Peroxisomal Localization of Enzymes Related to Fatty Acid β -Oxidation in an *n*-Alkane-grown Yeast, *Candida tropicalis*

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In *Candida tropicalis* cells grown on *n*-alkanes (C_{10} – C_{13}), the levels of the activities of the enzymes related to fatty acid β -oxidation—acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase—were found to be higher than those in cells grown on glucose, indicating that these enzymes were induced by alkanes. The enzymes were first confirmed to be localized only in peroxisomes, while none of these enzymes nor acyl-CoA dehydrogenase, which is known to participate in the initial step of mitochondrial β -oxidation in mammalian cells, were detected in yeast mitochondria under the conditions employed.

The significance of the peroxisomal β -oxidation system in the metabolism of alkanes by the yeast was also discussed.

Candida tropicalis that assimilates *n*-alkanes is one of the valuable microorganisms for studying fatty acid metabolism in eukaryotic cells.¹⁾ Peroxisomes (microbodies) that profusely appear in the *n*-alkane-grown yeast cells^{2,3)} have revealed to participate in the metabolism of alkanes, based on the subcellular localization of various enzymes in the cells.^{4~6)} We have first demonstrated that the fatty acid β -oxidation system was localized in the yeast peroxisomes of alkane-grown *C. tropicalis* cells, by means of a stoichiometric study on the reduction of NAD, the formation of acetyl-CoA and hydrogen peroxide, and the consumption of oxygen depending on fatty acyl-CoA in the presence or absence of sodium azide, an inhibitor of catalase.⁷⁾ Under the conditions employed, the β -oxidation system was not found in mitochondria. Although acyl-CoA oxidase (EC 1.3.99.3) was purified from whole cells of *C. tropicalis* and characterized,⁸⁾ no information is available on the induction and the localization of the respective enzymes of the fatty acid β -oxidation system in the yeast.

The β -oxidation system has been demonstrated in microbodies of castor bean,⁹⁾ *Tetrahymena*¹⁰⁾ and *Euglena*.¹¹⁾ Many studies on peroxisomal and mitochondrial β -oxidation systems have been reported with mammalian cells treated with peroxisome-proliferators such as clofibrate.^{12~14)} The peroxisomal system plays a distinct role in degrading long chain fatty acids to middle chain fatty acids, and the mitochondrial system in degrading middle chain fatty acids, transported from peroxisomes, to acetyl-CoA. In addition, three enzymes constituting the peroxisomal fatty acid β -oxidation system—enoyl-CoA hydratase (EC 4.2.1.17), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and 3-ketoacyl-CoA thiolase (EC 2.3.1.16)—have been demonstrated to be immunochemically distinguishable from those of the mitochondrial system. There are also differences between the peroxisomal system and the mitochondrial system, that is, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase in peroxisomes constitute a bifunctional protein, while these enzymes in mito-

chondria are different proteins, and the initial step of the β -oxidation in peroxisomes is mediated by acyl-CoA oxidase but that in mitochondria is mediated by acyl-CoA dehydrogenase.

This paper deals with the induction and subcellular localization of the enzymes constituting the fatty acid β -oxidation system in the alkane-grown yeast, *C. tropicalis*. The results obtained indicated that the respective enzymes were peroxisomal.

MATERIALS AND METHODS

Cultivation of the yeast. *Candida tropicalis* pK 233 (ATCC 20336) was cultivated at 30°C in a medium containing 16.5 g/liter of glucose or 10 ml/liter of an *n*-alkane mixture (C_{10} – C_{13}) as the sole source of carbon and energy, as described previously.¹⁵⁾

Preparation of enzyme sources. Cell-free extracts were prepared from cells harvested at the exponential growth phase by disintegrating the cells with a Braun homogenizer.¹⁵⁾

Subcellular fractionation. To obtain intact peroxisomes and mitochondria, the previous method¹⁾ was modified as to the preparation of protoplasts and the protoplast homogenate, and to the fractionation of subcellular particles. Yeast cells (1.5 g dry weight) harvested at the exponential growth phase were suspended in 200 ml of 40 mM potassium phosphate buffer (pH 7.2) containing 0.65 M sorbitol, and treated with 40 mg of Zymolyase 20T for 70–80 min at 30°C until the degree of lysis reached about 75%. Thereafter all the procedures were carried out at 0–4°C. The protoplasts so formed were collected by centrifugation at $3,000 \times g$ for 10 min, suspended in 20 ml of 2.5 mM potassium phosphate buffer (pH 7.2) containing 0.65 M sorbitol and 0.5 mM EDTA, and homogenized for 10 min (20 times up and down) in a teflon homogenizer under cooling. The homogenate was centrifuged at $3,000 \times g$ for 10 min to remove heavy particles (P_1). The supernatant (S_1) was subjected to centrifugation again at $20,000 \times g$ for 15 min to obtain the supernatant fraction (S_2) and the particulate fraction (P_2) containing peroxisomes and mitochondria. The P_2 fraction was suspended in 8.0 ml of 2.5 mM potassium phosphate buffer (pH 7.2) containing 20.0% sucrose and 0.5 mM EDTA, and an aliquot (2.5 ml) was layered over a discontinuous sucrose density gradient composed of the same buffer containing 30.0, 40.0, 41.3, 42.5 and 50.0% sucrose (each 2.5 ml). Intact peroxisomes and mitochondria were isolated by centrifugation at $49,600 \times g$ for 2 hr. The fraction containing catalase was the peroxisomal fraction and that con-

