Plant Physiol. (1993) 102: 957–965

Purification and Characterization of 3-Methylcrotonyl-Coenzyme A Carboxylase from Higher Plant Mitochondria[1]

Claude Alban, Pierre Baldet, Stella Axiotis, and Roland Douce*

Laboratoire Mixte, Centre National de la Recherche Scientifique/Rhône-Poulenc UMR-41, Rhône-Poulenc Agrochimie, 14–20 rue Pierre Baizet, 69263 Lyon, France

3-Methylcrotonyl-coenzyme A (CoA) carboxylase was purified to homogeneity from pea (*Pisum sativum* L.) leaf and potato (*Solanum tuberosum* L.) tuber mitochondria. The native enzyme has an apparent molecular weight of 530,000 in pea leaf and 500,000 in potato tuber as measured by gel filtration. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate disclosed two nonidentical subunits. The larger subunit (B subunit) is biotinylated and has an apparent molecular weight of 76,000 in pea leaf and 74,000 in potato tuber. The smaller subunit (A subunit) is biotin free and has an apparent molecular weight of 54,000 in pea leaf and 53,000 in potato tuber. The biotin content of the enzyme is 1 mol/133,000 g of protein and 1 mol/128,000 g of protein in pea leaf and potato tuber, respectively. These values are consistent with an A_4B_4 tetrameric structure for the native enzyme. Maximal 3-methylcrotonyl-CoA carboxylase activity was found at pH 8 to 8.3 and at 35 to 38°C in the presence of Mg^{2+}. Kinetic constants (apparent K_m values) for the enzyme substrates 3-methylcrotonyl-CoA, ATP, and HCO_3^- were: 0.1 mM, 0.1 mM, and 0.9 mM, respectively, for pea leaf 3-methylcrotonyl-CoA carboxylase and 0.1 mM, 0.07 mM, and 0.34 mM, respectively, for potato tuber 3-methylcrotonyl-CoA carboxylase. A steady-state kinetic analysis of the carboxylase-catalyzed carboxylation of 3-methylcrotonyl-CoA gave rise to parallel line patterns in double reciprocal plots of initial velocity with the substrate pairs 3-methylcrotonyl-CoA plus ATP and 3-methylcrotonyl-CoA plus HCO_3^- and an intersecting line pattern with the substrate pair HCO_3^- plus ATP. It was concluded that the kinetic mechanism involves a double displacement. Purified 3-methylcrotonyl-CoA carboxylase was inhibited by end products of the reaction catalyzed, namely ADP and orthophosphate, and by 3-hydroxy-3-methylglutaryl-CoA. Finally, as for the 3-methylcrotonyl-CoA carboxylases from mammalian and bacterial sources, plant 3-methylcrotonyl-CoA carboxylase was sensitive to sulfhydryl and arginyl reagents.

MCase (EC 6.4.1.4) is a biotin-containing enzyme that catalyzes the carboxylation of 3-methylcrotonyl-CoA to form 3-methylglutaconyl-CoA according to the following reaction:

3-methylcrotonyl-CoA + HCO_3^- + MgATP ↔ 3-methylglutaconyl-CoA + ADP + Pi.

This enzyme has been identified as part of the Leu catabolic pathway in mammals and various bacteria (for a review, see Wood and Barden, 1977) and has been implicated as a component enzyme of the "mevalonate shunt" (Popjak, 1971). For the last 15 years, the study of MCase has focused the interest of numerous scientists. Indeed, in humans, the inherited disorder 3-methylcrotonylglycinuria has been shown to be the result of a deficiency of MCase (Gompertz et al., 1973; Finnie et al., 1976; Weyler et al., 1977). However, studies concerning plant MCase have been limited. Wurtele and Nikolau identified several biotin-containing enzymes, including MCase, in cell-free extracts of monocot and dicot plant species (Wurtele and Nikolau, 1990) and in somatic carrot embryos (Wurtele and Nikolau, 1992). Assigning a specific role to this enzyme activity in plant cells requires the purification and characterization of the enzyme. We recently characterized MCase activity in highly purified pea (*Pisum sativum* L.) leaf mitochondria (Baldet et al., 1992). Here, we report for the first time the purification of MCase to homogeneity from pea leaf and potato (*Solanum tuberosum* L.) tuber mitochondria and characterize some biochemical parameters of these purified enzymes.

MATERIALS AND METHODS

Reagents

ATP, DTT, acetyl-CoA, butyryl-CoA, CoA, hexanoyl-CoA, 3-hydroxy-3-methylglutaryl-CoA, 3-methylcrotonyl-CoA, propionyl-CoA, avidin, D-biotin, mevalonate, *N*-ethylmaleimide, and phenylglyoxal were obtained from Sigma Chimie SARL (la Verpillière, France). $NaH^{14}CO_3$ (53.1 mCi/mmol) was purchased from Amersham. Horseradish peroxidase color development reagent and peroxidase-labeled streptavidin were purchased from Bio-Rad Laboratories (Munich, Germany). Sepharose CL-4B and Sephadex G-25 were obtained from Pharmacia. Cyanogen bromide was obtained from Jansen Chemica. Sethoxydim and diclofop-methyl were obtained from BASF Wyandotte Corp. and Hoechst, respectively. All other chemicals were of analytical grade.

Plant Materials

Pea (*Pisum sativum* L. var Douce provence) plants were grown from seeds in soil under a 12-h photoperiod of white light from fluorescent tubes (10–40 $\mu E\ m^{-2}\ s^{-1}$) at 18°C. The

[1] This study has been conducted under the Bioavenir program financed by Rhône-Poulenc with the contribution of the Ministère de la Recherche et de l'Espace and the Ministère de l'Industrie et du Commerce Extérieur.

* Corresponding author; fax 33-72-29-22-97.

Abbreviation: MCase, 3-methylcrotonyl-CoA carboxylase.

plants were watered every day with tap water. Potato tubers (*Solanum tuberosum* L.) were obtained from a local market.

Preparation of Mitochondria

Mitochondria were isolated and purified from 4 to 5 kg of fully expanded pea leaves as described by Douce et al. (1987) using self-generating Percoll gradients and a linear gradient of 0 to 10% (w/v) PVP-25 (Kca 25, Serva) (top to bottom). The mitochondria (>95% intact as judged by their impermeability to Cyt *c* [Douce et al., 1972]) were subsequently concentrated by differential centrifugation.

