A SPECIFIC INACTIVATOR OF GLYOXYSOMAL ISOCITRATE LYASE FROM SUNFLOWER (*HELIANTHUS ANNUUS* L.) COTYLEDONS

Roland R. THEIMER

Abteilung Zellphysiologie, Botanisches Institut der Univ., Menzingerstr. 67, D 8000 München 19, West Germany

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1. Introduction

At present, two alternative mechanisms of the change of microbody function in greening cotyledons of fatty seedlings are discussed: (a) selective destruction of glyoxysomes and concomitant de novo synthesis of leaf peroxisomes [1] and (b) formation of leaf peroxisomes from pre-existing glyoxysomes [2–4]. We have recently presented evidence against a massive de novo production of leaf peroxisomes in greening sunflower and cucumber cotyledons [5] confirming electron microscopic and biochemical observations published previously [2–4,6]. In the present paper we report on the presence in crude homogenates of a proteinaceous component which selectively degrades isocitrate lyase activity in vitro and appears at stages of seedling development when glyoxysomal enzyme activities disappear from the tissue.

2. Materials and methods

Sunflower seeds (*Helianthus annuus* L., var. 'Spanners Allzweck') were grown as described previously [5]. Crude homogenates of the cotyledons were prepared as reported elsewhere [7,8] with the exception that sucrose was omitted from the grinding medium.

Partial purification of soluble glyoxysomal enzymes was achieved by osmotic shocking [9,10] of crude particulate fractions [8] of 4 day old sunflower cotyledons. Unbroken particles were sedimented by centrifugation at 27 000 *g* for 30 min and the supernatant fluid was used. When compared to crude homogenates the increase in specific activity of the enzymes was as follows: isocitrate lyase 5–10-fold, malate dehydrogenase 3–5-fold, catalase 3–5-fold, glycolate oxidase 5–8-fold, and hydroxypyruvate reductase 3–8-fold.

Inactivation of isocitrate lyase in vitro was achieved by incubation of either crude homogenates or a mixture of 4 parts of crude homogenate (10 mg protein/ml) and 1 part of partially purified enzyme solution (5 mg protein/ml) in evacuated Thunberg tubes in a water bath at 28°C. The reaction was stopped by freezing the mixtures in prechilled tubes in a deep freezer. Controls were frozen immediately after mixing.

Precipitation of proteins of crude homogenates was performed by addition of appropriate amounts of solid $(NH_4)_2SO_4$. The precipitates were dissolved in 0.1 M tricine buffer, pH 7.5. All fractions, and in some experiments, crude homogenates were de-salted by Sephadex G-50 gel filtration using dextrane blue and flavine mononucleotide as indicators.

Enzyme activities were determined as described previously [5,9]. Malate synthetase and citrate synthetase [10] were assayed after removal of dithiothreitol from the enzyme solutions by Sephadex G-50 gel filtration. One unit of isocitrate lyase activity is defined as 1 nmole glyoxylate phenylhydrazone produced per min per ml enzyme solution.

3. Results and discussion

When crude homogenates of cotyledons of 5 day old sunflower seedlings were incubated under

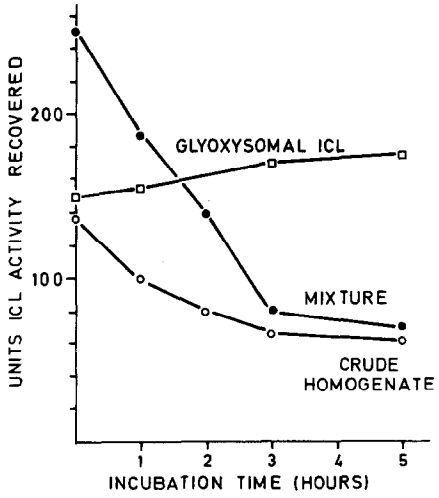

Fig.1. Inactivation of partially purified glyoxysomal isocitrate lyase (ICL) by crude homogenates of cotyledons of 5 day old etiolated sunflower seedlings.

anaerobic conditions at 28°C about 50% of the initial isocitrate lyase activity was lost within 3 h (fig.1) but the activity of none of the other microbody marker enzymes tested was affected by such treatment (table 1). Thus it seems unlikely that the inactivation of isocitrate lyase is due to the activity of unspecific proteases presumably present in the homogenates. Also, inactivation cannot be explained in terms of high thermolability of the enzyme since under identical conditions partially purified glyoxysomal isocitrate lyase was not inactivated (fig.1).

