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ISOCITRATE LYASE IN GERMINATING SPORES OF THE FERN *ANEMIA PHYLLITIDIS*

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Abstract—Isocitrate lyase was partially purified from germinating spores of the fern *Anemia phyllitidis*. The enzyme requires Mg^{2+} and thiol compounds for maximal activity and has a pH optimum between 6.5 and 7.5. The K_m of the enzyme for threo-Δ_s-isocitrate is 0.5 mM. Succinate inhibits the enzyme non-competitively (K_i 1.8 mM). The increase of isocitrate lyase activity is closely correlated with the induction of the germination process. The fall of enzyme activity during germination is associated with the decline in triglyceride reserves.

INTRODUCTION

Like many seeds of higher plants [1], fern spores contain lipids as storage material [2]. In spores of the fern *Anemia phyllitidis*, more than 80% of these lipids are accounted for by triglycerides which are degraded during germination [3]. In lipid-rich germinating seeds, storage-triglycerides are catabolized providing the growing embryo with energy and acetate units. The conversion of storage-lipids to sugars in seeds has been intensively studied [4]. The initial degradation steps involve lipases producing glycerol and free fatty acids. The latter, in turn, are degraded by β-oxidation to acetyl-CoA which is utilized via the glyoxylate cycle for carbohydrate synthesis. During the first days of germination, the activities of isocitrate lyase and malate synthetase, the key enzymes of the glyoxylate cycle, rise dramatically and then decline gradually, concomitant with the decrease of the triglyceride reserves. The enzymes of both β-oxidation and the glyoxylate cycle are located in discrete organelles, the glyoxysomes [5, 6]. During glyoxysome formation, both key enzymes probably are synthesized *de novo*, however, the involvement of stable *m*RNA or *de novo*-synthesized *m*RNA is not clearly established.

Isocitrate lyase (ICL, threo-D_s-isocitrate glyoxylate lyase, EC 4.1.3.1) was demonstrated for the first time in germinating castor beans [7]. Subsequently, this enzyme has been found in a variety of lipid storing seeds [8–12]. ICL has further been described in fungi [13, 14], algae [15, 16], bacteria [17, 18] and nematodes [19]. In micro-organisms, where ICL is an adaptive enzyme, the regulatory mechanisms involved have been investigated [16, 17].

The present study was undertaken to investigate the function of the glyoxylate cycle during fern spore germination. The occurrence and properties of ICL in ferns are reported for the first time. The relation between lipolysis and the development of ICL activity during the germination process is also reported.

RESULTS

Partial purification of ICL

The enzyme was extracted from 10-day-old prothalli grown in continuous white light. Because of the high concentration of phenolic compounds, an active enzyme preparation was obtained only when the extraction buffer was supplemented with PVP and 2-mercaptoethanol. The results from a representative purification experiment are summarized in Table 1. Due to the extreme instability of the enzyme, the purification procedure had to be carried out within one day. The purification was carried out as follows. The crude extract was heated at 50° for 10 min. After centrifugation, the supernatant was brought to pH 5.3 by dropwise addition of 0.5 M HOAc. Subsequent ion exchange chromatography was carried out at pH 7 using DEAE-Sepharose CL-6B which had been equilibrated in 0.01 M Tricine buffer, pH 7, supplemented with 1 mM EDTA and 1 mM 2-mercaptoethanol (TEM buffer). ICL was eluted with 0.1 M NaCl in this buffer. The most active fraction was layered on top of a linear sucrose gradient (5–20%; w/w) in TEM buffer. Samples were centrifuged for 4 hr at 39000 rpm (180000 *g*) in a swinging bucket rotor (SB-283) in a IEC/B-60 ultracentrifuge [20]. ICL activity was found at a concentration of 7% sucrose.

Properties

Stability. In crude extracts a loss of 70% of enzyme activity was observed within 6 hr at 4°; freezing resulted in complete loss of enzyme activity. The partially purified

Table 1. Purification of isocitrate lyase from *Anemia phyllitidis*

Step	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)	Yield (%)	Purification factor
Crude extract	65	14.2	0.22	100	1
Heat treatment 50°	46	12.0	0.26	85	1.2
Acid treatment pH 5.2	22	16.6	0.75	117	3.4
DEAE-Sepharose CL-6B	2.3	4.4	1.9	31	8.8
Sucrose gradient centrifugation	0.19	1.8	9.5	13	43

enzyme was completely inactivated during storage at −20° within 48 hr. The crude enzyme was relatively insensitive to heat; exposure to 50° for 10 min yielded 80–90% residual activity.

Effect of pH. A broad pH optimum (phosphate buffer) was found between 6.5 and 7.5 with a sharp decrease to pH 5.5 and a gradual decrease to pH 9. Enzyme activity was proportional to enzyme concentration between 5 and 500 μg protein (48–725 pkat).

