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STUDIES ON CARNITINE ACETYLTRANSFERASE FROM *n*-ALKANE-UTILIZING YEAST, *CANDIDA TROPICALIS*

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Department of Industrial Chemistry
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PREFACE

This is a thesis submitted by the author to Kyoto University for the degree of Doctor of Engineering. The studies collected here have been carried out under the direction of Professor Saburo Fukui and Professor Shosaku Numa in the Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engineering and in Department of Medical Chemistry, Faculty of Medicine, Kyoto University, during 1978-1983.

It is the author's great pleasure to express hearty gratitude to Professor Saburo Fukui and Professor Shosaku Numa for their continuous guidance and encouragement throughout this work.

The author is also grateful to Lecturer Dr. Atsuo Tanaka, Dr. Saburo Horikawa (Department of Medical Chemistry, Faculty of Medicine, Kyoto University) and Dr. Susumu Kawamoto (Chiba University) for their invaluable suggestions and to Professor Masako Osumi (Department of Biology, Japan Women's University) for her kind partnership.

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INTRODUCTION

Today, rapid progresses on biotechnology, including genetic engineering, bioreactor, utilization of biomass and tissue culture, have amazed us. Many biologically useful materials have been produced by microorganisms which have remarkable ability to utilize cheap raw materials. The genes for production of very important human proteins such as insulin, growth hormone, interferon and specific antibodies have been cloned in bacteria, especially in Escherichia coli. This allowed us to produce such substances which are now virtually impossible to produce on a large scale by traditional means.

Eukaryotic microorganisms, yeast and fungi, as well as prokaryotic cells also play very important roles industrially, agriculturally, and medically.[1] For practical applications, it has been believed that the exploitation of the function of these eukaryotic cells can furnish some solutions of many problems, such as the shortage of food and the deficiency of various substances (medicines and vitamins etc.). While yeast cells are valuable microorganisms for the fundamental studies, because they can be a model of mammalian cells as the most simple eukaryotic cells.

As unconventional carbon compounds, especially aliphatic and aromatic hydrocarbons,[2] and C_1-compounds (methanol etc.)[3-6]

are considered to be promising carbon sources instead of carbohydrates, the yeast cells which can utilize such non-carbohydrates as the sole carbon and energy sources have many potential advantages and specific features; production of biomass and single cell protein, biosyntheses of biologically useful compounds (amino acids, fatty acids, nucleic acids, organic acids, antibiotics, enzymes, hormones, carbohydrates, vitamins and coenzymes).[1,7] Moreover, yeasts utilizing hydrocarbons have offered a physiologically important model of fatty acid metabolism in mammalian cells.[8-12]

The author's studies deal with the metabolic pathway of n-alkanes and the physiological roles and biogenesis of peroxisomes (microbodies) in n-alkane-utilizing Candida yeasts not only to realize the physiological significance of peroxisomes but also to develop the ability of these organelles in the industrial and medical fields.

The author hopes that the information described in this thesis will contribute to the progress of cell technology in the field of industrial biochemistry and to the progress of further understanding of cooperative functions among different cellular organelles.

Peroxisomes

Peroxisomes are characterized as subcellular organelles,

which have homogeneous matrix surrounded by a single, boundary lipid bilayer membrane. Crystalloids (core) structure is often observed in them.

The organelles were first observed in rat kidney by electron microscopy[13] and isolated from rat liver by de Duve et al,[14] who named the organelles as peroxisomes because the organelles contained hydrogen peroxide-generating oxidases and hydrogen peroxide-degrading catalase.[15,16] Occurrence of peroxisomes has been reported in many eukaryotic cells in biological world; animals[17,18] including human beings,[19] plants,[15,20] protozoa,[21] algae,[22] fungi,[23] and yeasts.[24,25] In rat liver, peroxisomes have been found to be proliferated by hypolipidemic chemicals (for example, clofibrate)[26-30] and a kind of plasticizer (di-2-ethylhexyl phthalate).[31] Development of the organelles is also observed under a certain condition of diets, for example, high-fat diets[32] and in tumor cells.[33,34] So, the investigation of these organelles in mammalian cells has been approached from the stand point of the metabolism of fatty acids.[35] On the other hand, physiological functions of peroxisomes in leaves of higher plants[16] and in castor bean endosperm[15] have also been studied intensively. The former is called "leaf peroxisomes" and the latter "glyoxysomes" from functional viewpoints. Leaf peroxisomes participate in photorespiration in connection with mitochondria and chloroplasts.[16] Glyoxysomes play important roles in fatty

acid degradation and gluconeogenesis from fats stored in endosperm during germination.[15,36] Yeast peroxisomes were first found in Saccharomyces cerevisiae.[37,38] However, there were few reports about their physiological roles and metabolic functions.

