Induction of β-methylcrotonyl-coenzyme A carboxylase in higher plant cells during carbohydrate starvation: evidence for a role of MCCase in leucine catabolism

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Abstract Induction of β-methylcrotonyl-coenzyme A carboxylase (MCCase) activity was observed during carbohydrate starvation in sycamore cells. In mitochondria isolated from starved cells, we noticed a marked accumulation of the biotinylated subunit of MCCase, of which the apparent molecular weight of 74 000 was similar to that of the polypeptide from mitochondria of potato tubers. Our results provide evidence for a role of MCCase in the catabolic pathway of leucine, a branched-chain amino acid which transiently accumulates in carbon-starved cells in relation to a massive breakdown of proteins. Furthermore, when control sycamore cells were incubated in the presence of exogenous leucine, this amino acid accumulated in the cells and no induction or accumulation of MCCase was observed, indicating that leucine is not responsible for the induction of its catabolic machinery. Finally, MCCase is proposed as a new biochemical marker of the autophagic process triggered by carbohydrate starvation.

Key words: β-Methylcrotonyl-CoA carboxylase; Leucine catabolism; Plant mitochondria; Carbon starvation; Autophagy; [^{13}C]NMR; *Acer pseudoplatanus*

1. Introduction

Carbohydrate starvation is a stress that often occurs in plants. Indeed, frequent variations of environmental factors, such as light, water or temperature, limit the efficiency of photosynthesis, and thus reduce the supply of carbohydrates which represent the main respiratory substrates in plant cells [1]. Using isolated cells as a model, Journet et al. [2], Dorne et al. [3] and Génix et al. [4] demonstrated that plant cells can cope for more than 6 days without a supply of carbohydrates. When almost all the intracellular carbohydrate pools have disappeared, the cell protein content declines progressively, probably via macroautophagy (i.e. bulk degradation of cytoplasm including organelles, with a parallel increase in their content in asparagine and other free amino acids, including leucine). These accumulations have been correlated with the induction of proteolytic activities [5]. Such results strongly suggest that amino acids released during the course of protein breakdown can be metabolized for respiratory purposes. Indeed, the catabolism of most amino acids generally leads to compounds capable of entering the tricarboxylic acid cycle [6]. However, little is known concerning the degradation of amino acids in plants (for review, see [7]). Most of our knowledge comes from studies in bacteria and mammals. In particular, the degradation pathway(s) of branched-chain amino acids (leucine, isoleucine, and valine) in plants is(are) poorly understood. The first step leads by transamination to branched-chain 2-oxo acids (2-oxoisocaproate in the case of leucine), whose oxidative decarboxylation (to isovaleryl-CoA in the case of leucine) and further oxidation (to β-methylcrotonyl-CoA in the case of leucine) have been shown to take place in peroxisomes [8–10]. These authors suggested that further steps could also involve peroxisomal enzymes. However, the fate of β-methylcrotonyl-CoA was not clearly identified. In mammalian mitochondria and in bacteria this metabolite is carboxylated to β-methylglutaconyl-CoA by the biotin-containing β-methylcrotonyl-coenzyme A carboxylase (MCCase; EC 6.4.1.4) [11]. Gerbling and Gerhardt [9] suggested that an extraperoxisomal pathway is involved in leucine catabolism, since they reported that peroxisomes are unable to carry out the biotin-dependent carboxylation of β-methylcrotonyl-CoA. Interestingly plant MCCase has been purified and located in mitochondria [12,13]. MCCase, present in all plant organs, is usually the most prevalent of the biotin-containing enzymes [14], and its activity can be regulated by biotinylation of the apoenzyme [15]. Recently, genes and cDNAs coding for its biotinylated subunit have been isolated [16,17]. Despite these characterizations, the metabolic function(s) of plant MCCase remain(s) unclear. MCCase may be involved in the mitochondrial catabolism of leucine, and/or in the 'mevalonate shunt' [18].

In this article, we present evidence for a role of MCCase in the catabolism of leucine in plant cells. We report an induction of the activity of this enzyme in sycamore cells submitted to carbohydrate starvation, correlated with the accumulation of the enzyme in the mitochondria. We further show that MCCase is not regulated by the intracellular levels of leucine. Finally, we propose this enzyme as a new biochemical marker of the autophagic process triggered by carbohydrate starvation.