Michaelis constant. K_m was determined with threo-Δ_S-isocitrate, whereas threo-DL-isocitrate was used throughout in routine measurements. From Lineweaver–Burk plots, a K_m value of 0.5 mM was determined.

Inhibitors. The effects of product inhibition and of sulfhydryl antagonists on the cleavage reaction were assayed with the standard enzyme assay. 50% inhibition at isocitrate saturation was observed with 50 mM malate. For succinate, which was found to inhibit non-competitively, a K_i value of 1.8 mM was determined. Preincubation of the samples (substrate omitted) for 5 min in the presence of 0.8 mM *p*-chloromercuribenzoate, 20 mM iodoacetamide or 10 mM hydroxylamine resulted in 91, 96 or 80% inhibition, respectively.

Development of ICL

White light. Activity of ICL during germination and early development of the prothalli from 100 mg spores was measured and compared to cell number and lipid content (Fig. 1). Only trace amounts of ICL activity could be detected in the dry spore. With the outgrowth of the first protonema cell between the third and fourth day of germination, ICL activity increases dramatically. The maximal enzyme activity coincides with the maximum of lipid degradation, and the subsequent decrease in ICL activity is associated with the depletion of the lipid reserves. No residual enzyme activity could be detected after 22 days when the storage lipids had been degraded completely. An identical time course of ICL activity has been observed when spores were germinated in white light of reduced intensity (25 μW cm^{-2}) resulting in a reduced cell number. This shows that the ICL activity is not correlated with cell number or growth rate.

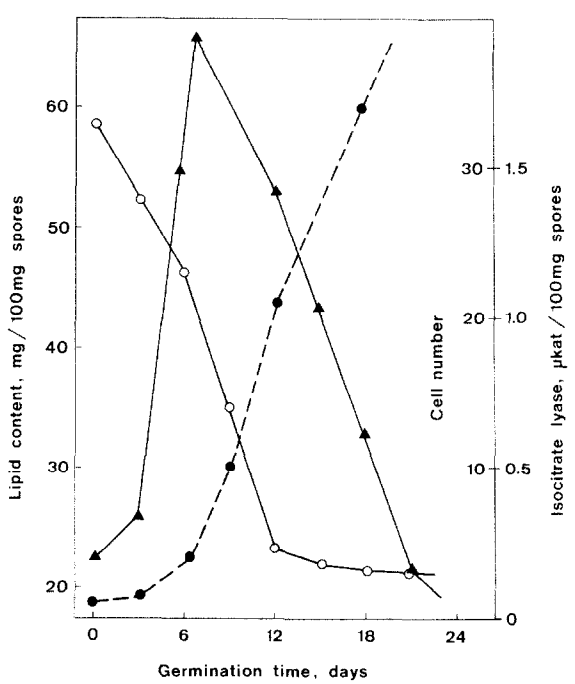

Fig. 1. Changes in isocitrate lyase activity (▲—▲), lipid content (○—○) and cell number (●- - -●) in spores of *Anemia phyllitidis* during germination.

Table 2. Effect of different light qualities, phytohormones and inhibitors on spore germination and induction of isocitrate lyase activity in *A. phyllitidis*

Treatment	Cell No.	ICL activity (%)
White light (1 mW cm^{-2})	10	100
White light + cycloheximide (5 μg/ml)	ng	0
Darkness	ng	0
Darkness + GA_3 (10^{-5} g/ml)	4	80
Darkness + kinetin (10^{-4} g/ml)	ng	0
Blue light	ng	0
Far-red light	ng	0
Red light	6	100

In each experiment, 100 mg spores were cultivated 10 days at 21°. Cell numbers are expressed as the average of 100 prothalli. ng = No germination.

Different light qualities. Further experiments on the induction of ICL activity revealed a close connection with the induction of the germination process. The data from Table 2 show that ICL activity increased only in those spores which had germinated, i.e. in red light or in darkness when the red light requirement of spores for germination had been replaced by gibberellic acid [21]. In red light, ICL activity followed the same time course as in white light. In contrast to gibberellic acid, there is no effect of kinetin in stimulating ICL activity in the dark. Furthermore, it was shown that ICL activity does not increase in spores kept under light qualities which do not induce germination, nor in spores in which germination is inhibited by cycloheximide.