In a mixture of glyoxysomal isocitrate lyase and crude homogenates of 5 day old cotyledons the enzyme was inactivated with a rate of 60–90 units/h during the first 3 h of incubation (fig.1). But when glyoxysomal isocitrate lyase was treated with boiled homogenates no inactivation was observed. The degree of inactivation was proportional to the amount of homogenate protein added to the mixture (fig.2). The inactivator was precipitated mainly between 0% and 30% saturation of crude homogenates with $(NH_4)_2SO_4$, and was eluted from Sephadex G-50 gel with the protein peak. These results strongly suggest that the inactivator contains or is a protein.

In homogenates of cotyledons of imbibed seeds (fig.3) and of 1 or 2 day old etiolated seedlings no inactivator was detected. Inactivator activity was highest between day 4 and day 5 of germination closely corresponding to the onset of isocitrate lyase degradation in vivo [7]. Illumination of 4 day old cotyledons enhances the in vivo destruction of isocitrate lyase activity [2]. Accordingly, homogenates

Table 1
Effect of incubation of crude homogenates at 28°C on glyoxysomal and leaf-peroxisomal enzyme activities

Enzyme	Enzyme activity recovered after pre-incubation of crude homogenates for different times (hours)		
	0	1	3
	μmol substrate utilized/min/ml		
Isocitrate lyase	0.175	0.120	0.072
Malate synthetase	0.039	0.029	0.036
Malate dehydrogenase	5.57	4.49	4.94
Citrate synthase	0.015	0.015	0.019
Catalase	824.1	835.0	916.2
Glycolate oxidase	0.104	0.078	0.079
Hydroxypyruvate reductase	0.37	0.41	0.38

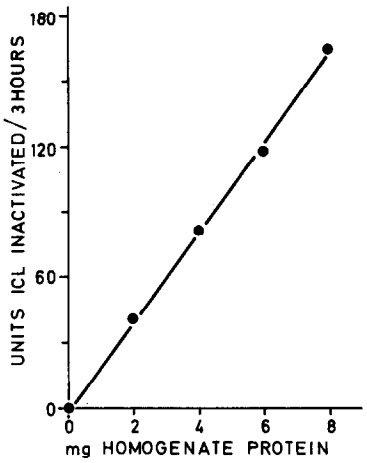

Fig.2. Proportionality of inactivation of isocitrate lyase to the amount of homogenate protein added to the mixtures. Incubation time was 3 h at 28°C.

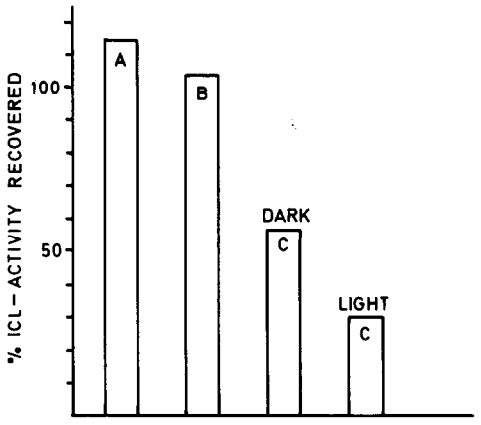

Fig.3. Recovery of glyoxysomal isocitrate lyase after incubation for 3 hours with crude homogenates of sunflower cotyledons. (A) partially purified glyoxysomal isocitrate lyase, no additions. (B), (A) plus 8 mg homogenate protein of cotyledons of seeds which had been imbibed for 12 h. (C), (A) plus 8 mg homogenate protein of cotyledons of seedlings which had been grown for 5 days in the dark (Dark) or for 4 days in the dark plus 1 day in the light (Light) – incident light intensity about 1000 lux (Philips TL 15 W/32 Warmton de Luxe).

of illuminated cotyledons showed maximum inactivator activity (fig.3). These data clearly demonstrate a temporal and physiological correlation between the in vivo pattern of development of isocitrate lyase and the occurrence of the inactivator activity in the cotyledons.

In conclusion, the results presented in this communication provide further evidence that distinct microbody enzymes are regulated by subtle mechanisms rather than by bulk degradation of entire glyoxysomal organelles [1]. Evidently isocitrate lyase is the sole glyoxysomal *matrix* enzyme which is not present in leaf peroxisomes. Other glyoxysomal enzymes are either found in both types of microbody, e.g. catalase, malate dehydrogenase, glycolate oxidase, and hydroxypyruvate reductase [13], or they are bound to the glyoxysomal membrane, e.g. malate synthetase and citrate synthetase [9,10]. Therefore one might expect either net preservation of these enzymes or distinct mechanisms such as increased membrane turnover to inactivate membrane bound enzymes.

Our results give no information on the nature of the inactivation reaction which could involve hydrolysis [14], (de-)phosphorylation [15] or (de-)acylation [16] of the enzyme protein. Attempts to isolate and characterize the inactivator of isocitrate lyase and to elucidate the type of inactivation reaction are presently undertaken in this laboratory.

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