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Involvement of Carnitine Acyltransferases in Peroxisomal Fatty Acid Metabolism by the Yeast *Pichia guilliermondii*

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This article provides information about peroxisomal fatty acid metabolism in the yeast *Pichia guilliermondii*. The existence of inducible mitochondrial carnitine palmitoyltransferase and peroxisomal carnitine octanoyltransferase activities was demonstrated after culture of this yeast in a medium containing methyl oleate. The subcellular sites and induction patterns were studied. The inhibition of carnitine octanoyl- and palmitoyltransferases by chlorpromazine to a large extent prevented the otherwise observed metabolism-dependent inactivation of thiolase by 2-bromofatty acids in vivo. We concluded that the metabolism of long- and medium-chain fatty acids in the peroxisome of this yeast involved carnitine intermediates.

The metabolic pathway of peroxisomal β-oxidation in yeasts has been extensively studied and is now well documented. Because of its great inducibility and the high levels of enzymatic activities observed, it is of great interest to industry. It is, for example, responsible for the biotransformation of ricinoleic acid [12(*R*)-hydroxy-9(*Z*)-octadecenoic acid] into γ-decalactone, a compound with a peachy odor (3, 12). The penetration of fatty acids into the peroxisome is a potentially rate-limiting step for this biotransformation (13).

The above-mentioned fatty acid penetration was first described as a non-rate-limiting and carnitine-independent process in rat hepatocytes. Researchers also observed the presence of a nonselective pore-forming protein (8, 18, 21). However, the fatty acid derivatives found inside the cell are amphiphilic molecules that probably do not enter the organelle via such a pore. The pH gradient observed between the cytosol and the peroxisomal lumen in yeasts (11) is inconsistent with the existence of a nonselective pore in vivo.

Two peroxisome-specific acylcarnitine transferases (carnitine octanoyltransferase [COT] and carnitine acetyltransferase [CAT] [9]) in rat hepatocyte peroxisomes have been described. These organelles also contain a carnitine biosynthesis system (14). Leighton et al. (7) demonstrated the specific inhibition of peroxisomal β-oxidation by chlorpromazine (CPZ), an inhibitor of COT (20). The metabolism-dependent inactivation of thiolase activity by 2-bromofatty acids was used to monitor the rate of uptake of these fatty acid analogs by peroxisomes in vivo. Tetradecylglycidic acid, a highly specific inhibitor of carnitine acyltransferases, protected peroxisomal 3-ketoacyl coenzyme A (CoA) thiolase activity in vivo from time-dependent inactivation by 2-bromododecanoic acid (2). The presence of CPZ in a culture medium decreased the specific rate of production of γ-decalactone from methyl ricinoleate by the yeast *Pichia guilliermondii*. This effect was directly dependent on the amount of CPZ present in the culture medium (13).

The aim of our study was to gain further knowledge about peroxisomal fatty acid metabolism and its role in the biotransformation of methyl ricinoleate into γ-decalactone by the yeast *P. guilliermondii*. CAT, COT, and carnitine palmitoyltransferase (CPT) activities were measured after culture on media containing glucose or methyl oleate. Their subcellular distribution between peroxisomes and mitochondrias and their role in the peroxisomal metabolism of fatty acid analogs were studied. 2-Bromofatty acids of long (palmitoyl- and dodecanoyl-CoA)- and medium-chain (octanoyl-CoA) lengths were incubated with yeast cells in the presence of CPZ, and their rates of metabolism were estimated from the degree of inhibition of thiolase activity.

Spheroplasting with Zymolyase (Seikagaku, Tokyo, Japan) was performed before cell fractionation. 2-Bromofatty acids were purchased from Fluka Chemical Co. (Buchs, Switzerland). Malt extract agar and yeast autolytic extract were obtained from Prolabo (Paris, France). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Biomass production was performed as previously described (3). Cells were recovered by centrifugation (6,000 × *g*, 5 min, 20°C) during the exponential growth phase and transferred to the induction medium. Peroxisomal β-oxidation induction was performed for 48 h in the same basal medium supplemented with 1 g of methyl oleate per liter or 1.8 g of glucose (carbon equivalent quantity; control cultures) per liter in the presence of 1 g of Tween 80 (emulsifier) per liter before cell fractionation or enzymatic analysis. The cells were then transferred to incubation media as described above. The incubation media were similar to the induction medium but contained various concentrations of 2-bromofatty acids as the sole carbon sources. Incubations were carried out for 15 min to 2 h with or without 0.5 mM CPZ. 2-Bromofatty acids were saponified by heating in an ammoniacal solution, dried under nitrogen, and solubilized in aqueous solutions prior to analysis.