Mitochondria were isolated and purified from potato tubers as described by Douce et al. (1987) using self-generating Percoll gradients. The mitochondria were subsequently concentrated by differential centrifugation. To remove all of the contaminating peroxisomes, the purification procedure on Percoll gradients was repeated once. Under these conditions, we have verified that catalase (EC 1.11.1.6) activity with substrate (H_2O_2) concentrations up to 200 μM was almost negligible.

Mitochondria were lysed by 10-fold dilution in a buffer containing 50 mM Hepes (pH 8), 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 1 mM benzamidine-HCl, and 5 mM ϵ-aminocaproic acid (buffer A). After vortex mixing, the mitochondria were freeze-thawed three times to ensure complete lysis. Membranes were removed by centrifugation at 100,000*g* for 20 min (Ti 50 rotor, Beckman). The resulting supernatant fraction (matrix proteins) contained most of the MCase activity (Baldet et al., 1992).

Preparation of Crude Extracts

Pea leaves (3–5 g) were frozen in liquid nitrogen and finely ground using a mortar and pestle. The powder was then homogenized in two volumes of buffer A. The homogenate was filtered through a 20-μm nylon mesh and the filtrate was centrifuged at 40,000*g* for 30 min. The supernatant comprised the crude extract and was used immediately for MCase activity, SDS/PAGE, and western blot analyses.

Electrophoretic Analyses of Proteins

Polypeptides from the different fractions were separated by SDS/PAGE in gels containing a 7.5 to 15% (w/v) acrylamide gradient. The experimental conditions for gel preparation, sample solubilization, electrophoresis, and gel staining were as detailed by Chua (1980). In some experiments, polypeptides were also transferred electrophoretically onto nitrocellulose sheets (Bio-Rad), essentially according to Towbin et al. (1979). Biotin-containing polypeptides were detected using horseradish peroxidase-labeled streptavidin as described previously (Baldet et al., 1992).

Purification of MCase

Crystalline ammonium sulfate was added to a mitochondrial matrix fraction (prepared as described above) with slow stirring to yield a 50% saturated solution. The solution was stirred for 20 min at 4°C and the resulting precipitate was collected by centrifugation (40,000*g*, JA 20 rotor, Beckman, 10 min) and resuspended in a minimal volume of a buffer containing 20 mM Hepes (pH 8), 10% (w/v) glycerol, 0.5 M KCl, 1 mM EDTA, 5 mM DTT, 1 mM benzamidine-HCl, and 5 mM ϵ-aminocaproic acid (buffer B). The protein suspension was clarified by centrifugation (40,000*g*, JA 20 rotor, Beckman, 20 min) and subjected to purification by affinity chromatography.

A monomeric avidin affinity chromatography column (1.5 × 20 cm; 35 mL) was prepared by covalent attachment of tetrameric avidin (10–15 units/mg protein, Sigma) to Sepharose CL-4B previously activated with cyanogen bromide, essentially following the procedure reported by Kohansky and Lane (1985). The column, connected to a Pharmacia fast protein liquid chromatography system, was prepared for use by washing with elution buffer containing 20 mM Hepes (pH 8), 10% (w/v) glycerol, 0.5 M KCl, 1 mM EDTA, 5 mM DTT, 2 mM biotin, 1 mM benzamidine-HCl, and 5 mM ϵ-aminocaproic acid (buffer C), followed by a wash with 0.1 M Gly, 0.2 M KCl (pH 1.5) to remove exchangeable biotin. The exchangeable biotin binding capacity was 18 nmol/mL packed volume as determined using [^{14}C]biotin (Kohansky and Lane, 1985). The MCase-enriched fraction from the ammonium sulfate fractionation step was loaded (flow rate 0.1 mL/min) on the column previously equilibrated in buffer B. Unbound proteins were removed by washing with 50 mL of the same buffer at a flow rate of 0.2 mL/min. The enzyme was finally eluted from the column with 15 mL of buffer C. Active fractions were pooled, dialyzed overnight against buffer B without KCl, concentrated to a final concentration of about 0.5 mg/mL with Macrosep-10 tubes (Filtron), and stored at −80°C until use.

Determination of Native MCase M_r

The M_r of native purified MCase was estimated by gel filtration on a prepacked Superdex 200 column (1.6 × 60 cm, 120 mL of gel [Pharmacia]) equilibrated in buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.2 M KCl. The column, connected to a Pharmacia fast protein liquid chromatography system, was eluted at a flow rate of 1 mL/min. Fractions of 1.5 mL were collected. The M_r was calculated from a plot of ln M_r against elution volume.

Assay for MCase

All assays were optimized with respect to the concentration of each reaction component and to the pH of the reaction mixture. The activity of MCase was measured as the incorporation of radioactivity from $NaH^{14}CO_3$ into an acid-stable product using a modification of the assay described by Wurtele and Nikolau (1990). The standard assay consisted of 50 mM Hepes (pH 8), 2.5 mM $MgCl_2$, 1 mM ATP, 2 mM DTT, 10 mM $NaH^{14}CO_3$ (1 mCi/mmol), 20 mM KCl, 0.4 mM 3-methylcrotonyl-CoA, and 1 to 50 μg of protein in a final volume of 200 μL. The assays were initiated by the addition of 3-methylcrotonyl-CoA. After 2 to 20 min of incubation at 30°C in a shaking water bath, 150-μL aliquots of the reaction mixture were mixed vigorously with 40 μL of 12 N HCl to stop the reaction. The solution was then taken to dryness under nitrogen gassing, and the acid-stable radioactivity was

quantified in a liquid scintillation counter. Duplicate assays without 3-methylcrotonyl-CoA were run as controls. One unit of activity corresponds to the amount of enzyme that catalyzed the incorporation of 1 μmol of bicarbonate per min.

