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Peroxisomal and Mitochondrial Citrate Synthase in CAM Plants

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Citrate synthase was studied for the first time in peroxisomes and mitochondria of crassulacean acid metabolism plants. Cellular organelles were isolated from *Agave americana* leaves by sucrose density gradient centrifugation and characterized by the use of catalase and cytochrome oxidase as marker enzymes, respectively. 48,000 × *g* centrifugation caused the breakdown of the cellular organelles. The presence of a glyoxylate cycle enzyme (citrate synthase) and a glycollate pathway enzyme (catalase) in the same organelles, besides the absence of another glyoxylate cycle enzyme (malate synthase) is reported for the first time, suggesting that peroxisomal and glyoxysomal proteins are synthesized at the same time and housed in the same organelle.

Citrate synthase (EC 4.1.3.7) catalyzes the reaction of acetyl-CoA and oxalacetate (OAA) to citric acid. It is the first and a key enzyme of the tricarboxylic acid cycle. The presence of citrate synthase in various organisms and its properties have been reviewed by SRERE (17) and WEITZMAN and DANSON (22). In eukaryotic cells it occurs chiefly in the mitochondria, but if the cells contain glyoxysomes it is also found in these organelles. Its metabolic role in mitochondria is a part of the Krebs cycle and as such the control of its activity has been postulated as a key regulatory step of the cycle. Glyoxysomal citrate synthase functions in the glyoxylate shunt in which two acetyl units are converted to succinate. No other subcellular location of citrate synthase has been reported.

Since seed germination of fat-storing species requires a functional glyoxylate cycle to effect net gluconeogenesis from acetyl-CoA derived by β-oxidation of storage triglycerides (4), most of the studies on glyoxysomal citrate synthase have been carried out in these plant sources. Data by AXELROD and BEEVERS (2) and BARBARECHI *et al.* (3) seem to show that the citrate synthases of mitochondria and glyoxysomes are widely different in many basic properties.

In a previous paper (1), we have studied some characteristics of the citrate synthase

from *Agave americana* leaves, a plant with crassulacean acid metabolism (CAM), using as enzyme preparation the supernatant of 7,000 × *g* centrifugation. In leaves of CAM plants, peroxisomes have been observed electron-microscopically (7) and isolated by isopycnic centrifugation in sucrose gradients (6). They have been recognized as organelles containing the majority of the enzymes of the glycollate patway metabolism and, thus, of photorespiration (14, 19). Bearing in mind the absence of glyoxysomes in CAM plants and the relationship between glyoxysomes and peroxisomes, it would be of interest to know if the citrate synthase activity is associated to the mentioned organelles in these plants.

Materials and Methods

Locally-grown *Agave americana* plants were used. Acetyl-CoA and OAA were obtained from Sigma. Other reagents were of the highest purity available from commercial sources.

Cell-free extracts from *Agave americana* leaves were obtained as previously described (12). For differential centrifugation, the homogenate was squeezed through a cloth and centrifuged at 10,000 × *g* for 20 min at 4° C resulting in a supernatant ($S_{10,000}$) and a particulate fraction ($P_{10,000}$). $S_{10,000}$ was centrifuged at 48,000 × *g* yielding a supernatant ($S_{48,000}$) and a pellet ($P_{48,000}$). Discontinuous sucrose gradients (55 to 30 %) were prepared manually. Samples (3 ml) were layered onto the top of the gradient and centrifuged at 48,000×*g* for 4 h at 4° C in a Beckman L3-50 Ultracentrifuge. The peroxisomal and mitochondrial bands were collected by means of a Pasteur pipette. Protein contents were determined by the method of LOWRY *et al.* (9) using bovine albumin as standard. All enzyme assays were performed in a Pye Unicam Sp. 1700 Ultraviolet Spectrophotometer with an Unicam Sp. 1,085 Programme Controller and an Unicam AR 25 Linear Recorder.

Citrate synthase activity was measured by the method of SRERE *et al.* (18). Unless otherwise specified, reactions were carried out as previously described (1). Cytochrome oxidase (EC 1.9.3.1) was determined by the method of SOTTOCASA *et al.* (16). Catalase (EC 1.11.1.6) was assayed according to LÜCK (10) modified by TOLBERT *et al.* (20), malate dehydrogenase (EC 1.1.1.37) according to OCHOA (11) and malate synthase (EC 4.1.3.2) according to DIXON and KORNBERG (5).