2. Materials and methods

2.1. Materials

Cell suspensions were chosen as a model whose incubation conditions can be easily controlled, including the carbohydrate supply. Sycamore cells (*Acer pseudoplatanus* L.) used in the present study were grown at 20°C as a suspension in liquid nutrient media containing sucrose [19]. The culture medium was kept at a volume of 0.3 l

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Abbreviations: MCCase, β-methylcrotonyl-coenzyme A carboxylase.

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and stirred continuously at 60 rpm. Under these conditions the cell number doubling time was 40–48 h after a lag phase of approx. 2 days and the maximum density of sycamore cells was attained after 7–8 days of growth, when the stationary phase was reached. The cell suspensions were maintained in exponential growth by every 7 days subcultures. The age of cells is referred to the day of subculturing. The cell fresh weight was measured after straining culture aliquots onto a glass-fiber filter.

Cells harvested from the culture medium were rinsed three times by successive resuspensions in fresh culture medium devoid of sucrose and incubated at zero time into flasks containing sucrose-free culture medium. The fresh weight remained constant during the course of carbohydrate starvation (since growth was stopped, see [3]), so it was used as a reference for quantitative comparisons between control and starved cells.

2.2. Perchloric extract

For perchloric acid extraction, cells (9 g fresh weight) were quickly frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle with 1 ml of 70% (v/v) perchloric acid. The frozen powder was then placed at −10°C and thawed. The thick suspension thus obtained was centrifuged at 10000 × *g* for 10 min to remove particulate matter, and the supernatant was neutralized with 2 M $KHCO_3$ to about pH 5.5. The supernatant was then centrifuged at 10000 × *g* for 10 min to remove $KClO_4$, the resulting supernatant being lyophilized and stored in liquid nitrogen. For the NMR measurements, this freeze-dried material was redissolved in 2.5 ml water containing 10% D_2O (perchloric acid extract). [^{13}C]NMR spectra of neutralized perchloric acid extracts were obtained using a Bruker NMR spectrometer (AM 400, narrow bore) equipped with a 10-mm multinuclear probe tuned at 100.6 MHz. Acquisition used 90° pulses at 6-s intervals. The deuterium resonance of D_2O was used as a lock signal, and the spectra were recorded over a period of 90 min under conditions of broadband proton decoupling. 900 scans were taken at a sweep-width of 6000 Hz and an exponential multiplication (0.2 Hz line width) was used to increase the signal-to-noise ratio. Perchloric acid extract spectra are referenced to hexamethyldisiloxane resonance at 2.7 ppm.

The assignment and the quantification of carbon metabolites were carried out according to previous publications (see for instance [20]), and from spectra of perchloric acid extracts prepared after the addition of known amounts of the authentic compounds.

2.3. Isolation and purification of mitochondria

Protoplasts were first prepared, and after gentle rupturing mitochondria were isolated and purified. Washed cells (50 g wet wt) were suspended in their culture medium containing 0.5 M mannitol, 1% (w/v) cellulase and 0.1% pectolyase Y-23 (both enzymes from Seishin Pharmaceutical Co., Nishinomiya, Japan) adjusted to pH 5.7. The cells were incubated with constant shaking (20 cycles/min) at 30°C. This high temperature increases considerably the yield of intact protoplasts. After digestion for 1 h, the suspension was filtered through one layer of Miracloth (Krantex, Alforville, France) which retained any undigested aggregates. The protoplasts were then collected by centrifugation (150 × *g* for 10 min) and washed twice with 150 ml of suspension medium containing 0.5 M mannitol, 5 mM phosphate buffer (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$, 1% PVP ($M_r \sim 25000$, Serva) (w/v) and 0.1% BSA (w/v).

Since sycamore protoplasts have an average diameter of 20–30 μm, a rapid and effective procedure for the gentle rupture of intact protoplasts (i.e. for stripping the cell membrane) was to pass them through a fine nylon mesh (Nybolt PA, 20 μm) affixed to the cut end of a 100 ml disposable syringe [2]. The lysate was then homogenized and centrifuged for 20 min at 15000 × *g* (SS-34 rotor, Sorvall) and the mitochondrial pellet was resuspended in ~4 ml of suspension medium.