Biotin Determination

The biotin content of MCase was determined as described previously (Baldet et al., 1992). Aliquots (20 μg) of pure enzyme were treated with 15% (w/v) TCA for 1 h at 4°C and centrifuged for 20 min at 12,000*g*. Precipitated protein pellets containing all the bound biotin and no free biotin were then heated in 4 N H_2SO_4 for 2 h at 120°C and suspensions were passed through a 0.2-μm filter. After neutralization with 10 N NaOH, samples were lyophilized. The lyophilized powder was resuspended in a minimum volume of water. Then, 20-μL aliquots of the samples were spotted into wells on agar plates containing the minimal medium M9 (Maniatis et al., 1982) and 2,3,5-triphenyltetrazolium chloride (100 mg/L) that had been seeded with the *bioB105* strain of *Escherichia coli* (Eisenberg, 1975). After incubation at 37°C for 18 to 20 h, biotin was detected as growth of bacteria, which resulted in the appearance of reddish color. The total amount of biotin present in the sample was determined as the square of the diameter of the spot and was referred to a standard curve constructed from known amounts of free D-biotin.

Protein Determination

Protein was measured by the method of Bradford (1976) using the Bio-Rad protein assay reagent with γ-globulin as the standard.

RESULTS

MCase Activity during Pea Leaf Formation

In a previous publication, we demonstrated that pea leaf mitochondria contain in their matrix space a very active biotin-dependent MCase (Baldet et al., 1992). However, the activity of MCase increased considerably during pea leaf development. Thus, MCase activity measured in young pea leaf crude extracts (4 d after germination) was very low (0.19 nmol min^{-1} mg^{-1} protein) and increased rapidly during leaf development to reach values nearly 10 times higher in the fully expanded leaf (Fig. 1A). The MCase activity in pea leaf is associated with a biotinyl polypeptide of M_r 76,000 (Baldet et al., 1992). The appearance of this polypeptide during leaf formation paralleled the evolution of MCase activity (Fig. 1B), suggesting that the increase in activity was linked to an increase in the synthesis of MCase.

Isolation of MCase from Pea Leaf and Potato Tuber Mitochondria

Pea leaf mitochondria used in our experiments were extracted from 16-d-old leaves, where MCase activity was found to be high. Pea leaf and potato tuber mitochondria were purified on Percoll gradients (Douce et al., 1987). The amount of extramitochondrial contamination in the Percoll-purified mitochondrial fraction was determined by measuring the activity of various marker enzymes of cytosol (pyrophosphate:Fru-6-P-1-phosphotransferase; EC 2.7.1.90), chloroplasts (glyceraldehyde-3-P dehydrogenase; EC 1.2.1.13), and peroxisomes (catalase, hydroxypyruvate reductase; EC 1.1.1.81). This analysis showed that extramitochondrial contaminations were negligible (Lunn et al., 1990; not shown). The isolated intact mitochondria were lysed (see "Materials and Methods") and soluble proteins (matrix) were separated from membranes by centrifugation. Over 90% of MCase activity was recovered in the soluble protein fraction. The purification procedure described in "Materials and Methods" provides MCase in good yield and at a high level of purity in 1 working day. Table I shows a representative purification of MCase from pea leaf mitochondria. Ammonium sulfate fractionation was utilized to concentrate protein. Typically, 60 to 90% of the total MCase activity was recovered in the 0 to 50% ammonium sulfate fraction with a 2- to 3-fold enrichment. Thereafter, the ammonium sulfate fraction was directly

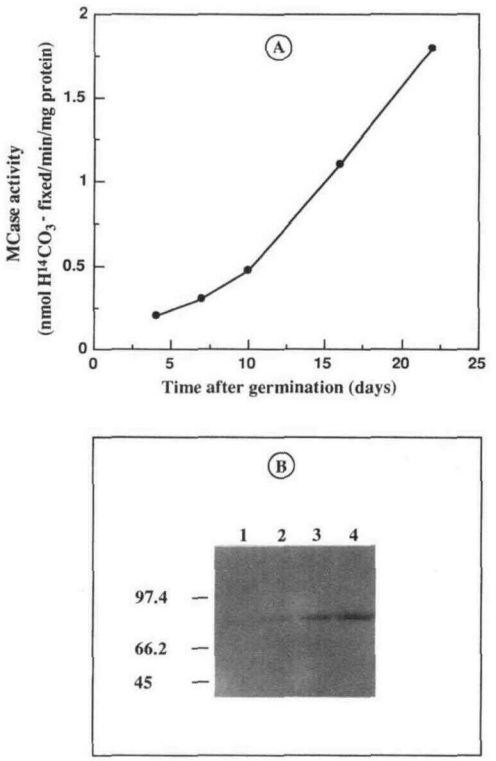

Figure 1. Evolution of MCase activity during the development of pea leaves. A, At the time indicated, pea leaves (3–5 g) were harvested, ground in liquid nitrogen, and resuspended in buffer A (see "Materials and Methods"). After centrifugation on an Eppendorf centrifuge, supernatants (leaf extracts) were used for MCase activity as described in "Materials and Methods." B, Extracts from 4- (1), 7- (2), 10- (3), and 16-d-old (4) pea leaves were separated on SDS-PAGE gels, the polypeptides were transferred onto nitrocellulose sheets, and biotinylated polypeptides were labeled with streptavidin-peroxidase and revealed with the color development reagent 4-chloro-1-naphtol as described in "Materials and Methods." Protein loaded on each lane was equivalent to 1 mg. Molecular masses on the left are given in kD.

Table I. *Purification of MCase from pea leaf mitochondria*

Step	Total Protein	Total Activity	Specific Activity	Recovery	Purification
	mg	*units*	*units/mg protein*	%	*-fold*
Matrix	132	1.8	0.013	100	1
Ammonium sulfate 0–50%	47.5	1.2	0.025	66	1.9
Monomeric-avidin-Sepharose	0.1	0.45	4.5	25	346

chromatographed on a monomeric avidin-Sepharose column without previous desalting. This affinity column is specific for biotin proteins (Henrikson et al., 1979; Kohansky and Lane, 1985). The procedure resulted in an overall 350-fold purification. The yield obtained from 130 mg of matrix protein was about 25%. Furthermore, it must be remembered that preparation of the mitochondrial matrix represents an important purification of the enzyme. Therefore, the actual purification of MCase from crude leaf extract is more than 4000-fold. On the other hand, attempts to purify MCase from crude extracts using the same procedure were unsuccessful because of the presence of other biotin proteins (i.e. acetyl-CoA carboxylase) in these extracts and the lack of protection of the enzyme by the mitochondrial membranes from proteases released from the vacuolar space during the course of tissue grinding.

