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SUCCINYL-CoA SYNTHETASE IN GREENING MAIZE LEAVES

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Abstract—In extracts of greening maize leaves succinyl-CoA synthetase was present in both a particulate and a soluble fraction. Aqueous and non-aqueous fractionation together with determination of chlorophyll content and cytochrome oxidase activity indicated that the enzyme was neither located, nor originated in plastids. Pre-illumination of leaves caused only small increases in the activity of either the particulate or the soluble enzyme. The soluble enzyme was ATP specific and had a low affinity for succinate (Km = 63 mM).

INTRODUCTION

It has been generally accepted that δ-aminolevulinic acid (ALA), a key substrate in the synthesis of chlorophyll, is formed by the condensation of succinyl-CoA and glycine, catalysed by ALA synthetase [1]. Succinyl-CoA could be formed in the mitochondria by the action of α-ketoglutarate oxidase or of succinyl-CoA synthetase [succinate: CoA ligase (ADP) (EC 6.2.1.5)]. This would however, pose considerable problems with regard to the diversion of large amounts of substrate from the citric acid cycle and the translocation of the product to the plastids in greening tissues [2]. The possible existence of a plastid succinyl-CoA synthetase, which is involved in the massive synthesis of chlorophyll during the greening process, has been suggested [2]. Although the enzyme has been demonstrated in leaves of higher plants [3–6], its reported location in plastids [4] has been questioned [2,7]. We have studied the activity of succinyl-CoA synthetase in greening maize leaves, and have attempted to determine its subcellular location and examine its possible relation to the greening process.

RESULTS AND DISCUSSION

Crude extracts of etiolated 10-day-old maize leaves synthesized succinyl-CoA when added to a reaction mixture containing buffer, succinate, $MgCl_2$, ATP, CoA and GSH. Differential centrifugation of the crude extract showed that activity was absent in the fractions sedimenting at up to 2000 *g* and almost all the activity could be recovered in the 20000 *g* precipitate and supernatant. Protochlorophyll could be detected only in the fractions precipitating at and below 2000 *g*. The low activity found in the particulate fractions precipitating at a lower centrifugal force can be ascribed to contamination by mitochondria (Table 1). Fractionation in the presence or absence of 0·8 M sucrose had no significant effect on the distribution of activity between fractions. The particulate enzyme synthesizing succinyl-CoA appears to be located in the mitochondria

Table 1. Succinyl-CoA synthetase activity in various fractions obtained from etiolated maize leaves

Fraction	Succinyl-CoA synthetase activity μmol/g fr. wt/hr	μmol/mg protein/hr
Crude homogenate	7·28 ± 0·91*	0·18
200 *g* for 5 min, ppt.	0·00	0·00
1000 *g* for 5 min, ppt.	0·14	0·20
2000 *g* for 5 min, ppt.	0·12	0·20
20000 *g* for 30 min, pp	3·24 ± 0·40	1·15
20000 *g* for 30 min, supernatant	4·60 ± 0·79	0·13

* s.e.

Table 2. Succinyl-CoA synthetase, cytochrome *c* oxidase, and chlorophyll in fractions isolated from greening maize leaves in a non-aqueous medium

Fraction	Succinyl-CoA synthetase μmol/ml/hr	Cytochrome oxidase 10^2 (K/ml)	Chlorophyll A_{663}/ml
Upper layer, green	1·10	1·47	0·086
Middle layer, pale green	2·60	6·03	0·069
Bottom layer, white	0·70	1·87	0·007

10-day-old etiolated leaves were illuminated for 7 hr, freeze-dried and subjected to non-aqs fractionation as described in Experimental, using a continuous gradient of CCl_4:*n*-hexane (sp gr 1·35–1·45). Three particulate fractions were collected and either extracted with 80% acetone for chlorophyll determination or suspended in buffer and assayed for enzyme activities.

and there is no activity in plastids. However, the observation that more than 50% of the total activity was found in the soluble fraction could result from leakage of an enzyme from plastids during fractionation. We therefore subjected the tissue to non-aqueous fractionation and examined the distribution of enzyme activity between the particulate and soluble fractions. The total activity found in the particulate fractions after non-aqueous isolation was 3·34 μmol succinyl-CoA/g fr wt/hr compared to 3·55 μmol/g/hr found in the precipitate obtained at 20000 *g* for 30 min, by isolation in an aqueous medium.