The suspension thus obtained (washed mitochondria) was purified by centrifugation in self-generated Percoll density gradients (Pharmacia Fine Chemicals) [21]. The washed mitochondria were layered on Percoll medium [24% Percoll (v/v), 0.5 M mannitol, 10 mM phosphate buffer (pH 7.5), EDTA 1 mM, 1% PVP ($M_r \sim 25000$, Serva) (w/v) and 0.1% BSA (w/v)] in Sorvall SS-34 tubes. After 35 min centrifugation at 40000 × *g* the purified mitochondria were collected and washed twice with the same medium devoid of Percoll. This procedure resulted in mitochondria with better than 90% intact outer membranes as judged by their impermeability to cytochrome *c* [22].

2.4. Electrophoretic analysis of proteins and Western blotting

Polypeptides from purified mitochondria 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 [23]. Polypeptides were transferred electrophoretically onto nitrocellulose sheets, essentially according to Towbin et al. [24]. Biotin-containing polypeptides were detected using horseradish peroxidase-labelled streptavidin as described previously [12].

2.5. Crude extract preparation and determination of MCCase activity

The preparation of crude cell extracts, and the determination of MCCase activity were made according to the procedure of Alban et al. [13], using $NaH^{14}CO_3$ as a substrate. The standard assay consisted of 50 mM Hepes (pH 8), 2.5 mM $MgCl_2$, 1 mM ATP, 2 mM DTT, 10 mM $NaH^{14}CO_3$ (37 MBq/mmol), 20 mM KCl, 0.4 mM β-methylcrotonyl-CoA, and 20 μg of protein in a final volume of 200 μl. The assays were initiated by the addition of β-methylcrotonyl-CoA. After 2–20 min of incubation at 30°C in a shaking bath, 150-μl aliquots of the reaction mixture were mixed 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.

2.6. Protein determination

Mitochondrial proteins were prepared in the presence of deoxycholate (0.04%, w/v), and determined according to the method of Lowry et al. [25] using BSA as a standard.

3. Results

3.1. Effects of carbohydrate starvation on MCCase

Fig. 1 illustrates the evolution of the MCCase activity in

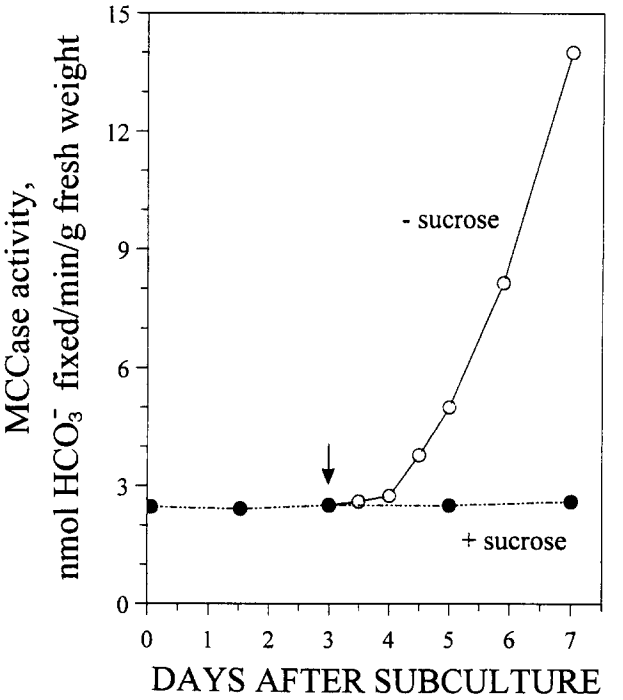


Fig. 1. Induction of MCCase activity during the course of sucrose starvation in sycamore cells. Evolution of MCCase activity in sucrose-supplemented cells (dotted curve) and sucrose-starved cells (unbroken curve). The arrow indicates the time at which sucrose was omitted from the culture medium. Crude cell extract preparation and determination of enzymatic activity were carried out according to the procedure described in section 2. MCCase activities are expressed as nmol HCO_3^- incorporated/min per g fresh weight. These data are from a representative experiment and the experiment has been reproduced five times.