Using this method, we also purified MCase from purified potato tuber mitochondria. The final enrichment of the preparation and the yields of purified enzyme obtained were similar to those of the pea enzyme (Table II). MCase was found to be unstable in the absence of glycerol and thiols such as DTT or β-mercaptoethanol. Therefore, all purification media contained 10% glycerol and 5 mM DTT. In addition, the presence of protease inhibitors in the lysis buffer and chromatography media protected the enzyme against limited proteolysis (not shown). Under these conditions, the purified enzyme could be stored for prolonged periods at −80°C with essentially no loss of activity.

Structure of the Purified MCase

On the basis of SDS-PAGE, pea leaf MCase contained two nonidentical subunits present in equal proportions: a smaller biotin-free subunit (A subunit) and a larger biotin-containing subunit (B subunit) with apparent M_r of 54,000 and 76,000, respectively (Fig. 2). Potato tuber MCase, analyzed under the same conditions, was also composed of two different subunits, but with slightly lower M_rs than those of the pea enzyme (Fig. 3, Table III). The apparent M_r of the native enzyme was estimated by gel filtration chromatography on a Superdex 200 column (Pharmacia) and was found to be 530,000 for the pea MCase and 500,000 for the potato MCase (MCase emerged from the column between the standards thyroglobulin [M_r of 668,700] and apoferritin [M_r of 443,000]). These values are consistent with a tetrameric structure of the native MCase, A_4B_4, for the two plant enzymes.

The biotin content of purified MCase was determined after acid hydrolysis of the protein and biological determination of biotin released, as described in "Materials and Methods."

Table II. *Purification of MCase from potato tuber mitochondria*

Step	Total Protein	Total Activity	Specific Activity	Recovery	Purification
	mg	*units*	*units/mg protein*	%	*-fold*
Matrix	524	3.3	0.006	100	1
Ammonium sulfate 0–50%	175	2.5	0.014	75	2.3
Monomeric-avidin-Sepharose	0.18	0.5	2.77	15	461

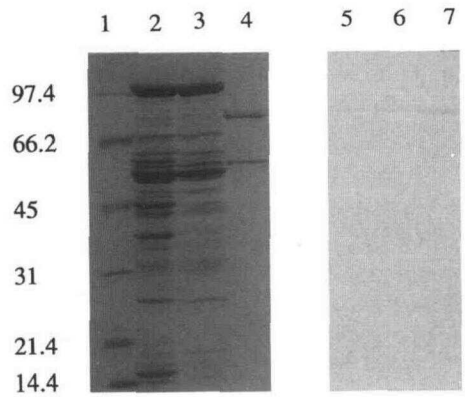

Figure 2. Documentation of purification procedure for pea leaf MCase by SDS-PAGE. Polypeptides were separated on SDS/PAGE gels and stained with Coomassie brilliant blue R-250 (lanes 1–4) or labeled with streptavidin-peroxidase and revealed with the color development reagent 4-chloro-1-naphtol after electrotransfer onto a nitrocellulose sheet (lanes 5–7) (see "Materials and Methods"). Lane 1, Molecular mass markers, 10 μg each; lanes 2 and 5, matrix extract, 100 μg; lanes 3 and 6, ammonium sulfate 0 to 50% fraction, 100 μg; lanes 4 and 7, affinity-purified MCase, 10 μg. Molecular masses on the left are given in kD.

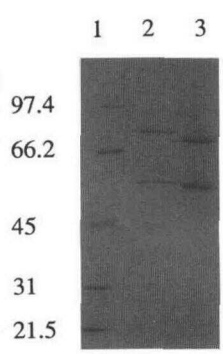

Figure 3. Analysis by SDS-PAGE of purified MCase from pea leaf and potato tuber mitochondria. Polypeptides were separated on SDS-PAGE gels and stained with Coomassie brilliant blue R-250 as described in "Materials and Methods." Lane 1, Molecular mass markers, 10 μg each; lane 2, purified MCase from pea leaf mitochondria, 6 μg; lane 3, purified MCase from potato tuber mitochondria, 10 μg. Molecular masses on the left are given in kD.

Table III. *Summary of the physicochemical and kinetic properties of mitochondrial MCase from pea leaves and potato tubers*

	Pea MCase	Potato MCase
Native M_r (gel filtration)	530,000	500,000
Subunit M_r	A: 54,000	A: 53,000
	B: 76,000	B: 74,000
Biotin content (μg/mg protein)	1.83	1.9
Structure	A_4B_4	A_4B_4
K_m for 3-methylcrotonyl-CoA	0.1 mM	0.1 mM
K_m for ATP	0.1 mM	0.07 mM
K_m for HCO_3^-	0.9 mM	0.34 mM
Optimal pH	8.3	8.0–8.2
Optimal temperature	35°C	38°C

This assay gave values of 1.83 μg of biotin/mg of protein for pea leaf MCase and 1.9 μg of biotin/mg of protein for potato tuber MCase (average of triplicate determinations). These values corresponded to 1 mol of biotin per 133,000 g of protein and 1 mol of biotin per 128,000 g of protein for pea leaf and potato tuber MCase, respectively. Assuming M_rs of 530,000 and 500,000, these results indicate the presence of about four biotin residues per enzyme molecule in the two plant MCases. These data confirm the A_4B_4 structure proposed above for these enzymes, where each B subunit contains one biotin prosthetic group. The pea leaf MCase purified in this study appears to be similar to the potato tuber MCase based on its elution profile from monomeric-avidin-Sepharose and its apparent subunit composition.

Biochemical Properties

It has been previously shown that mammalian MCase is activated (5- to 7-fold) by inclusion of 50 to 100 mM K^+ ions in the assay medium (Weyler et al., 1977; Lau et al., 1980). We found that this was not the case for purified plant MCase. Pea as well as potato enzymes were stimulated only 1.1- to 1.2-fold by the presence of 20 mM KCl. Furthermore, KCl concentrations higher than 50 mM inhibited enzyme activity severely (not shown).

The activity of purified MCase was assayed as a function of pH by buffering the reaction mixture with either Mops or Tris-HCl from pH 6 to 9. The optimum pH range measured at 30°C was quite narrow, with a maximum at pH 8.3 for the pea enzyme and 8.0 to 8.2 for the potato enzyme (Table III). In this respect, plant MCase resembles MCase from *Achromobacter* (Schiele and Lynen, 1981) and bovine kidney (Lau et al., 1980).

