized this enzyme during plant development in two dicot species (soybean and carrot) and one monocot species (maize).
GCCase is present in all plant organs examined. GCCase activity is highest in non-photosynthetic tissues, especially roots, for example, activity is about 20-fold greater in the root than in the primary leaf in the 15 day old soybean plant. During development of soybean cotyledons post-imbibition, peaks of GCCase activities occur in 12 day old cotyledons and in

cotyledons at senescence. During embryogenesis, GCCase activity is highest in highly embryogenic cells. Differential centrifugation indicates that 99% of the GCCase activity is associated with the organelle fraction (plastids and mitochondria) in carrot. Sucrose and percoll density gradient centrifugation indicate that in carrot at least 84% of this activity is plastidic. In maize, sucrose density gradient fractionation of subcellular organelles indicates almost all the activity occurs in the plastids. A metabolic function for this enzyme in plants is proposed.

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