Results

As a first assay of the subcellular distribution of citrate synthase in *Agave americana* leaves, the crude extracts were subjected to a differential centrifugation and the specific activities of citrate syn-

Table I. *Enzymatic activities on differential centrifugation of an extract from* Agave americana *leaves*

Details of assay methods are given in the text. Specific activities are given in nmol/min/mg protein. Total activities are given in nmol/min.

	Citrate synthase		Cytochrome oxidase		Malate dehydrogenase		Catalase	
	Sp. act.	Total act.	Sp. act.	Total act.	Sp. act.	Total act.	Sp. act.	Total act.
10,000 × *g* supernatant ($S_{10,000}$)	8.6	788	3.9	351	4.0	360	0.24	21.6
10,000 × *g* pellet ($P_{10,000}$)	6.8	8	1.9	2	2.0	2	0.12	0.1
48,000 × *g* supernatant ($S_{43,000}$)	10.2	724	2.4	170	4.5	319	0.25	17.8
48,000 × *g* pellet ($P_{43,000}$)	4.3	38	18.3	165	2.0	18	0.14	1.3

Table II. *Enzymatic activities on sucrose density gradient centrifugation of 10,000 × g supernatant from* Agave americana *leaves.*
Details of assay methods are given in the text. Specific activities are given in nmol/min/mg protein. Total activities are given in nmol/min.

	Citrate synthase		Cytochrome oxidase		Catalase	
	Sp. act.	Total act.	Sp. act.	Total act.	Sp. act.	Total act.
10,000 × *g* supernatant ($S_{10,000}$)	8.5	1,105	3.9	507	0.24	31.2
1.12 g/ml density	14.0	1,498	0.0	0	0.26	27.8
1.25 g/ml density	7.0	21	162.0	486	0.00	0.0

thase, cytochrome oxidase, malate dehydrogenase, malate synthase, and catalase determined in each of the pellet and supernatant fractions. Cytochrome oxidase, which was regarded as an exclusively mitochondrial enzyme (13), was found chiefly in $S_{10,000}$ but also in $S_{48,000}$ and $P_{48,000}$, both in similar amounts, thus indicating a possible breakage of mitochondria during the 48,000 × *g* centrifugation (table I). The distribution of catalase, an enzyme localized in the leaf peroxisomes of the CAM plants (6), seems to corroborate the breakage of cell organelles. A similar pattern was found in the distribution of citrate synthase and malate dehydrogenase activities. Both enzymes were reported to be localized within the matrix of mitochondria and microbodies. Their abundance in $S_{48,000}$ was probably due to the presence in peroxisomes and to mitochondria breakdown. In any case, it is interesting to note that no malate synthase activity was found in any fraction.

In order to avoid as much as possible the breakage of cellular organelles, the 10,000 × *g* supernatants were centrifuged on a sucrose density gradient. In all the experiments, two main visible bands were observed in the sucrose gradient, with equilibrium densities of about 1.12 and 1.25 g/ml. Using the recovery of protein as an index, the percentage of recovery in both fractions was about 90 %. Both fractions were collected and assayed for citrate synthase, cytochrome oxidase, catalase and malate synthase activities. Distribution of these enzymes is shown in table II. No malate synthase activity was observed in any of the preparations assayed. Citrate synthase was found chiefly in the band of 1.12 g/ml density, which contained no cytochrome oxidase activity but most of the catalase activity. Because of this pattern, this band was taken to correspond to the peroxisomal region, without mitochondrial contamination. The fraction having 1.25 g/ml density showed

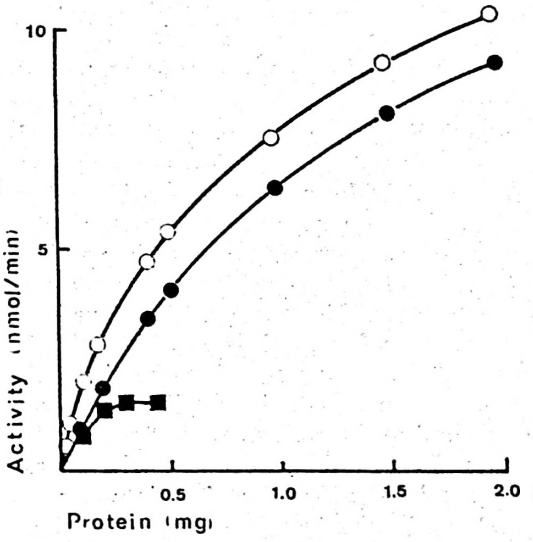

Fig. 1. *Influence of protein concentration on the citrate synthase activity.*
(●), citrate synthase in supernatant of 10,000 × *g* centrifugation; (○), peroxisomal citrate synthase; (■), mitochondrial citrate synthase.

less citrate synthase activity but all the cytochrome oxidase activity was observed in this band. In contrast, no catalase activity was found. It seemed clear that this 1.25 g/ml density band corresponded to the mitochondrial fraction, without peroxisomal contamination.