Professor Fukui's research group has revealed electron-microscopically the profuse appearance of peroxisomes in n-alkane-grown cells of Candida yeasts, as shown in Fig. 1.[11,12,39] In methanol-utilizing yeasts,[40] they have also demonstrated the presence of peroxisomes, which are larger in size and smaller in number than those in n-alkane-utilizing yeasts and contain the crystalline core.[24]

Assimilation of n-alkanes by Candida yeasts

n-Alkane assimilation by Candida yeasts is divided into several steps[41] : (1) Incorporation of n-alkanes into cells; (2) oxidation of n-alkanes to the corresponding fatty acids; (3) activation of fatty acids to their CoA esters; (4) subsequent metabolism of fatty acyl-CoAs—degradation to acetyl-CoA or incorporation of fatty acyl moieties into cellular lipids; (5) synthesis of tricarboxylic acid (TCA) cycle intermediates from acetyl-CoA via the glyoxylate cycle; (6) gluconeogenesis; (7) syntheses of amino acids, nucleic acids, and others including a portion of cellular fatty acids. First, n-alkanes

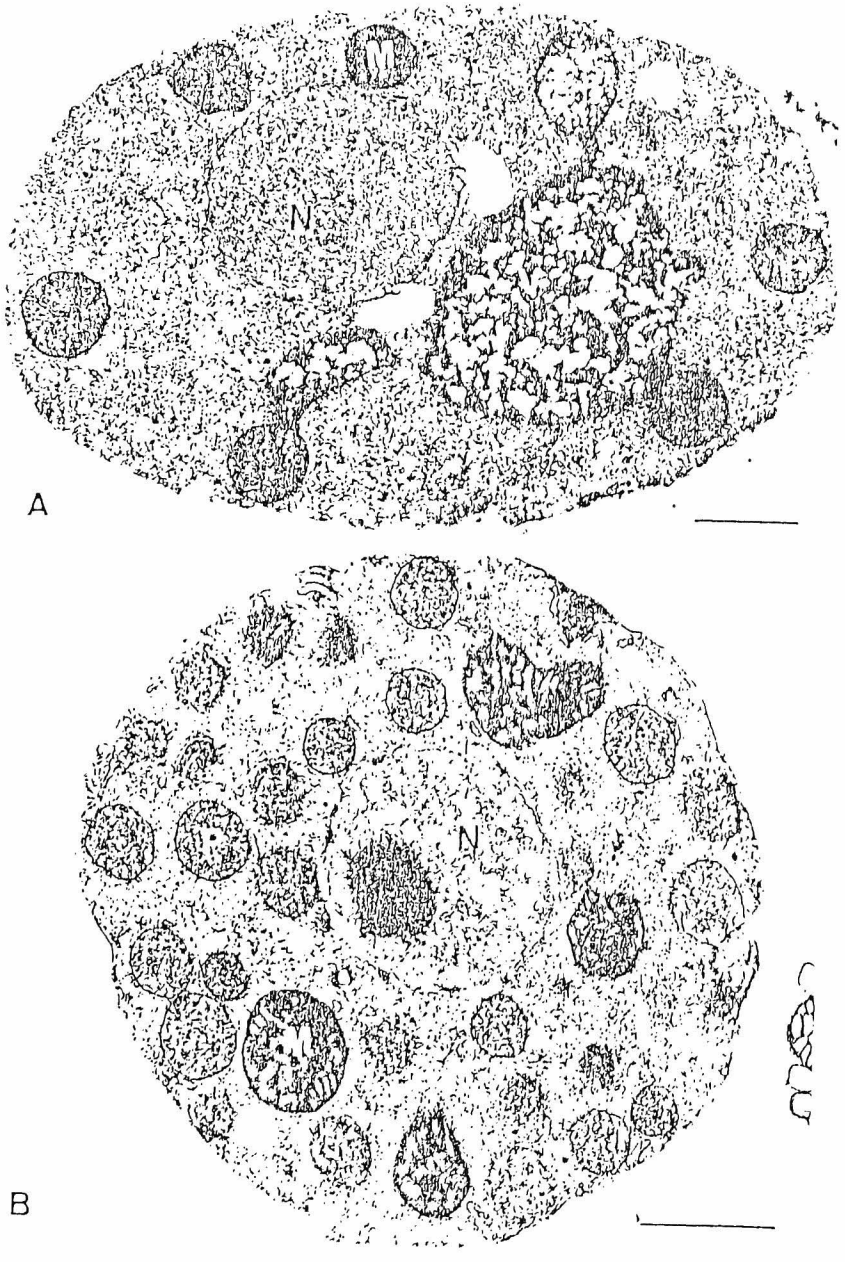


Fig. 1. Ultrastructure of Candida tropicalis pK 233 grown Glucose [A] and alkanes [B].

Spheroplasts were prepared with a lytic enzyme, Zymolyase. Glutaraldehyde-OsO_4 fixed. M, mitochondrion; N, nucleus; P peroxisome; V, vacuole. Bar = 1 µm.

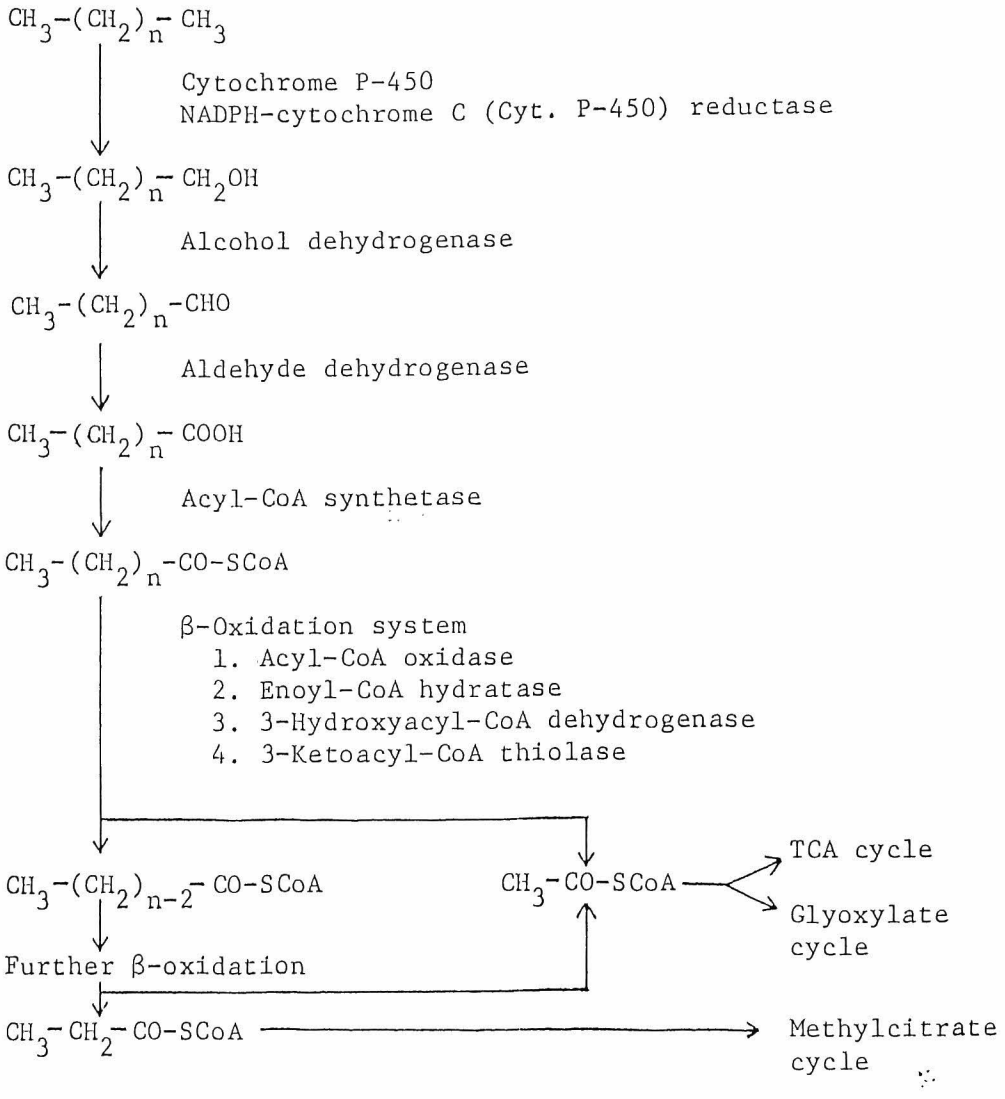


Fig. 2. Metabolic pathway of n-alkanes in yeast.

