ENZYME ACTIVITIES OF THE β-OXIDATION PATHWAY IN SPINACH LEAF PEROXISOMES

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1. Introduction

The subcellular localization of the β-oxidation pathway in plant cells has been studied using primarily fatty tissues of germinating seeds. For those tissues it is established that the fatty acyl-CoA oxidizing system is housed within the glyoxysomes (peroxisomes) which play a key role in the conversion of the stored fat to sucrose at germination [1,2]. Apart from the fatty seedling tissues the enzymes of the β-oxidation pathway are generally thought to be localized in mitochondria in plant tissues [3]. However, there appear to be no reports unequivocally demonstrating mitochondrial localization of the β-oxidation pathway in non-fatty plant tissues. These results show that enzyme activities of the β-oxidation pathway in leaf tissues are associated with peroxisomes.

2. Materials and methods

2.1. *Organelle isolation*

Young leaves (15 g) from spinach (*Spinacia oleracea* L. var. Früremona) grown in water culture [4] were homogenized in 15 ml medium consisting of 170 mM tricine (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 1mM EDTA, 10 mM mercaptoethanol, 1 M sucrose, and 9 mg BSA/ml. Organelles were pelleted from the homogenate by differential centrifugation [5] and then separated by centrifugation (2 h, 83 000 × g_{av}) on density gradients composed of 60% (3 ml), 57% (5 ml), 51% (8 ml), 47% (8 ml), 42% (6 ml), and 35% (3 ml) sucrose (w/w) made up in 1 mM EDTA (pH 7.5). The mitochondrial as well as the peroxisomal bands, identified by their marker enzymes, were collected as whole fractions. In various experiments these fractions were recentrifuged (16 h, 83 000 × g_{av}) on linear flotation gradients from 30–60% sucrose.

2.2. *Assays*

The mitochondrial and peroxisomal fractions were assayed either directly or after dilution with an equal volume of 20 mM Tris (pH 8.0) and following concentration by ultrafiltration (Diaflo membrane PM10, Amicon) to 1/5th of their original volume. Acyl-CoA oxidase activity was determined spectrophotometrically by measuring the palmitoyl-CoA-dependent H_2O_2 production as in [6–8]. The assay medium (1 ml; 20°C) contained 50 mM potassium phosphate (pH 7.5), 5.5 units peroxidase (Sigma type II), 12.5 mM *p*-hydroxybenzoic acid, 1 mM 4-aminoantipyrine, 50 μM FAD, 1 mM NaN_3, and 50 μM palmitoyl-CoA. Acyl-CoA oxidase activity was also measured polarographically with a YSI Clark-type electrode (Yellow Springs Instr.) by the rate of palmitoyl-CoA-dependent oxygen uptake. The assay medium (2 ml, air saturated, 25°C) contained 50 mM potassium phosphate (pH 7.5) and 50 μM palmitoyl-CoA. All other enzyme activities were assayed, with only minor modifications: enoyl-CoA hydratase [9]; 3-hydroxyacyl-CoA dehydrogenase [9]; thiolase [10]; catalase [5]; glycolate oxidase [11]; fumarase [12]; cytochrome oxidase [13]. Enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and thiolase were assayed in the presence of 2 mM KCN. Protein was determined by the Lowry method.

3. Results and discussion

Mitochondrial as well as peroxisomal fractions isolated from spinach leaves showed activity when tested for the presence of 3-hydroxyacyl-CoA dehydrogen-

Table 1
Activity of 3-hydroxyacyl-CoA dehydrogenase (nmol . min^{-1} . fraction^{-1}) peroxisomal and mitochondrial marker enzymes in the peroxisomal and mitochondrial fraction from spinach leaves

	Peroxisomal fraction (I)	Mitochondrial fraction (II)	Ratio I/II
Protein/fraction	2.5 mg	21.9 mg	
3-Hydroxyacyl-CoA dehydrogenase	52	12	4.33
Catalase	1060 × 10^3	190 × 10^3	5.58
Glycolate oxidase	1160	275	4.22
Cytochrome oxidase	53	325	0.16
Fumarase	200	1163	0.17

ase. The specific activity of the enzyme was 10–50-times lower in the mitochondrial fraction than in the peroxisomal fraction. This difference did not result from contamination of the mitochondrial fraction by non-mitochondrial protein only (thylakoids of broken chloroplasts) but was also due to a higher total activity of the enzyme in the peroxisomal fraction (table 1). On the average, this fraction contained a 5-fold higher total activity of 3-hydroxyacyl-CoA dehydrogenase than the mitochondrial fraction.