Cells were disrupted after 2 days of culture in the induction medium and incubation as previously described (3). An aliquot of the resulting mixture was used to measure CAT, COT, and CPT activities. Cell debris was removed by centrifugation (6,000 × *g*, 4°C, 5 min). Soluble proteins were obtained from the supernatant as previously described (13). The protein concentration in the resulting extract was determined as described by Bradford (1) with bovine serum albumin as the standard and then adjusted to 4 mg/ml. Acyl-CoA oxidase activity was measured as described by Endrizzi et al. (3). Succinate dehydrogenase activity was measured according to the method of Singer et al. (17). Thiolase activity was measured as described by Raaka and Lowenstein (15), with aceto-acetyl-CoA as the

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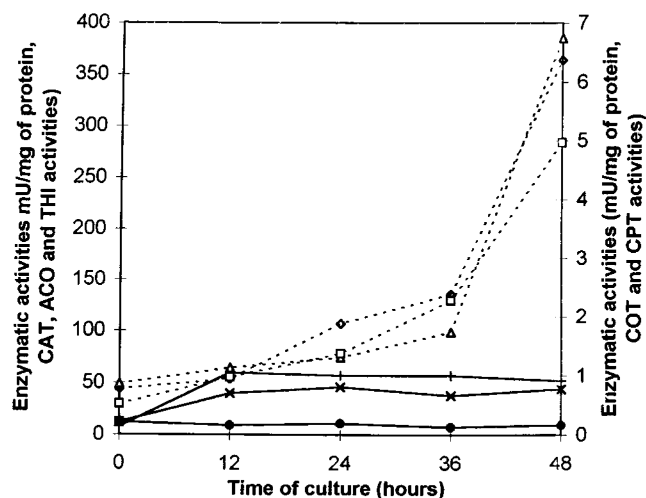

FIG. 1. Induction of carnitine acyltransferase and β-oxidation enzyme activities in *P. guilliermondii* during culture on a medium containing 1 g of methyl oleate per liter as the sole carbon source. —+—, acyl-CoA oxidase (ACO); —•—, acyl-CoA oxidase (control culture on glucose medium); —×—, thiolase (THI); --◇--, CAT; --△--, COT; --□-- CPT.

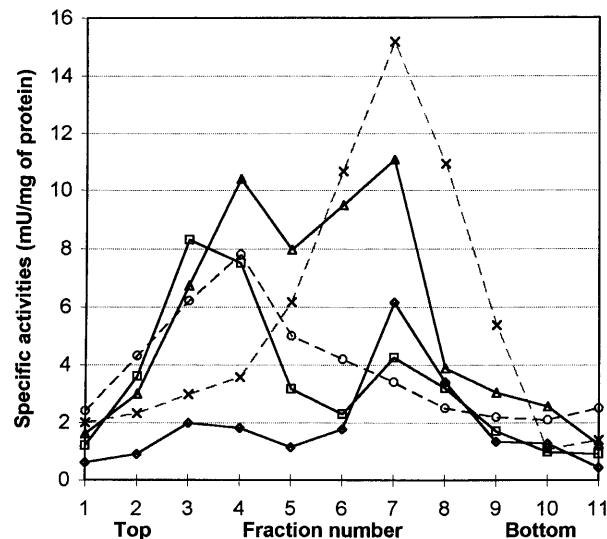

FIG. 2. Subcellular distributions of carnitine acyltransferase activities between peroxisomes and mitochondria after fractionation on a discontinuous Nycodenz gradient (12 to 50%). Marker enzymes include succinate dehydrogenase (mitochondria) (—○—) and acyl-CoA oxidase (peroxisomes; values are 100-fold higher than those on the graph) (—×—). Carnitine acyltransferase activities were measured with palmitoyl-CoA (CPT) (—□—), octanoyl-CoA (COT) (—◇—), and acetyl-CoA (CAT; values are 25-fold higher than those on the graph) (—△—) as the substrates.

substrate. Carnitine acyltransferase activity was measured in the forward direction as described by Schäfer et al. (16). Overall β-oxidation activities were measured according to the method of Lazarow (5). The incubation buffer used to measure β-oxidation, supplemented with 2 mM ATP (to allow the activation of 2-bromofatty acids into acyl-CoAs), was also used to check the inhibitory effect of 2-bromofatty acids on thiolase activity in vitro. 2-Bromofatty acids at a final concentration of 1 mM were incubated at 30°C in 1 ml of the incubation buffer in the presence of 100 μl of protein extract or of enriched fractions (see below). Thiolase activities in the assay were measured after 1 h of incubation. The peroxisomes were separated from the mitochondria after the induction of peroxisomal β-oxidation activities for 48 h in induction medium with a 12 to 50% Nycodenz gradient as described by Moreno de la Garza et al. (10). The resulting fractions were subjected to enzyme analysis as described above.