The effect of temperature on MCase activity was measured in the range 15 to 55°C using standard assay conditions. Maximal enzyme activity occurs at 35°C in the case of pea leaf MCase and at 38°C in the case of the potato tuber enzyme (Table III). These optimum temperatures were in the same order as those reported for mammalian and bacterial MCase (Lau et al., 1980; Schiele and Lynen, 1981); however, plant MCase, unlike *Pseudomonas citronellolis* MCase (Hector and Fall, 1976), was not inactivated, even partially, upon heating to 50°C for 1 min.

In previous work, we demonstrated that plant MCase, like other biotin-dependent carboxylases described so far, required the presence of Mg^{2+} ions for activity (Baldet et al., 1992). Maximal activity was obtained at 2.5 and 3 mM Mg^{2+} ions with purified pea leaf and potato tuber MCase, respectively, when ATP was included at a concentration of 1 mM. Inhibition of enzyme activity at Mg^{2+} ion concentrations exceeding 5 mM (a concentration of Mg^{2+} well above the physiological levels found in cells) was also noticed, and, as shown before, an accurate K_m value for Mg^{2+} could not be determined because Michaelis-Menten plots produced a sigmoidal curve (Baldet et al., 1992).

Kinetic experiments were carried out under steady-state conditions at saturating Mg^{2+} concentrations (2.5–3 mM) with varied concentrations of substrates. Purified MCase from pea leaf and potato tuber mitochondria exhibited Michaelis-Menten kinetics with respect to ATP, methylcrotonyl-CoA, and $NaHCO_3$. The apparent K_m values for these substrates were determined by using the double-reciprocal plot method (Lineweaver-Burk equation) to be 0.1 mM, 0.1 mM, and 0.9 mM, respectively, for the pea enzyme, and 0.07 mM, 0.1 mM, and 0.34 mM for the potato enzyme (Table III). Double-reciprocal plots of initial velocity versus 3-methylcrotonyl-CoA concentration at different fixed levels of ATP or HCO_3^- gave rise to parallel patterns (Fig. 4, A and B). However, when HCO_3^- and ATP levels were both varied, linear intersecting lines were obtained, with the intersection point on the abscissa (Fig. 4C). These results are consistent with a "Bi Bi Uni Uni ping-pong" mechanism, i.e. a double-displacement kinetic mechanism, for pea leaf MCase. The substrate specificity of plant MCase was also assessed using the substrates of other known biotin enzymes, acetyl-CoA, propionyl-CoA, and pyruvate, and different acyl-CoA esters such as butyryl-CoA and hexanoyl-CoA. At concentrations of 0.4 mM, none of these substrates were carboxylated, indicating, first, that purified MCase fractions were not contaminated by other biotin enzymes and, second, that plant MCase exhibited a high substrate specificity for 3-methylcrotonyl-CoA.

The influence of various metabolites on the activity of purified MCase from pea leaf mitochondria was studied as one possible mechanism for its regulation (Table IV). We analyzed two classes of compounds: (a) end products of the MCase reaction and related compounds, and (b) inhibitors of monocot acetyl-CoA carboxylase. ADP and Pi (1.0 mM), which are products of the reaction, inhibited the enzyme activity substantially (Table IV). It is interesting that the addition of both ADP and Pi inhibited the enzyme activity in an additive mode. CoA, acetyl-CoA, and mevalonate at concentrations up to 1 mM had little or no effect on MCase activity. On the other hand, 3-hydroxy-3-methylglutaryl-CoA, the direct precursor of mevalonate synthesis, strongly inhibited MCase activity at 1 mM concentration (more than 80% inhibition). Approximately 40% inhibition was observed at 0.1 mM 3-hydroxy-3-methylglutaryl-CoA. Cyclohexanedione and aryloxyphenoxypropionate herbicides are strong inhibitors of monocot acetyl-CoA carboxylase (Rendina and Felts, 1988; Rendina et al., 1988), another biotin enzyme exhibiting a functional mechanism similar to that of MCase. Therefore, it was interesting to determine the effect of these herbicides on the activity of purified plant MCase. Neither the cyclohexanedione herbicide (sethoxydim) nor the arylox-