Further support for the conclusion that there is no succinyl-CoA synthetase activity in plastids came from a series of fractionation experiments. In these experiments succinyl-CoA synthetase activity in fractions obtained by either aqueous or non-aqueous fractionation was compared to chlorophyll content and cytochrome oxidase, activity of the fractions. Table 2 is an example of such a comparison, using density gradient centrifugation in a non-aqueous medium. Succinyl-CoA synthetase activity showed better correlation with cytochrome oxidase activity than with chlorophyll content. The separation between plastids and mitochondria was incomplete. However, succinyl-CoA synthetase activity in the particulate fractions always showed a better correlation with cytochrome oxidase than with plastid markers in all the fractionation experiments, using various density ranges and analysis of small fractions along the gradient. Failure to obtain satisfactory separation with maize leaf tissue, contrary to other tissues used, was reported by Ting *et al.* [8]. A relatively good correlation between succinyl-CoA synthetase activity and cytochrome oxidase activity in the particulate fractions was also observed after fractionation in 0·8 M sucrose 0·05 M tris buffer pH 7·5 (Table 3). This further supports the view that the particulate activity of succinyl-CoA synthetase in maize leaves is located in mitochondria. The mitochondrial enzyme has been studied by several workers [3,9]. We therefore attempted to partly characterise the enzyme from the soluble fraction.

The activity of succinyl-CoA synthetase is expressed as the difference in the amount of hydroxamate produced in the presence and absence of added succinate. The colorimetric readings for

Table 3. Succinyl-CoA synthetase and cytochrome c oxidase activities in subcellular fractions isolated in an aqueous medium from etiolated maize leaves

Fraction	Succinyl-CoA synthetase activity Total	Specific	Cytochrome *c* oxidase activity Total	Specific
200 *g* for 5 min, supernatant	5·74	0·67	11·25	1·32
1000 *g* for 5 min, ppt.	0·12	0·30	0·72	1·87
5000 *g* for 10 min, ppt.	1·17	1·96	6·07	10·22
20000 *g* for 30 min, ppt.	1·58	1·73	4·88	5·33
20000 *g* for 30 min, supernatant	3·91	0·43	0·0	0·0

Ten-day-old etiolated leaves were ground in 0·8 M sucrose–0·05 M Tris buffer pH 7·2 containing 5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. Fractions were isolated by centrifugation, re-suspended in buffer and dialysed. Succinyl-CoA synthetase—total activity as μmol/g fr. wt/hr; sp. act. as μmol/mg protein/hr. Cytochrome *c* oxidase—total activity as 10^2 K/g fr. wt; sp. act. as 10^2 K/mg protein.

Table 4. The response of soluble succinyl-CoA synthetase to added ATP and CoA

Enzyme preparation	Reaction mixture	Succinyl-CoA synthetase μmol/g fr. wt/hr Before gel filtration	After gel filtration
a. Supernatant, 20000 *g* for 30 min.	Complete	7·60	4·16
	−CoA and GSH	4·41	0·39
	−ATP	3·81	0·00
b. Supernatant, 20000 *g* for 30 min after dialysis and Dowex-1 column	Complete	3·35	
	−CoA and GSH	0·00	
	−ATP	0·07	
	±GTP, no ATP	0·57	
	±CTP, no ATP	0·47	

The 20000 *g* for 30 min supernatant fraction, isolated from etiolated maize leaves was used for the experiments, a—4 ml of the supernatant fraction (equivalent to 2·5 g fr. wt of leaves) were passed through a Sephadex G-25 column (25 ml bed volume) and the protein containing fractions were assayed for enzyme activity b—The supernatant fraction was dialysed for 8 hr and then passed through a column of Dowex-1 (x4) Cl before assaying for enzyme activity.

the particulate fractions in the absence of succinate were relatively low, under our experimental conditions. However, this was not the case for the soluble fraction. The high background readings are probably due to endogenous esters and to the presence of endogenous substrate. In addition, the soluble fraction showed a relatively low response to the addition of ATP or CoA to the reaction mixture (Table 4a). As this could considerably affect the significance of the results obtained we attempted to reduce the background readings. Passing the 20000 *g* supernatant through a Sephadex G-25 column and collecting the enzymically active fractions resulted in a considerable (90%) reduction in the background readings and in a marked increase in the response to ATP and CoA (Table 4a). Dialysis for 8 hr also resulted in a considerable reduction (75%) of the background readings. The specificity of the enzyme for ATP was demonstrated with the soluble enzyme after dialysis and passage through a Dowex-1 column to remove endogenous cofactors (Table 4b). The soluble fraction after dialysis gave a linear rate of succinyl-CoA formation for at least 1 hr on incubation at 25°. The activity of the fraction was linear with enzyme concentration, with the amounts of enzyme used in the experiments. The *Km* of the soluble enzyme for succinate was 63 mM.