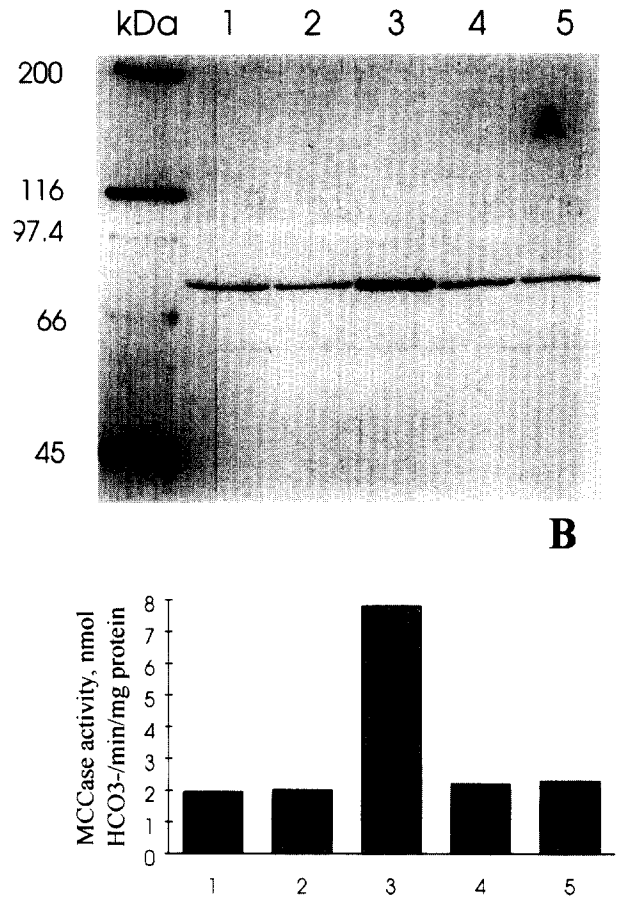


Fig. 2. Detection of MCCase in mitochondria from sycamore cells (A), and corresponding activities (B). The data are from a representative experiment and the experiment has been reproduced three times. Proteins from purified mitochondria were separated on SDS-PAGE gels, transferred onto nitrocellulose sheets, and biotinylated polypeptides were labelled with streptavidin-peroxidase and revealed with the color development reagent 4-chloro-1-naphthol as described in section 2. Each lane was loaded with 100 μg protein. In parallel, the corresponding MCCase activities in the mitochondria were determined and expressed as nmol HCO_3^- incorporated/min per mg protein. Mitochondria were isolated and purified from sucrose-supplemented cells (lanes 1 and 2 corresponding to 3-and 7-day-old cells), from cells starved for 4 days (lane 3), from control cells incubated with 1 mM leucine for 4 days (lane 4), and from control cells treated with sulfometuron methyl 1 nM for 4 days (lane 5).

control sycamore cells and in cells starved in carbohydrate. The values are expressed on the basis of fresh weight which remains constant throughout sucrose starvation [3], in contrast with total cell protein which markedly declines [2,4]. MCCase activity remained low in control cells (i.e. sucrose-supplied cells) during the week following subculturing (Fig. 1, dotted curve), at values of nearly 2.5 nmol HCO_3^- fixed/min per g fresh weight. In contrast, we observed an increase of MCCase activity from 36 h of sucrose starvation (Fig. 1, unbroken curve). After 4 days of starvation, values nearly 6-times higher than in control cells were obtained.

Plant MCCase has been clearly characterized as a mitochondrial enzyme [12]. We thus purified mitochondria from control and starved sycamore cells to investigate the evolution of the biotinylated subunit of the MCCase, which can be easily detected on Western blots using horseradish peroxidase-labelled streptavidin. Fig. 2A indicates that a major biotinylated polypeptide with an apparent molecular weight of 74 000 was detected in sycamore mitochondria. It was assumed to be the biotinylated subunit of MCCase, as its apparent molecular weight was identical to that found in potato tubers [13]. In mitochondria from control cells a basal level of MCCase was observed, which remained constant (Fig. 2A, lanes 1 and 2 corresponding to 3-and 7-day-old cells, respectively). In contrast, when cells were incubated for 4 days in the absence of carbon source a marked accumulation of the MCCase occurred in the mitochondria (Fig. 2A, lane 3). Based on mitochondrial proteins, the activity of MCCase was 4-times higher in mitochondria purified from starved cells than that in the control mitochondria (7.8 nmol HCO_3^- fixed/min per mg protein and 1.9 nmol HCO_3^- fixed/min per mg protein, respectively, Fig. 2B).