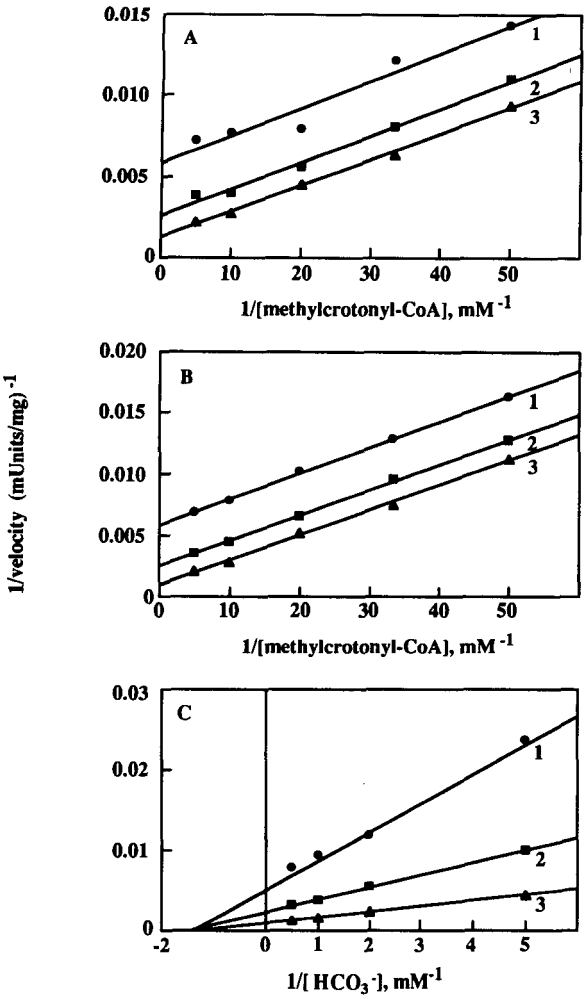

Figure 4. Steady-state kinetic experiments with purified pea leaf MCase. A, Double-reciprocal plot of initial velocities with variable 3-methylcrotonyl-CoA concentrations and different fixed concentrations of ATP. Concentrations of 3-methylcrotonyl-CoA used were 0.02 mM, 0.03 mM, 0.05 mM, 0.1 mM, and 0.2 mM. Concentrations of ATP used were 0.05 mM (1); 0.1 mM (2); and 0.2 mM (3). B, Double-reciprocal plot of initial velocities with variable 3-methylcrotonyl-CoA concentrations and different fixed concentrations of HCO_3^-. Concentrations of 3-methylcrotonyl-CoA used were as in A. Concentrations of HCO_3^- used were 0.5 mM (1); 1 mM (2); and 2 mM (3). C, Double-reciprocal plot of initial velocities with variable HCO_3^- concentrations and different fixed concentrations of ATP. Concentrations of HCO_3^- used were 0.2 mM, 0.5 mM, 1 mM, and 2 mM. Concentrations of ATP used were 0.05 mM (1); 0.1 mM (2); and 0.2 mM (3). Each assay was initiated by addition of 2 μg of purified pea leaf MCase to 200 μL of the reaction mixture described in "Materials and Methods." Other conditions were as described in "Materials and Methods."

yphenoxypropionic acid herbicide (diclofop-methyl) affected MCase activity at concentrations up to 0.1 mM.

Human and bovine MCase were found to be sensitive to sulfhydryl inhibitors such as *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, or iodoacetamide and to phenylglyoxal. Previous studies (Wolf et al., 1979; Lau et al., 1980) showed that

Table IV. *Effect of various metabolites on the activity of purified MCase from pea leaf mitochondria*

MCase activity was assayed at 30°C as described in "Materials and Methods." The activity of the native enzyme was 4.5 units/mg of protein. These data are from a representative experiment repeated four times.

Compounds	Concentration	Relative Activity
	mM	%
End metabolites and related compounds		
Pi	0.1	78
Pi	1	56
ADP	0.1	68
ADP	1	29
ADP + Pi	1	17
3-Hydroxy-3-methylglutaryl-CoA	0.1	59
3-Hydroxy-3-methylglutaryl-CoA	1	18
CoA	1	90
Mevalonate	1	95
Acetyl-CoA	1	90
Inhibitors of monocot acetyl-CoA carboxylase		
Sethoxydim	0.1	98
Diclofop-methyl	0.1	100

phenylglyoxal interacts specifically with the guanidinium group of Arg. The inhibitory effect of *N*-ethylmaleimide and phenylglyoxal at various concentrations on plant MCase is shown in Table V. Both products inhibited purified pea leaf MCase. In both cases, the inhibition was time (not shown) and concentration dependent (Table V). Therefore, at a con-

Table V. *Inhibition by N-ethylmaleimide and phenylglyoxal of MCase activity purified from pea leaf mitochondria*

Aliquots of 20 μg of pure enzyme were passed through small Sephadex G-25 columns equilibrated with 50 mM borate (pH 8). Desalted fractions were then incubated for 15 min at 25°C in 50 mM borate (pH 8) with or without various concentrations of phenylglyoxal or *N*-ethylmaleimide in a final volume of 40 μL. Thereafter, 20 μL of these reaction mixtures were added to the mixture used for measuring enzyme activity, and MCase activity was assayed at 30°C for 20 min as described in "Materials and Methods." These data are from a representative experiment repeated three times.

Inhibitor	Concentration	Inhibition
	mM	%
N-Ethylmaleimide	0	0
	0.01	5
	0.05	15
	0.1	20
	0.5	75
	1	100
Phenylglyoxal	0	0
	1	30
	2	50
	3	80
	5	95
	10	100

centration of 0.01 mM and after 15 min of incubation with purified MCase, *N*-ethylmaleimide affected the enzyme activity only very slightly. However, the inhibition was complete at an *N*-ethylmaleimide concentration of 1 mM. Similarly, phenylglyoxal partially inhibited the MCase activity at 1 mM (30% inhibition) and almost completely inhibited it at concentrations of 5 to 10 mM. Preincubation (10 min) of the enzyme with ATP or 3-methylcrotonyl-CoA, the natural substrates of MCase, protected the enzyme against *N*-ethylmaleimide and phenylglyoxal inactivation: ATP (5 mM) and 3-methylcrotonyl-CoA (2 mM) afforded nearly complete protection (70–100% protection) against inhibition by phenylglyoxal (2.5 mM) and *N*-ethylmaleimide (0.5 mM).

DISCUSSION

In this study, we have identified, purified, and characterized, for the first time, an MCase from plant tissues. The purification procedure described in this article provides mitochondrial MCase from pea leaves and potato tubers in good yield and at a high level of purity in 1 working day. MCase has been isolated from total extracts of mammalian and bacterial sources (Apitz-Castro et al., 1970; Fall and Hector, 1977; Lau et al., 1980; Oei and Robinson, 1985). The protocols employed by these authors involved classical chromatographic techniques, including ion-exchange chromatography, gel filtration, dye-ligand chromatography, and hydroxylapatite chromatography (Apitz-Castro et al., 1970; Fall and Hector, 1977; Lau et al., 1980) or affinity chromatography using monomeric avidin gels (Oei and Robinson, 1985), as developed by Henrikson et al. (1979) and Kohansky and Lane (1985). If we take into consideration the great lability of plant MCase and the relatively low levels of the enzyme in mitochondria (1 mg/330 mg of matrix protein in the case of pea leaf mitochondria and 1 mg/640 mg of matrix protein in the case of potato tuber mitochondria), the use of pure mitochondria and affinity chromatography on a monomeric avidin-Sepharose column appeared to be the most convenient way to purify plant MCase to homogeneity.

SDS-PAGE analyses of plant MCase revealed two distinct bands of equal density, A and B subunits, corresponding to M_rs of about 54,000 and 76,000 for the pea enzyme and 53,000 and 74,000 for the potato enzyme. The larger subunit, as judged by streptavidin peroxidase labeling, contained the biotin prosthetic group. As previously demonstrated, the presence of biotin covalently linked to the enzyme was essential for MCase activity (Baldet et al., 1992). A similar subunit composition was reported for mammalian and bacterial MCases, with some variations in the size of the two subunits. Thus, M_rs in the range of 60,000 to 78,000 for the A subunit and 73,000 to 96,000 for the B subunit have been reported from mammalian tissues (Lau et al., 1980; Oei and Robinson, 1985) and bacteria (Schiele et al., 1975; Fall and Hector, 1977). In all cases, the larger subunit was biotinylated.