The effect of protein concentration of both fractions on enzyme activity has been examined and compared with that obtained working with $S_{10.000}$. A higher activity was observed in the peroxisomal fraction with respect to that of $S_{10.000}$, with a comparable profile in both cases (fig. 1). However, enzyme activity in the mitochondrial fraction seemed to reach a maximum at lower protein concentration.

Discussion

Although much is already known about the developmental physiology of the glyoxylate cycle enzymes and the glyoxysomal compartment in which they are localized, few studies have been carried out on the enzyme activities associated with peroxisomes in CAM plants, probably due to the difficulty of obtaining cell organelles from these plants. HERBERT *et al.* (6) have reported that isolated peroxisomes from different CAM plants contained activities of glycollate oxidase, catalase, hydroxypyruvate reductase, glycine aminotransferase, serine-glyoxylate aminotransferase and aspartate aminotransferase, comparable to activities found in spinach leaf peroxisomes. This clearly indicated a function of peroxisomes in CAM plants in the glycollate pathway metabolism. However, the presence of citrate synthase in these organelles is reported for the first time in this paper.

Several attempts to separate various cell organelles from *Agave americana* crude extracts by differential centrifugation have been done without success. In all the experiments a breakdown of organelles was observed by the recovery of the marker enzymes in different fractions. Cytochrome oxidase and catalase were especially used as markers of mitochondria and peroxisomes, respectively. Since malate synthase has been considered a marker enzyme for glyoxysomes, its activity was tested. No malate synthase activity was observed in any fraction.

On the other hand, centrifugation in sucrose gradient was recognized as a powerful tool for separating cell organelles in a variety of plants. In our experiments, the two main bands observed contained most of the protein and marker activities. Therefore, these bands were used for the study of citrate synthase activity. Distribution of marker enzymes indicated that the band with 1.12 g/ml density corresponded to the peroxisomal region whereas the band with 1.25 g/ml density corresponded to the mitochondrial fraction.

The presence in the same fraction of a glyoxylate cycle enzyme (citrate synthase) and a glycollate pathway enzyme (catalase) and the absence of another glyoxylate cycle enzyme (malate synthase) seem to suggest the presence of enzymes with different metabolic functions in the same organelles. It is well known that in the cotyledon of germinated fatty seedling a change occurs in metabolism during postgerminative growth that directly involves enzymes contained within microbody particles. Three models have been postulated to explain the succession from glyoxysomes to peroxisomes found in photosynthetic stages (21). More recently, SCHOPFER *et al.* (15) reported that peroxisomes of greening cotyledons are ultimately originated as products of the gradual replacement of glyoxysomes by microbodies of intermediary character — glyoxyperoxisomes — and then by organelles of predominantly peroxisomal properties. The results of KÖLLER and KINDL (8) proved that peroxisomal and glyoxysomal proteins are synthesized at the same time during the phase of greening; they also indicated that these proteins are housed

in the same organelle. Our data would also be in agreement with this hypothesis. Another interpretation may be raised by the fact that both peroxisomes and glyoxysomes exhibit the same over all density, but this is extremely improbable (8).

Resumen

Por vez primera se estudia la citrato sintasa en peroxisomas y mitocondrias de plantas crasuláceas. Los orgánulos celulares se aislaron a partir de hojas de *Agave americana* mediante centrifugación en gradiente de sacarosa y se caracterizaron usando catalasa y citocromo oxidasa como enzimas marcadoras, respectivamente. La presencia de una enzima del ciclo del glioxílico (citrato sintasa) y de una enzima de la vía del glicólico (catalasa) en los mismos orgánulos, junto con la ausencia de otra enzima del ciclo glioxílico (malato sintasa) se cita por vez primera, y sugiere que las proteínas de los peroxisomas y glioxisomas se sintetizan al mismo tiempo y en los mismos orgánulos.

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