3.2. *Effects of intracellular leucine content on MCCase*

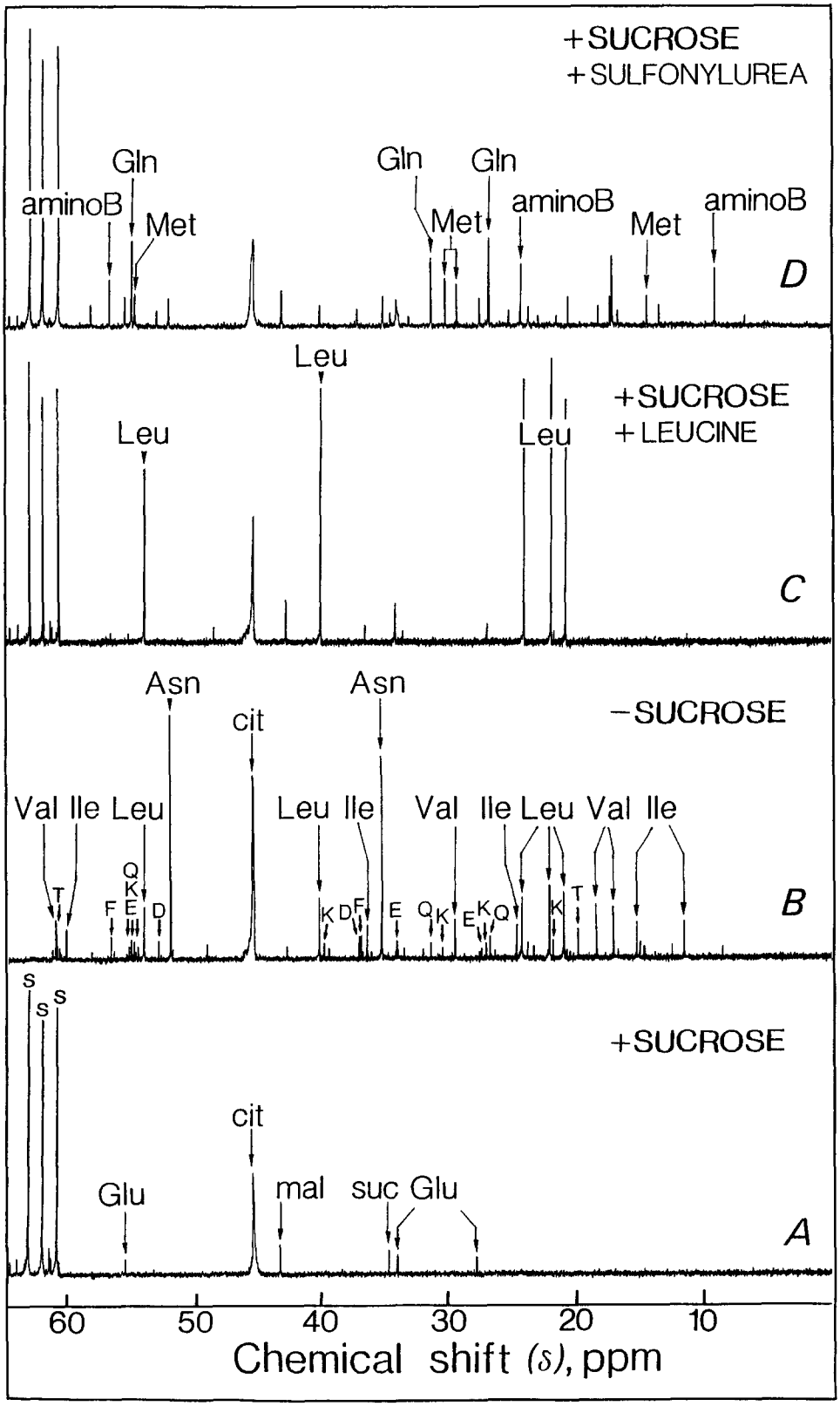
In animal cells the autophagic degradation of proteins has been shown to be regulated by amino acids, in particular leucine [26]. In plant cells carbohydrate starvation triggers a transient accumulatation of several amino acids, leucine being the second most abundant after asparagine (Fig. 3B; see also [4]). We therefore hypothesized that leucine could regulate the enzymes involved in its own degradation. Experiments were thus carried out to study the effects of variations of intracellular leucine contents on MCCase.