taining cytochrome oxidase was the mitochondrial fraction.

Enzyme assays. Catalase, cytochrome oxidase and protein were assayed by the methods described previously.¹⁶⁾ Overall activity of the β -oxidation system was measured by following the change in absorbance of NADH at 340 nm by the method reported previously⁷⁾ with a slight modification. The reaction mixture contained 27.5 mM potassium phosphate buffer (pH 7.2), 5 μ M palmitoyl-CoA or decanoyl-CoA, 5 μ M L-carnitine, 0.3 mM CoA, 1.0 mM NAD, 1.0 mM $MgCl_2$, 3.3 mM NaN_3 , 10 mM dithiothreitol and the enzyme preparation in a final volume of 2.0 ml.

Acyl-CoA oxidase activity was determined by following the increase in absorbance of a quinoneimine dye depending on palmitoyl-CoA or decanoyl-CoA at 500 nm.¹⁷⁾ The reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.4), 0.83 mM 4-aminoantipyrine, 10.6 mM phenol, 10 μ M FAD, 0.1 mM palmitoyl-CoA or decanoyl-CoA, 45 U peroxidase (from horseradish) and the enzyme preparation in a final volume of 1.5 ml.

Acyl-CoA dehydrogenase activity was assayed by following the decrease in absorbance of dichlorophenolindophenol depending on palmitoyl-CoA or decanoyl-CoA at 600 nm.¹⁸⁾ The reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.5), 30 μ M L-carnitine, 1.0 mM *N*-ethylmaleimide, 3.0 μ M palmitoyl-CoA or decanoyl-CoA, 35 μ M dichlorophenolindophenol, 1.6 mM phenazine methosulfate and the enzyme preparation in a final volume of 1.5 ml.

FIG. 1. Novel Assay System for Enoyl-CoA Hydratase.

The initial reaction mixture contained 83.2 mM potassium phosphate buffer (pH 8.0), 25 μ M decanoyl-CoA and 1.68 ng of acyl-CoA oxidase in a final volume of 1.4 ml. The reaction was started by the addition of acyl-CoA oxidase (at A). After the absorbance of enoyl-CoA at 263 nm had reached a plateau, the mixture was boiled for 10 min and cooled to 30°C, and then the enzyme preparation from *Candida tropicalis* (0.1 ml) was added to the mixture (at B). Then, the decrease in absorbance at 263 nm was followed. The extinction coefficient for crotonyl-CoA¹⁹⁾ was used for the estimation of the enzyme activity. In this experiment, the P_2 fraction was used as the enzyme preparation. When palmitoyl-CoA was used as substrate, similar results were obtained.

Enoyl-CoA hydratase activity was measured by following the decrease in absorbance of crotonyl-CoA at 263 nm.¹⁹⁾ The reaction mixture contained 83.4 mM potassium phosphate buffer (pH 8.0), 25 μ M crotonyl-CoA and the enzyme preparation in a final volume of 1.5 ml. Enoyl-CoAs with long carbon chains are not available commercially. Therefore, an alternative novel method with acyl-CoA oxidase was devised (Fig. 1). Palmitoyl-CoA or decanoyl-CoA was first dehydrogenated to the corresponding enoyl-CoA with acyl-CoA oxidase (as judged by the increase in absorbance at 263 nm). After the reaction mixture had been boiled and cooled, the enzyme preparation from *C. tropicalis* was added to the mixture and the decrease in absorbance of enoyl-CoA was followed at 263 nm. Formation of enoyl-CoA during the second reaction was not taken into account because the activity of acyl-CoA oxidase in the enzyme preparation from *C. tropicalis* was lower than that of enoyl-CoA hydratase. As this method gave a similar result to that with crotonyl-CoA, it seemed to be much more valid to measure the activity of enoyl-CoA hydratase with enoyl-CoAs of different carbon chain lengths. Unless mentioned otherwise, this novel method was applied for the assaying of enoyl-CoA hydratase in this study.