Contamination of the peroxisomal fraction by mitochondria could not account for the activity of 3-hydroxyacyl-CoA dehydrogenase associated with this fraction. The total activity of two mitochondrial marker enzymes, membrane-bound cytochrome oxidase and soluble fumarase, was considerably lower in the peroxisomal fraction than in the mitochondrial fraction while the reverse was the case with respect to 3-hydroxyacyl-CoA dehydrogenase (table 1). The ratio of 3-hydroxyacyl-CoA dehydrogenase activity to the activity of peroxisomal marker enzymes, catalase and glycolate oxidase, did not significantly differ, according to the Mann-Whitney U-test ($\alpha = 0.1$, one-sided test), between the peroxisomal and mitochondrial fraction (table 2).

Besides 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase and thiolase are associated with the peroxisomal fraction from spinach leaves (table 3). Since enoyl-CoA hydratase showed a relatively high activity, the distribution of this enzyme was determined on a linear flotation gradient, following isopycnic centrifugation (16 h, 83 000 × g_{av}) of a crude organelle fraction. The gradient was fractionated into 1 ml fractions and each fraction assayed for enoyl-CoA hydratase and marker enzymes. As fig.1 shows the distribution of enoyl-CoA hydratase was virtually identical to that of glycolate oxidase, demonstrating a peroxisomal localization.

Finally, fractions of leaf peroxisomes were tested for the presence of acyl-CoA oxidase, the initial enzyme of the β-oxidation pathway located in glyoxysomes [10] and rat liver peroxisomes [7,8,14]. In contrast to the acyl-CoA dehydrogenase of the mammalian mitochondrial β-oxidation system tightly coupled to the electron-transport chain [15], the acyl-CoA oxidase transfers electrons directly to O_2, producing H_2O_2 [7,10,14]. Acyl-CoA oxidase could be demonstrated in the leaf peroxisomal fraction, measuring the palmitoyl-CoA dependent H_2O_2 production or O_2-uptake (table 3). Addition of cyanide increased

Table 2
Ratio of 3-hydroxyacyl-CoA dehydrogenase activity to the activity of peroxisomal marker enzymes in the peroxisomal and mitochondrial fraction from spinach leaves

	Peroxisomal fraction	Mitochondrial fraction
3-Hydroxyacyl-CoA DH / Glycolate oxidase	(3.6 ± 0.7) × 10^{-2}	(4.7 ± 0.3) × 10^{-2}
3-Hydroxyacyl-CoA DH / Catalase	(4.5 ± 1.1) × 10^{-5}	(5.6 ± 1.0) × 10^{-5}

Results represent means ± SE for 8 expt

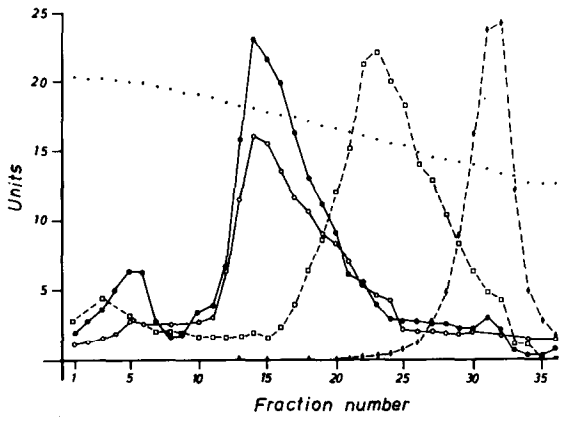

Fig.1. Distribution of enzyme activities after isopycnic centrifugation of a crude organelle fraction on a flotation gradient. Ten units correspond to: 118 nmol glyoxylate formed . min^{-1} . $fraction^{-1}$ for glycolate oxidase (•), 322 nmol NADH formed . min^{-1} . $fraction^{-1}$ for enoyl-CoA hydratase (○), 83 nmol fumarate formed . min^{-1} . $fraction^{-1}$ for fumarase (□), 168 μg chlorophyll/fraction (♦), and 30% sucrose concentration for gradient density (••). Enoyl-CoA hydratase was assayed in the presence of 0.02% Triton X-100.

the measurable O_2-uptake due to the inhibition of the breakdown of H_2O_2 catalyzed by the peroxisomal catalase (table 3). However, the theoretical doubling of the rate of O_2-uptake was not completely reached. Salicylhydroxamic acid (2 mM), an inhibitor of the cyanide-insensitive electron transport in plant mitochondria [16], did not influence the palmitoyl-CoA-dependent O_2-uptake whether cyanide (2 mM) was present or absent. These results demonstrate that activity of each of the enzymes involved in peroxisomal fatty acid oxidation is associated with spinach leaf peroxisomes. The specific activity of the enzymes, however, is 10–100-times lower than in the glyoxysomal (peroxisomal) fractions isolated from fatty seedling tissues [2].

Table 3
Activity of the β-oxidation pathway enzymes associated with the peroxisomal fraction from spinach leaves

	(nmol . min^{-1} . mg $protein^{-1}$)
Acyl-CoA oxidase	
spectrophotometric assay	22
polarographic assay	37
+2 mM KCN	64
Enoyl-CoA hydratase	671
3-Hydroxyacyl-CoA DH	32
Thiolase	8

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