DISCUSSION

The present work provides evidence for the function of ICL during germination of the lipid storing spores of *A. phyllitidis*. Since malate synthetase, the second key enzyme of the glyoxylate cycle, is active during the same phases of development (Gemmrich, A. R., unpublished results) it is suggested that, in germinating spores, the storage triglycerides are also converted to carbohydrates via the glyoxylate cycle as has been established for lipid-storing seeds of higher plants. The ICL from *A. phyllitidis* is similar to the ICLs from seeds in several aspects, e.g. in their pH optimum, K_m for isocitrate, and requirement of thiol compounds and Mg^{2+} for maximal activity [22]. In contrast to castor bean ICL, the *Anemia* enzyme is not sensitive to heat. The inhibitory effect of sulfhydryl antagonists indicates that, as also reported for yeast ICL [23], the maintenance of reduced sulfhydryl groups is necessary for enzyme activity. However, in purified enzyme of *Pseudomonas indigofera* no inactivation of ICL was achieved with 0.2–1 M hydroxylamine, indicating that no thioester is involved in the catalytic action [24]. As in microorganisms [25], succinate inhibits

ICL in a non-competitive manner. This supports the hypothesis that ICL activity can be controlled by the reaction product in which the levels of succinate might regulate the activity of ICL.

The sharp rise and fall of ICL activity during germination which is associated with the decline in lipid content seems to be typical for the single-celled fern spore as well as for the complex seed [9, 22]. The fact that the development of ICL does not depend on the cell number of the prothalli but rather on their age suggests that ICL may be localized within the spore and not in the protonema cells. This is further supported by the fact that the storage lipids are also located in the spore. In seeds of higher plants, the enzymes of the glyoxylate cycle are compartmented in microbodies [4–6] or mitochondria [26]. By analogy with the established existence of microbodies in the lipid-storing fern spores of *Polypodium vulgare* [27], it seems likely that ICL is also located in microbodies in *Anemia* spores. However, ultrastructural studies which are in progress will solve this question.

From the view of the process of spore germination, the present results show that the development of ICL activity is dependent on the previous induction of germination. The mode of induction, e.g. by white or red light (phytochrome control) or in darkness by gibberellic acid (hormone control) has no influence on the development of ICL activity. Thus, it is possible that the same factors which induce germination would also be responsible for the rise of ICL activity. However, it seems unlikely that in fern spores this regulation mechanism would be realized since in cotton seeds [11] and mustard seedlings [31] no phytochrome response on ICL activity has been found. As the development of ICL activity reflects the function of the glyoxylate cycle, the regulatory site might be expected early in lipolysis pathways. Studies on microorganisms [15, 17] have yielded evidence that the glyoxylate cycle is induced by the entering substrate acetate. In lipid-utilizing fern spores, acetyl-CoA is generated via β-oxidation by the degradation of fatty acids. Under the assumption that this model is realized in fern spores too, it seems likely that the onset of lipid degradation through lipases might be the regulatory factor, providing acetyl-CoA via β-oxidation as primer for the glyoxylate cycle. The fall of ICL activity, which depends on the decline in triglyceride content, might cease when acetyl-CoA is no longer available in the glyoxysomes. It is not clear, however, whether the enzymes involved in these steps are synthesized *de novo* during the early germination phase, yet there is evidence that the formation of ICL in seeds results from *de novo* synthesis [28–30]. Since cycloheximide inhibits the increase of ICL in *Anemia* spores, it is suggested that the formation of ICL during germination results from protein synthesis.

We tentatively conclude that the glyoxylate cycle is regulated by substrate induction since the rise and fall of ICL activity occurs only in germinating spores in which lipolysis is active. However, the mode of regulation of lipolysis is not yet understood.

EXPERIMENTAL

Plant material. Spores were collected from plants grown in the greenhouse at the University of Ulm. Culture conditions have been described previously [3].

Enzyme extraction. Enzyme was extracted from 8- to 10-day-old prothalli by grinding 10 g in a chilled mortar with 15 ml 0.1 M Tris–HCl buffer, pH 7, containing 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.1 g PVP/g fr. wt. The homogenate was squeezed through muslin and centrifuged for 20 min at 12000 *g*. The upper fat layer was discarded and the clear supernatant soln was used as the crude enzyme prepn. All operations were performed in a cold room at 4°.

Enzyme assay. ICL activity was determined according to the method of ref. [32]. The incubation mixture contained in a final vol. of 2.5 ml: 0.2 mmol Pi buffer, pH 6.8, 20 μmol $MgCl_2$, 2 μmol dithioerythretol, 10 μmol phenylhydrazine and enzyme. Reaction was started by adding 20 μmol of Na isocitrate. The formation of the glyoxylate phenylhydrazone was followed at 324 nm. The extinction coefficient of glyoxylate phenylhydrazone is 1.68×10^4/M/cm. One unit of enzyme is the amount of activity effecting the production of one mol of glyoxylate/sec. Protein was determined by the biuret method [33] and in column eluates by the method of ref. [34]. Lipid content was determined gravimetrically, as described in ref. [3].

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