The native enzyme was determined to have a tetrameric structure, i.e. A_4B_4. The arguments in favor of this conclusion are as follows. First, gel filtration experiments disclosed an M_r in the range of 500,000 to 530,000. Second, the biotin content of plant MCase was consistent with a stoichiometry of four biotin molecules per A_4B_4 tetramer, presumably with each B subunit containing one biotin prosthetic group. In this respect, plant MCase resembles bacterial MCase (Apitz-Castro et al., 1970; Fall and Hector, 1977) but differs from mammalian MCase, which seems to exhibit a hexameric structure (A_6B_6) (Lau et al., 1980). These differences are probably linked to different hydrophobic properties of MCase. Indeed, plant and bacterial MCases are soluble enzymes (Apitz-Castro et al., 1970; Fall and Hector, 1977; Baldet et al., 1992). In contrast, the mammalian MCase is associated with the inner mitochondrial membrane (Hector et al., 1980).

The specific activities we report for plant MCase, 2.7 to 4.5 units/mg of protein, are comparable with those reported for the bovine kidney enzyme (3.8 units/mg of protein; Lau et al., 1980), but are somewhat lower than those reported for *Achromobacter* MCase (9.3–9.9 units/mg of protein; Apitz-Castro et al., 1970; Schiele and Lynen, 1981), for *Pseudomonas citronellolis* MCase (10.5 units/mg of protein; Fall and Hector, 1977), or for rat liver MCase (24.2 units/mg of protein; Oei and Robinson, 1985). The K_m values measured for purified MCase from pea leaves and potato tubers for 3-methylcrotonyl-CoA, ATP, and bicarbonate (0.1 mM, 0.07–0.1 mM, and 0.34–0.9 mM, respectively) are in the range of values reported for this enzyme from mammals and bacteria (Lau et al., 1980; Schiele and Lynen, 1981). Steady-state kinetic experiments demonstrated that the plant MCase-catalyzed reaction, like that of other biotin enzymes described so far (Wood and Barden, 1977), probably proceeds by a double-displacement mechanism where ATP and bicarbonate bind to the enzyme in a first half-reaction and react to form carboxybiotinyl enzyme. Then, ADP and Pi produced by hydrolysis of ATP are released. In a second half-reaction, 3-methylcrotonyl-CoA binds to the carboxybiotinyl enzyme and is carboxylated to form 3-methylglutaconyl-CoA.

The activity of all the biotin enzymes studied to date has been shown to be dependent upon the presence of divalent metal cations. Furthermore, it has been demonstrated that MgATP is the actual substrate for biotin enzymes (for a review, see Knowles, 1989), and it is assumed that this is also the case for the plant MCase.

The activity of plant MCase was modulated by several metabolites. The purified enzyme from pea leaf mitochondria was inhibited by the reaction end products, namely ADP and Pi. Unfortunately, we could not examine the effect of the third end product of MCase, 3-methylglutaconyl-CoA, because this compound was not commercially available.

MCase has been implicated in the Leu catabolism pathway in a variety of organisms (Wood and Barden, 1977) and has been implicated as a component enzyme of the mevalonate shunt (Popjak, 1971). The possible involvement of plant MCase in these metabolic pathways is still unknown. On the other hand, propionyl-CoA carboxylase, another biotin enzyme involved in the breakdown of Ile, which has been detected recently in various plant tissues (Wurtele and Nikolau, 1990, 1992), was not detected in purified plant mitochondria (Baldet et al., 1992, and this work). The results of our work show that neither acetyl-CoA, the end product of Leu degradation, nor mevalonate affected plant MCase activity. However, 3-hydroxy-3-methylglutaryl-CoA, the direct

precursor of mevalonate synthesis, inhibited MCase activity significantly. Whether this inhibitory effect reflected true allosteric regulation of mevalonate synthesis or was simply due to a resemblance with the natural MCase substrate 3-methylcrotonyl-CoA remains to be established. MCase from pea leaf mitochondria was not affected by cyclohexanedione and aryloxyphenoxypropionic acid herbicides, which are strong inhibitors of monocot acetyl-CoA carboxylase (Rendina and Felts, 1988; Rendina et al., 1988). Thus, the structure of the MCase active site apparently differs greatly from that of acetyl-CoA carboxylase despite the similarity in the mechanism of carboxylation catalyzed by both enzymes.

Finally, we demonstrated that plant MCase was sensitive to *N*-ethylmaleimide, a sulfhydryl reagent, and to phenylglyoxal, a reagent known to modify Arg residues. These results suggest, but do not prove, that at least one Cys and one Arg residue are located within or near the active center of the enzyme. Preincubation of MCase with ATP or 3-methylcrotonyl-CoA led to protection against inhibition by these compounds. This last result is surprising, since it means that 3-methylcrotonyl-CoA is able to bind to the free enzyme, which is not in agreement with the double-displacement mechanism we have established for plant MCase. However, similar findings were reported for propionyl-CoA carboxylase and MCase from human fibroblast (Wolf et al., 1979). In contrast, inhibition of *Achromobacter* MCase by iodoacetamide was prevented by 3-methylcrotonyl-CoA but not by ATP (Schiele and Stürzer, 1975), whereas inhibition of bovine kidney MCase by *p*-hydroxymercuribenzoate, another sulfhydryl reagent, was prevented by ATP but not by 3-methylcrotonyl-CoA (Lau et al., 1980). On the other hand, bovine kidney MCase, which was inactivated by phenylglyoxal, was protected by ATP but not by 3-methylcrotonyl-CoA (Lau et al., 1980).

One possible explanation for the protection by 3-methylcrotonyl-CoA of MCase against inactivation by sulfhydryl and arginyl reagents would be the presence of another binding site on the enzyme for this compound that is not involved in the catalysis of the reaction but more probably is involved in the regulation of the enzyme activity. The fixation of 3-methylcrotonyl-CoA on this site would induce a modification of the enzyme conformation, preventing the binding of *N*-ethylmaleimide or phenylglyoxal on the active site. Moreover, such a regulatory site has been described for pyruvate carboxylase (EC 6.4.1.1), which is highly activated by acetyl-CoA (Attwood and Graneri, 1992). The differences in reactivities of Cys and Arg residues most probably reflect important structural differences between the mammalian, the bacterial, and the plant enzyme active sites and highlight the need for precise information regarding the plant system in order to better understand its regulation. In this respect, the routine purification of plant MCase that we developed in our laboratory will be helpful.