3-Hydroxyacyl-CoA dehydrogenase activity was determined by following the decrease in absorbance of NADH depending on acetoacetyl-CoA at 340 nm.²⁰⁾ The reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.4), 25 μ M acetoacetyl-CoA, 0.1 mM NADH and the enzyme preparation in a final volume of 1.5 ml.

3-Ketoacyl-CoA thiolase activity was assayed by following the decrease in absorbance of acetoacetyl-CoA depending on CoA at 303 nm.^{21,22)} The reaction mixture contained 100 mM Tris/HCl buffer (pH 8.3), 25 mM MgCl₂, 50 mM KCl, 40 μ M acetoacetyl-CoA, 50 μ M CoA and the enzyme preparation in a final volume of 1.5 ml.

All assays were carried out at 30°C.

Chemicals. NAD and CoA were donated by Kohjin Co. (Tokyo, Japan). Other CoA derivatives were obtained from Sigma (St. Louis, U.S.A.) and acyl-CoA oxidase was donated by Amano Seiyaku Co. (Nagoya, Japan). Zymolyase 20T was obtained from Seikagaku Kogyo Co. (Tokyo, Japan) and other chemicals were purchased from commercial sources.

RESULTS

Induction of enzymes related to the fatty acid β -oxidation system

The levels of the activities of the respective enzymes constituting the fatty acid β -oxidation system in *Candida tropicalis* cells grown to the exponential phase on *n*-alkanes and glucose are presented in Table I. High activity levels of acyl-CoA oxidase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase were observed in alkane-grown cells compared with those in glucose-grown cells. The level of 3-ketoacyl-CoA thiolase activity was about three times higher in the cells grown on alkanes. On the other hand, acyl-CoA dehydrogenase, which is known as an enzyme participating in the initial step of the mitochondrial β -oxidation in mammalian cells, was not detected in both types of cells.

Subcellular localization of enzymes related to the β -oxidation system

Alkane-grown cells of *C. tropicalis* exhibited activities of the enzymes related to the fatty

TABLE I. LEVELS OF ENZYMES RELATED TO THE FATTY ACID β -OXIDATION SYSTEM IN GLUCOSE-GROWN AND *N*-ALKANE-GROWN *Candida tropicalis* pK 233

Enzyme activities were measured with cell-free extracts prepared from cells harvested at the exponential growth phase.

Enzyme	Enzyme activity (nmol $\cdot$ min ⁻¹ $\cdot$ mg protein ⁻¹)		(B)/(A)
	Glucose-grown cells (A)	Alkane-grown cells (B)	
Acyl-CoA dehydrogenase	ND ^a	ND	—
Acyl-CoA oxidase	6.2	2500	403
Enoyl-CoA hydratase ^b	55	5700	104
3-Hydroxyacyl-CoA dehydrogenase	ND	240	—
3-Ketoacyl-CoA thiolase	67	190	2.8

^a Not detectable.

^b Crotonyl-CoA was used as substrate.

FIG. 2. Subcellular Localization of Enzymes in *n*-Alkane-grown *Candida tropicalis*.

The experimental procedures were described in MATERIALS AND METHODS. P₂, 20,000 × *g* pellet (peroxisomes and mitochondria); S₂, 20,000 × *g* supernatant (microsomes and cytoplasm). A, catalase; B, cytochrome oxidase; C, β -oxidation system assayed with decanoyl-CoA; D, acyl-CoA oxidase assayed with decanoyl-CoA; E, enoyl-CoA hydratase assayed as in Fig. 1; F, 3-hydroxyacyl-CoA dehydrogenase; G, 3-ketoacyl-CoA thiolase.

FIG. 3. Particulate Localization of Enzymes in P_2 .

The experimental procedures were described in MATERIALS AND METHODS. The volume of each fraction was as follows: 1, 3.75 ml; 2~5, 2.5 ml each; 6, 1.25 ml. A, protein; B, catalase; C, cytochrome oxidase; D, β -oxidation system; E, acyl-CoA oxidase; F, enoyl-CoA hydratase; G, 3-hydroxyacyl-CoA dehydrogenase; H, 3-ketoacyl-CoA thiolase. Enzyme activities were measured as in Fig. 2.

acid β -oxidation system, as described above, as well as the overall activity of the system. These activities were mainly found in particulate fraction P_2 ($20,000 \times g$ pellet) containing catalase, a marker enzyme of peroxisomes, and cytochrome oxidase, that of mitochondria (Fig. 2). The activities observed in the S_2 fraction ($20,000 \times g$ supernatant) might be derived from organelles that disintegrated during preparation of the subcellular fractions. Acyl-CoA dehydrogenase was detected neither in the P_2 fraction nor the S_2 fraction under the conditions employed. When decanoyl-CoA or palmitoyl-CoA was used as substrate to measure the activity of the overall β -oxidation system, acyl-CoA oxidase and acyl-CoA dehydrogenase, no difference was found between the results with these substrates.