In order to investigate the effect of leucine accumulation on MCCase, we incubated control cells (i.e. sucrose-supplied cells) with 1 mM leucine for 4 days. Cell growth was not significantly modified (not shown). Surprisingly, the [^{13}C]NMR spectrum on Fig. 3C showed that leucine massively accumulated in sycamore cells, at intracellular concentrations attaining 50 mM. However, no accumulation of MCCase was observed in the mitochondria isolated from these cells incubated with leucine for 4 days (Fig. 2A, lane 4). Similarly the activity of MCCase was not affected by the cellular accumulation of leucine (Fig. 2B, lane 4).

Conversely, in order to study the effects of a reduction of intracellular leucine contents we treated control cells with sulfometuron methyl. This herbicide is a potent inhibitor of the synthesis of branched-chain amino acids (leucine, isoleucine, valine) at the level of acetolactate synthase [27]. Cell growth was stopped and, in accord with the results of Shaner and Singer [28], we observed that treated cells accumulated α-aminobutyrate, the transamination product of α-ketobutyrate (the substrate of acetolactate synthase leading to isoleucine) (Fig. 3D). This accumulation reflects the inhibition of acetolactate synthase by sulfometuron methyl, and the arrest of leucine synthesis [28]. However, under these conditions, neither the content of MCCase protein nor the activity of the enzyme was significantly affected (Fig. 2A,B; lane 5).

4. Discussion

Little is known concerning the enzymes involved in the catabolism of amino acids in plants. Only a few enzymes have been purified, and their regulation is unknown. In this paper we present evidence for a role of MCCase in the degradation of leucine during the autophagic process triggered by carbohydrate starvation (for review on carbohydrate starvation, see [29]). Leucine, after asparagine, is the second most abundant amino acid which transiently accumulates during carbon starvation ([4]; Fig. 3B), in relation with the induction



of proteolytic activities [5] leading to a massive breakdown of proteins [2,30]. These amino acids, as well as the fatty acids deriving from the lipid breakdown [3], are used in place of sugars to fuel the respiration of the mitochondria spared by autophagy. Here we report that MCCase activity is induced in starved cells, in correlation with an accumulation of the enzyme in mitochondria. The suggestion that MCCase is involved in leucine catabolism during carbohydrate starvation is consistent with the observation that MCCase activity is 10-fold higher in senescing leaves than in young leaves [13]. In addition, MCCase activity is also induced in cotyledons during the mobilization of storage proteins for seedling growth [31]. In these develomental situations (senescence and germination), as well as during carbohydrate starvation, proteins

← Fig. 3. Representative in vitro [^{13}C]NMR spectra (perchloric extracts, expanded scale from 0 to 60 ppm), from sucrose-supplemented cells (A), sucrose-starved cells (B), cells incubated with leucine (C), or treated with sulfometuron methyl (D). (A) Control cells; (B) cells starved in sucrose for 4 days; (C) cells incubated with 1 mM leucine for 4 days; (D) cells treated with 1 nM sulfometuron methyl for 4 days. Each spectrum (900 transients, except spectrum B corresponding to 3600 transients) is representative of a perchloric acid extract obtained from 9 g fresh weight, and was recorded at 20°C. On spectra A, C, and D, the intracellular concentration of sucrose is estimated to 55–60 mM. On spectrum B. asparagine corresponds to an intracellular concentration of 20–25 mM; branched-chain amino acids (leucine, isoleucine, valine) correspond to intracellular concentrations of 6–8 mM each. The total of other free amino acids represents about 15–20 mM. Peak assignments: aminoB, α-aminobutyrate; Asn, asparagine; cit, citrate; Gln, glutamine; Glu, glutamate; Ile, isoleucine; Leu, leucine; mal, malate; Met, methionine; s, sucrose; suc, succinate; Val, valine. Capital letters refer to the single letter amino acid designations: D, aspartate; E, glutamate; F, phenylalanine; K, lysine; Q, glutamine; T, threonine.
←

are massively degraded and amino acids are used as carbon and energy sources [32].