CAT, COT, CPT, acyl-CoA oxidase, and thiolase activities were measured during culture in a medium containing methyl oleate (1 g/liter). The results are presented in Fig. 1. Acyl-CoA oxidase activities in control cultures are provided as markers of peroxisomal β-oxidation. The peroxisomal β-oxidation activities greatly increased during the first 12 h of culture (about four- and sixfold for thiolase and acyl-CoA oxidase, respectively) and then became stable. Carnitine acyltransferase activities on medium (octanoyl-CoA)- and long (palmitoyl-CoA)-chain fatty acyl-CoAs were detected and slightly increased during the first 36 h of culture (about twofold). This increase was even greater after 36 to 48 h of culture. The induction profile was the same for all three enzymes, although the activity of CAT was about 50-fold higher than those of COT and CPT. None of these activities was induced in control cultures.

Subcellular distribution of CAT, COT, and CPT activities. The results from determining the subcellular distribution of CAT, COT, and CPT activities are presented in Fig. 2. Succinate dehydrogenase and acyl-CoA oxidase were used as marker enzymes of mitochondrial and peroxisomal fractions, respectively. Peroxisome- and mitochondrion-enriched fractions had densities of 1.14 and 1.09, respectively. CAT activity was enriched in mitochondrial and peroxisomal fractions as expected. COT and CPT activities were present in both fractions, but COT activity was enriched in the peroxisomal fraction while CPT was enriched in mitochondrial fractions.

Effects of CPZ and 2-bromofatty acids on carnitine acyltransferase and thiolase activities in vitro. The results of assays to determine the effects of CPZ (0.5 mM) on carnitine acyltransferase (1 mM) and thiolase activities in vitro are presented in Table 1. CPZ (0.5 mM) did not have any effect on either thiolase activity or overall β-oxidation activity in vitro. After 1 h of incubation, 2-bromooctanoic acid and 2-bromododecanoic acid (1 mM) inhibited thiolase activity by about 65% and 2-bromopalmitic acid (1 mM) inhibited thiolase activity by about 85%. The effects of CPZ and 2-bromofatty acids on acylcarnitine transferase activities in vitro were estimated with enriched fractions after cell fractionation. Fractions 3 (mitochondrial) and 7 (peroxisomal) were used to measure CPT and COT activities, respectively. CAT activity was measured in both fractions. After 1 h of incubation, 0.5 mM CPZ inhibited about 55% of COT and CPT activities but had no effect on CAT activity. 2-Bromooctanoic and 2-bromododecanoic acids (1 mM) did not inhibit any of the three carnitine

TABLE 1. In vitro effects of β-oxidation inhibitors on carnitine acyltransferase and thiolase activities

Enzyme	Activity of indicated enzyme remaining (% of reference) with inhibitor[a]:			
	CPZ	2-BrC8	2-BrC12	2-BRC16
CAT	100	102	98	101
COT	48	99	97	31
CPT	43	101	101	16.5
THI[b]	96	32	34	17

[a] 2-BrC8, 2-bromooctanoic acid; 2-BrC12, 2-bromododecanoic acid; 2-BrC16, 2-bromopalmitic acid.

[b] THI, thiolase.