It has been suggested [2,6] that succinyl-CoA synthetase might be involved in the production of succinyl-CoA for ALA synthesis in greening leaves. The activity of the enzymes in etiolated maize leaves (Table 1) appears to be sufficient to account for the rate of ALA production by this tissue in light −0·14 μmol/g fr. wt/hr [10]. It has been reported that the activity of the enzyme from etiolated bean leaves was increased by 50–100% following a brief illumination [6]. Pre-illumination of maize leaves had only a small effect on

Table 5. The effect of pre-illumination on succinyl-CoA synthetase activity of etiolated maize leaves

Fraction		Pre-illumination (hr) 0	1·5	3·0	5·0	8·5
Particulate fraction (20000 *g* for 30 min ppt.)	Total activity	2·60	2·92		4·17	3·49
	Sp act	0·98	1·17		1·47	1·15
Soluble fraction (20000 *g* for 30 min supernatant, dialysed)	Total activity	2·54	2·54	2·97	2·48	
	Sp act	0·19	0·19	0·23	0·15	

Pre-illumination—860 lx of white, fluorescent light. Total activity as μmol/g fr wt/hr. Sp act as μmol/mg protein/hr.

the activity of succinyl-CoA synthetase (Table 5). An increase up to 60% was observed in the activity of the particulate enzyme. However, the increase observed seems to be small, considering the experimental error (Table 1) and the relatively high activity which already exists in non-illuminated leaves. In view of the large increase in ALA and chlorophyll production induced by light [10] it seems unlikely that the observed increase in succinyl-CoA synthetase is significant with regard to the control of ALA synthesis.

According to our results there is no significant activity of succinyl-CoA synthetase in the plastids, nor does it appear following illumination for up to 8·5 hr. The results of the fractionation experiments do not exclude the possible existence of a plastid succinyl-CoA synthetase. However, the apparent low level of such an enzyme makes it unlikely that plastids are able to produce succinyl-CoA in amounts sufficient to account for the δ-aminolevulinic acid synthesized by greening leaves. Similar conclusions were reached by Kirk and Pyliotis [7].

Recent observations suggest that the synthesis of ALA in higher plants does not involve the condensation of succinyl-CoA and glycine [11,17]. Our results on the subcellular location of succinyl-CoA synthetase in maize leaves, the low affinity of the soluble enzyme for succinate and the relatively small effect of pre-illumination on the activity of the enzymes tend to support these views.

EXPERIMENTAL

Maize (*Zea mays*, cv Neve Yaar 170) seeds were germinated and grown in vermiculite in the dark at 22°. Leaves of 9 to 10-day-old plants were collected under green safe-light or after illumination with 860 lx of white fluorescent light.

Isolation of subcellular fractions. Leaves were ground with a pestle and mortar in 50 mM tris buffer pH 7·2 containing 5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol, with or without 0·8 M sucrose. The homogenate was filtered through gauze and the fractions were collected by centrifugation and resuspended in buffer. For non-aqueous fractionation the particles were isolated as described in ref [12] and suspended in 50 mM tris buffer pH 7·2. When density gradient centrifugation was performed, the particles collected from the hexane layer after the first centrifugation were suspended in a CCl_4:hexane mixture of the appropriate density, layered over a density gradient and centrifuged at 13000 *g* for 20 min. Fractions were collected, dried under vacuum and either extracted with 80% Me_2CO for chlorophyll determination or suspended in buffer and assayed for enzyme activity.

Succinyl-CoA synthetase was assayed as described by Kaufman [13]. The reaction mixture contained in μmol: 50 tris–HCl buffer pH 7·2, 10 $MgCl_2$, 200 Na-succinate, 0·25 CoA, 5 GSH, 5 ATP and 960 salt-free NH_2OH in a final vol of 2 ml. Blanks lacking succinate were run in parallel in all determinations.

Salt-free NH_2OH was prepared according to Davie [14]. The activity of succinyl-CoA synthetase determined with salt-free NH_2OH was 2·5× higher than with neutralized NH_2OH. *Cytochrome c oxidase* was assayed spectrophotometrically according to Smith [15]. Enzyme activity is expressed as the first order velocity constant for the oxidation (K sec^{-1}). *Chlorophyll* was extracted with 80% Me_2CO and the *A* determined at 663 nm. *Protein* was determined according to Lowry *et al.* [16]. *Dialysis* was performed for 8 hr at 2° against 50 mM tris buffer pH 7·2, containing 5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. The buffer was replaced every 2 hr.

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