Surprisingly, the induction of the activity of MCCase assayed in purified mitochondria (Fig. 2B indicated a 4-fold increase after 4 days of starvation) was lower than that observed in intact cells (Fig. 1 indicated a 6-fold increase after 4 days of starvation). The opposite would have been expected. Indeed, Journet et al. [2] demonstrated that the autophagic process occurring in starved cells leads to a progressive decline of the number of mitochondria per cell, as determined from the quantification of the decline of cardiolipin (diphosphatidylglycerol) and cytochrome aa_3 (cytochrome oxydase), two specific mitochondrial markers: after 4 days of starvation sycamore cells only hold around 1/3 of their normal content in mitochondria. In mitochondria isolated from starved cells, we could thus have expected a $6\times3=18$-fold increase of MCCase activity, assuming that the protein content/mitochondria remains constant during starvation (protein are the reference usually used for quantitative purposes with mitochondria). However, the fact that we only observed a 4-fold increase of MCCase activity in starved mitochondria can be explained by several reasons. First, part of the activity of MCCase could have been lost during the preparation of the mitochondria. However, this hypothesis is unlikely since the techniques used for the isolation and purification of the mitochondria have previously been shown not to interfere with the MCCase activity [13]. Second, we can hypothesize that many soluble proteins accumulated in the matrix of the mitochondria of starved cells (in addition to MCCase). This hypothesis is supported by recent electron microscopy studies of autophagy in sycamore starved cells, which showed that mitochondria isolated from sucrose-deprived cells presented a darker matrix (I. Marty, personal communication). In addition, similar observations have been reported in mitochondria isolated from starved maize root tips [33]. Therefore, the 4-fold increase of MCCase activity observed in Fig. 2 (instead of the expected 18-fold increase) in starved mitochondria can be explained if we assume a 4.5 (18/4)-fold increase in the protein content/mitochondria after 4 days of starvation. Among the accumulated proteins are probably the enzymes involved in fatty acid β oxidation, the activity of which has been shown to increase in mitochondia from starved maize root tips [34]. According to this idea, mitochondria purified from carbon-starved cells could represent a good material for the characterization of induced enzymes involved in the catabolism of amino acids or fatty acids. Isolated cells represent a model which can be controlled more easily than senescing organs, which also present high degradative activities [32].

These observations raise the problem of the factors controlling the induction of MCCase during starvation. In animal cells autophagic proteolysis is regulated by amino acids, leucine in particular (for review, see [26]). In plant cells our results strongly suggest that leucine does not regulate its catabolism, since we observed that MCCase in sucrose-supplemented cells is not significantly affected by either accumulation or depletion of leucine (Fig. 3). Parenthetically, we observed that the incubation of control cells with leucine triggered huge accumulations of this amino acid in the cells. This observation suggests that sycamore cells, which are able to synthesize amino acids from nitrates, possess powerful transport system(s) for amino acid import. The significance of this equipment is still unknown. MCCase is likely to be coordinately regulated with other degradative enzymes induced during the autophagy triggered by carbon starvation. It has been shown that the transcription of α-amylases genes involved in starch breakdown [35] or genes involved in glyoxylate cycle [36] is stimulated by starvation. The recent cloning of the cDNAs coding for MCCase [16,17] will permit a further investigation of a possible regulation of MCCase at the level of gene expression.

Finally, we observed that the activity of MCCase remains constant during the cell cycle, the low basal activity being likely involved in the house-keeping turnover of proteins and amino acids. In contrast, the MCCase activity was induced during carbon starvation, with a pattern ressembling the accumulation of phosphocholine, the biochemical marker of the autophagic degradation of membrane phospholipids [3,37]. We consider therefore MCCase activity as a good enzymatic marker of the autophagic process triggered by carbon starvation in plant cells.

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