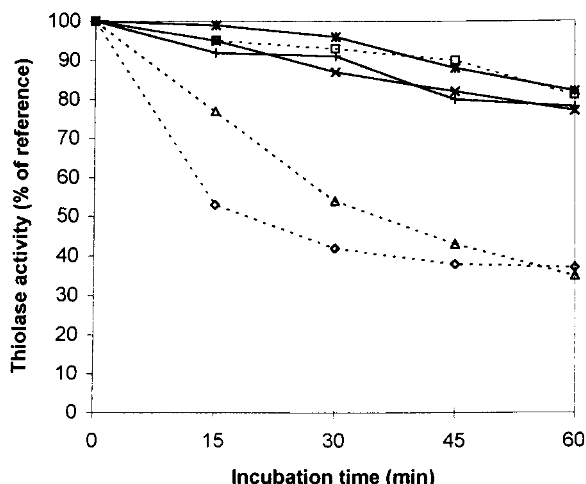

FIG. 3. Thiolase activity during incubation on a medium containing a 1 mM 2-bromofatty acid as the sole carbon source and 0.5 mM CPZ. --◇--, medium containing 2-bromooctanoic acid without CPZ; --△--, 2-bromododecanoic acid without CPZ; --□--, 2-bromopalmitic acid without CPZ; —+—, 2-bromooctanoic acid plus CPZ; —×—, 2-bromododecanoic acid plus CPZ; —✱—, 2-bromopalmitic acid plus CPZ.

acyltransferases, whereas 2-bromopalmitic acid (1 mM) appeared to be a potent inhibitor of COT and CPT activities but did not have any effect on CAT activity.

Thiolase and carnitine acyltransferase activities were monitored in vivo during cell incubation in media containing 2-bromofatty acids. The results are presented in Fig. 3. 2-Bromooctanoic and 2-bromododecanoic acids irreversibly inactivated thiolase activity in a time-dependent manner. Sixty to 65% of the thiolase activity was inactivated after 1 h of incubation. In contrast, the presence of 1 mM 2-bromopalmitic acid in the incubation medium produced only very slight inhibition. The addition of 0.5 mM CPZ to the incubation medium protected thiolase activity from the inactivation mediated by 1 mM 2-bromooctanoic and 2-bromododecanoic acids to a large extent. The slight inhibition of thiolase activity observed during incubation in medium containing 2-bromopalmitic acid was unaffected by 0.5 mM CPZ.

Two distinct β-oxidation systems (mitochondrial and peroxisomal) have been described for animal cells (6). The peroxisomal system transforms long-chain fatty acids into shorter-chain fatty acids. The resulting medium-chain fatty acids are exported towards mitochondria. COT activity may be involved in this phenomenon (9). Yeasts, in contrast, possess only one significant β-oxidation system (4, 13, 19), which is located in the peroxisome. This system produces the complete oxidation of fatty acids to acetyl-CoA. The role proposed for COT in rat hepatocytes is not relevant to yeasts.

We have demonstrated the existence of COT and CPT activities in the yeast *P. guilliermondii*. The subcellular distribution of these activities between peroxisomes and mitochondria was similar to that observed in rat hepatocytes (9). The enrichment patterns for different carnitine acyltransferase activities in the various subcellular fractions demonstrated the existence of two distinct enzymes, one located in mitochondria, the other located in peroxisomes. These activities can be induced during culture in a medium containing methyl oleate (1g/liter) as the sole carbon source. The induction profiles for COT and CPT were very similar but different from those observed for β-oxidation enzymes, suggesting the existence of distinct induction systems.

2-Bromofatty acids were irreversible inhibitors of 3-keto-acyl-CoA thiolase activity in vitro. 2-Bromopalmitic acid was also a potent inhibitor of COT and CPT in vitro, as observed in rat hepatocytes (2). The presence of CPZ in the culture medium prevented the inactivation of thiolase activity by 2-bromooctanoic and 2-bromododecanoic acid in vivo. This suggests that the inhibition of acylcarnitine transferases by CPZ at least partially prevents metabolism of these fatty acid analogs by a peroxisome. 2-Bromopalmitic acid inhibited thiolase activity only very slightly when added to the incubation medium. Because it is a strong inhibitor of COT and CPT, it would probably not be metabolized by a peroxisome.

These results taken together suggest that the peroxisomal metabolism of fatty acids in *P. guilliermondii* involves acylcarnitine transferase activities. Metabolism seems to be carnitine dependent, whatever the size (from C_8 to C_{16}) of the side chain of the fatty acid analog tested.

This involvement of carnitine acyltransferases in peroxisomal β-oxidation may explain the observed correlation between the concentration of CPZ in the culture medium and the decrease in the γ-decalactone production rate by *P. guilliermondii* (13). Furthermore, the linearity of this relationship suggests a potentially rate-limiting role for the step inhibited by CPZ (13). We conclude that peroxisomal fatty acid metabolism by the yeast *P. guilliermondii* involves carnitine intermediates. It is likely that, as in rat hepatocytes, these intermediates play a role in the peroxisomal uptake mechanism. This process may be a rate-limiting step for peroxisomal β-oxidation and thus for the biotransformation of methyl ricinoleate into γ-decalactone by the yeast *P. guilliermondii.*

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