incorporated into yeast cells are considered to be subjected to initial oxidation in microsomes with the most specific monooxygenase system which is composed of cytochrome P-450, NADPH-cytochrome P-450 reductase and a heat-stable lipid fraction.[42] Higher alcohols derived from n-alkanes are oxidized to the corresponding fatty acids via aldehydes by NAD-linked alcohol dehydrogenase and aldehyde dehydrogenase.[43,44] Higher fatty acids are further activated to the corresponding CoA esters by acyl-CoA synthetase[44,45] and degraded by the fatty acid β-oxidation system which is composed of acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. The final products of β-oxidation of fatty acids were acetyl-CoA (from even- and odd-chain n-alkanes) and propionyl-CoA (from odd-chain n-alkanes) . Acetyl-CoA is considered to be metabolized mainly through TCA cycle, energy-producing cycle, and through glyoxylate cycle, which participates in the synthesis of C_4-dicarboxylic acids, the precursors of cellular components including carbohydrates and amino acids. Propionyl-CoA is regarded to be further metabolized through methylcitric acid cycle.[46,47] (Fig. 2)

When cultivated on n-alkanes, Candida yeast cells contain conspicuous numbers of special organelles, which have been identified as peroxisomes by a cytochemical technique.[11,12,48] As such organelles can be rarely observed in glucose-grown

cells, peroxisomes have been supposed to play an indispensable role in n-alkane assimilation. The previous studies by Professor Fukui's research group have demonstrated that catalase, fatty acid β-oxidation system, part of glyoxylate cycle (isocitrate lyase and malate synthase), D-amino acid oxidase, uricase and NADP-linked isocitrate dehydrogenase are contained in peroxisomes from n-alkane-utilizing yeast, Candida tropicalis.[49-51)]

TCA, glyoxylate, and methylcitrate cycles in n-alkane-utilizing Candida yeasts

Acetyl-CoA, the final product of fatty acid β-oxidation, is condensed with oxalacetate by citrate synthase and yields citrate in TCA cycle. Through TCA cycle, citrate is again metabolized to oxalacetate. By the turnover of the cycle, C_2-units from acetyl-CoA are completely oxidized to two molecules of CO_2, and reducing powers like $FADH_2$ and NADH, which are used to generate ATP by oxidative phosphorylation connected to respiratory chain, are produced.

Oxalacetate, a key material for gluconeogenesis, is converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase. Moreover, intermediates in TCA cycle are utilized for the biosynthesis of amino acids. From these points, supply of extra oxalacetate to TCA cycle is essential for the biosyntheses

of carbohydrates and amino acids. In organisms assimilating pyruvate-donating substrate such as carbohydrates, one possible pathway is carboxylation of pyruvate to produce oxalacetate. However, in n-alkane-utilizing yeasts, glyoxylate cycle is an alternative pathway to synthesize oxalacetate. This cycle is composed of two key enzymes, isocitrate lyase which cleaves isocitrate into succinate and glyoxylate and malate synthase which condenses glyoxylate with acetyl-CoA to yield malate, and several enzymes common to TCA cycle. Succinate and malate are metabolized *via* TCA cycle to produce isocitrate again. Thus, the net biosynthesis of C_4-compound from two molecules of acetyl-CoA is accomplished in glyoxylate cycle, an anaplerotic pathway to replenish C_4-compounds in C_2-compound-utilizing microorganisms.[52] This pathway is expected to play an essential and important role in n-alkane assimilation by *Candida* yeast.[53-55] (Fig. 3)

On the other hand, being different from acetyl-CoA, propionyl-CoA derived from odd-chain alkanes is suggested to be metabolized *via* the methylcitrate cycle. As shown in Fig. 4, the key enzymes of this cycle are methylcitrate synthase and methylisocitrate lyase, these clearly differing from citrate synthase and isocitrate lyase, respectively.[46,47] C_4-compounds such as succinate formed in methylcitric acid cycle are considered to be further metabolized to synthesize various