To obtain more detailed information on the subcellular localization of these enzymes, it is necessary to isolate intact peroxisomes and mitochondria by sucrose density gradient centrifugation of P_2 . The buffer concentration in the protoplast homogenate and for the sucrose gradient was found to affect the separation of these organelles. With 2.5 mM potassium phosphate buffer, instead of the 50 mM potassium phosphate buffer employed before, good separation of peroxisomes and mitochondria and high reproducibility of the results were obtained (data not shown). This procedure enabled us to demonstrate that acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, the respective enzymes constituting the fatty acid β -oxidation system, were localized only in peroxisomes of alkane-grown cells of *C. tropicalis* (Fig. 3). Acyl-CoA dehydrogenase was again not detected in these organelles.

DISCUSSION

Peroxisomes and mitochondria have recently become very significant organelles for studying the biology of eukaryotic cells.²³⁾ Peroxisomes are one of the organelles whose development can be controlled under various conditions, and provide a model to investigate

the transportation of precursor proteins encoded by nuclear genes. In mammalian cells, in which peroxisomes develop after the administration of various hypolipidemic drugs including clofibrate *etc.*, catalase and fatty acid β -oxidation enzymes have been found in the organelles and the role of the peroxisomal β -oxidation system in the cells has been discussed in detail.

As to yeasts, the presence of the fatty acid β -oxidation system was first demonstrated by us in peroxisomes developed in cells grown on *n*-alkanes or higher fatty acids as the sole source of carbon and energy.^{7,24)} The yeast system was shown to be similar to that of mammalian peroxisomes by the results of a stoichiometric study on NAD reduction, oxygen consumption, acetyl-CoA formation and hydrogen peroxide formation. That is, initial step of the system in *Candida tropicalis* is catalyzed by acyl-CoA oxidase yielding hydrogen peroxide, which is degraded by peroxisomal catalase.⁷⁾ However, the individual enzymes constituting the system, except for acyl-CoA oxidase,⁸⁾ have not been detected in the yeast cells, and their localization in peroxisomes has not been clarified yet.

Peroxisomes and mitochondria were separated successfully by using potassium phosphate buffer of a low concentration for the preparation of the protoplast homogenate and for the sucrose density gradient centrifugation. The purity of the organelles seemed to be sufficient for a conclusion on the subcellular localization of the β -oxidation enzymes, judged from the distribution of the respective marker enzymes (catalase and cytochrome oxidase).

Thus, we could first demonstrate that four enzymes of the fatty acid β -oxidation system—acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase—were induced by alkanes and localized only in peroxisomes, that is, not in mitochondria, of alkane-grown *C. tropicalis*, and that acyl-CoA dehydrogenase, the enzyme catalyzing the initial step of the mitochondrial β -oxidation system in mam-

Enzymes: 1, cytochrome P-450; 2, NADPH-cytochrome P-450 (cytochrome *c*) reductase; 3, long-chain alcohol dehydrogenase; 4, long-chain aldehyde dehydrogenase; 5, acyl-CoA synthetase; 6, acyl-CoA oxidase; 7, enoyl-CoA hydratase; 8, 3-hydroxyacyl-CoA dehydrogenase; 9, 3-ketoacyl-CoA thiolase; 10, catalase; 11, carnitine acetyltransferase; 12, isocitrate lyase; 13, malate synthase; 14, citrate synthase; 15, aconitase; 16, NAD-linked isocitrate dehydrogenase; 17, malate dehydrogenase; 18, NADP-linked isocitrate dehydrogenase; 19, NAD-linked glycerol-3-phosphate dehydrogenase; 20, FAD-linked glycerol-3-phosphate dehydrogenase; 21, glycerol-3-phosphate acyltransferase. Abbreviations used: Acetyl-Car, acetylcarnitine; CA, citrate; DHAP, dihydroxyacetone phosphate; GA, glyoxylate; G3P, glycerol-3-phosphate; iCA, isocitrate; KG, 2-ketoglutarate; MA, malate; OAA, oxalacetate; OH-Acyl-CoA, 3-hydroxyacyl-CoA; Oxo-Acyl-CoA, 3-ketoacyl-CoA; SA, succinate.

nitine acetyltransferases²⁵⁾ for the operation of the TCA cycle in mitochondria and the glyoxylate cycle between peroxisomes and mitochondria (Fig. 4).

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