ACKNOWLEDGMENT

We are grateful to Dr. D. Job for helpful discussions and for critical reading of the manuscript.

Received January 25, 1993; accepted April 5, 1993.

LITERATURE CITED

Apitz-Castro R, Rehn K, Lynen F (1970) β-Methylcrotonyl-CoA carboxylase. Kristallisation und einige physikalische Eigenschaften. Eur J Biochem **16:** 71–79

Attwood PV, Graneri BDLA (1992) Bicarbonate-dependent ATP cleavage catalysed by pyruvate carboxylase in the absence of pyruvate. Biochem J **287:** 1011–1017

Baldet P, Alban C, Axiotis S, Douce R (1992) Characterization of biotin and 3-methylcrotonyl-coenzyme A carboxylase in higher plant mitochondria. Plant Physiol **99:** 450–455

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Chua NH (1980) Electrophoresis analysis of chloroplast proteins. Methods Enzymol **69:** 434–436

Douce R, Bourguignon J, Brouquisse R, Neuburger M (1987) Isolation of plant mitochondria: general principles and criteria of integrity. Methods Enzymol **148:** 403–415

Douce R, Christensen EL, Bonner WD (1972) Preparation of intact plant mitochondria. Biochim Biophys Acta **275:** 148–159

Eisenberg MA (1975) Mode of action of α-dehydrobiotin, a biotin analogue. J Bacteriol **123:** 248–254

Fall RR, Hector ML (1977) Acyl-coenzyme A carboxylases. Homologous 3-methylcrotonyl-CoA and geranyl-CoA carboxylases from *Pseudomonas citronellolis*. Biochemistry **16:** 4000–4005

Finnie MDA, Cottrall K, Seakins JWT, Snedden W (1976) Massive excretion of 2-oxoglutaric acid and 3 hydroxy-iso-valeric-acid in a patient with a deficiency of 3-methylcrotonyl coenzyme A carboxylase. Clin Chim Acta **73:** 513–519

Gompertz D, Goodey PA, Barlett K (1973) Evidence for the enzymatic defect in beta-methylcrotonylglycinuria. FEBS Lett **32:** 13–14

Hector ML, Cochran BC, Logue EA, Fall RR (1980) Subcellular localization of 3-methylcrotonyl-coenzyme A carboxylase in bovine kidney. Arch Biochem Biophys **199:** 28–36

Hector ML, Fall RR (1976) Evidence for distinct 3-methylcrotonyl-CoA and geranyl-CoA carboxylases in *Pseudomonas citronellolis*. Biochem Biophys Res Commun **71:** 746–753

Henrikson KP, George Allen SH, Lee Maloy W (1979) An avidin monomer affinity column for the purification of biotin-containing enzymes. Anal Biochem **94:** 366–370

Knowles JR (1989) The mechanism of biotin-dependent enzymes. Annu Rev Biochem **58:** 195–221

Kohansky RA, Lane DL (1985) Homogeneous functional insulin receptor from 3T3-L1 adipocytes. Purification using $N^{\alpha B1}$-(biotinyl-ε-aminocaproyl)insulin and avidin-Sepharose. J Biol Chem **260:** 5014–5025

Lau EP, Cochran BC, Fall RR (1980) Isolation of 3-methylcrotonyl-coenzyme A carboxylase from bovine kidney. Arch Biochem Biophys **205:** 352–359

Lunn JE, Droux M, Martin J, Douce R (1990) Localization of ATP sulfurylase and *O*-acetylserine(thiol)lyase in spinach leaves. Plant Physiol **94:** 1345–1352

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Oei J, Robinson BH (1985) Simultaneous preparation of the three biotin-containing mitochondrial carboxylases from rat liver. Biochim Biophys Acta **840:** 1–5

Popjak G (1971) Specificity of enzymes of sterol biosynthesis. Harvey Lect **65:** 127–156

Rendina AR, Felts JM (1988) Cyclohexanedione herbicides are selective and potent inhibitors of acetyl-CoA carboxylase from grasses. Plant Physiol **86:** 983–986

Rendina RR, Felts JM, Beaudoin JD, Craig-Kennard AC, Look LL, Paraskos SL, Hagenah JA (1988) Kinetic characterization, stereoselectivity, and species selectivity of the inhibition of plant acetyl-CoA carboxylase by the aryloxyphenoxypropionic acid grass herbicides. Arch Biochem Biophys **265:** 219–225

Schiele U, Lynen F (1981) 3-Methylcrotonyl-CoA carboxylase from *Achromobacter*. Methods Enzymol **71:** 781–791

Schiele U, Niedermeier R, Stürzer M, Lynen F (1975) Investigations

of structure of 3-methylcrotonyl-CoA carboxylase from *Achromobacter*. Eur J Biochem **60:** 259–266

Schiele U, Stürzer M (1975) Decreased dissociation of the 3-methylcrotonyl-CoA carboxylase complex from *Achromobacter* in the presence of 3-methylcrotonyl-CoA. Possible regulatory mechanism for the intracellular degradation of the enzyme. Eur J Biochem **60:** 267–269

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354

Weyler W, Sweetman L, Maggio DC, Nyhan WL (1977) Deficiency of propionyl coenzyme A carboxylase and methylcrotonyl coenzyme A carboxylase in a patient with methylcrotonylglycinuria. Clin Chim Acta **76:** 321–328

Wolf B, Kalousek F, Rosenberg LE (1979) Essential arginine residues in the active sites of propionyl CoA carboxylase and beta-methylcrotonyl CoA carboxylase. Enzyme **24:** 302–306

Wood HG, Barden RE (1977) Biotin enzymes. Annu Rev Biochem **46:** 385–413

Wurtele ES, Nikolau BJ (1990) Plants contain multiple biotin enzymes: discovery of 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase in the plant kingdom. Arch Biochem Biophys **278:** 179–186

Wurtele ES, Nikolau BJ (1992) Differential accumulation of biotin enzymes during carrot somatic embryogenesis. Plant Physiol **99:** 1699–1703