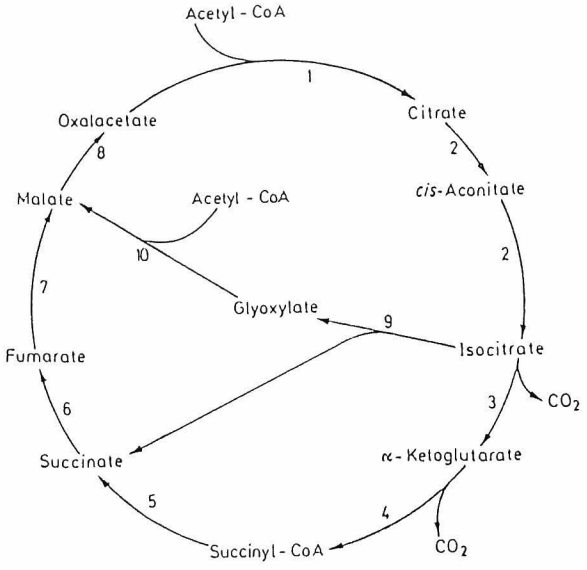


Fig. 3. TCA and glyoxylate cycles.

Enzymes: 1 = citrate synthase; 2 = aconitase; 3 = isocitrate dehydrogenase; 4 = α-ketoglutarate dehydrogenase; 5 = succinyl-CoA synthethase; 6 = succinate dehydrogenase; 7 = fumarase; 8 = malate dehydrogenase; 9 = isocitrate lyase; 10 = malate synthase.

cellular constituents.

In a n-alkane-utilizing yeast, Candida tropicalis, isocitrate lyase and malate synthase, the key enzymes of glyoxylate cycle,[56] have been demonstrated to be located in peroxisomes and the other glyoxylate cycle enzymes common to the TCA cycle, malate dehydrogenase, citrate synthase and

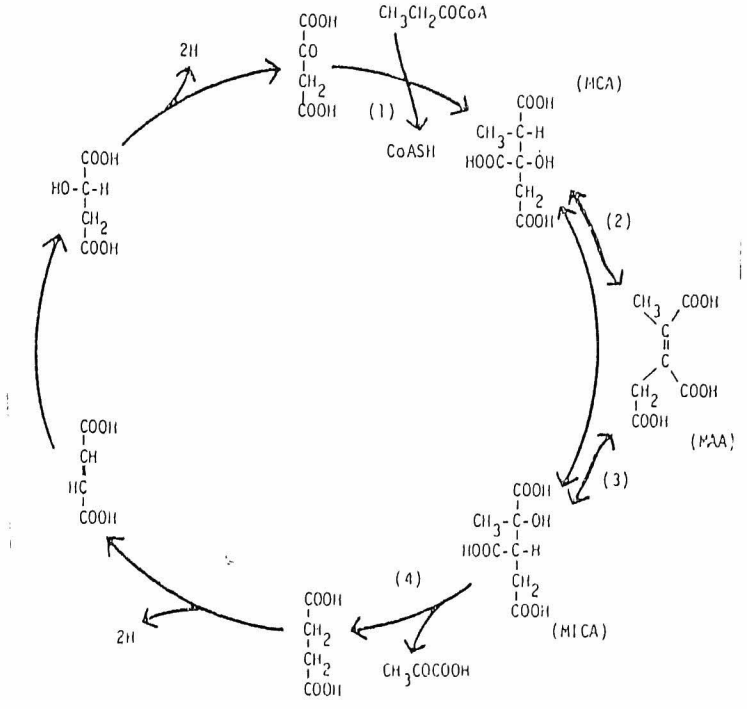


Fig. 4. Methylcitric acid cycle.

Abbreviations: MAA = methylaconitate; MCA = methylcitrate; MICA = methyisocitrate.

Enzymes: 1 = methylcitrate synthase; 2 = methylcitrate dehydratase; 3 = methylaconitate hydratase; 4 = methylisocitrate lyase.

aconitase, have been detected in mitochondria.[49] Furthermore, the fatty acid β-oxidation system, which participates in acetyl-CoA production from alkane substrates, has been proved to be exclusively peroxisome-associated under the experimental conditions employed.[51] These results strongly indicate that cooperation of peroxisomes with mitochondria is necessary for the complete operation of glyoxylate cycle and that acetyl-CoA

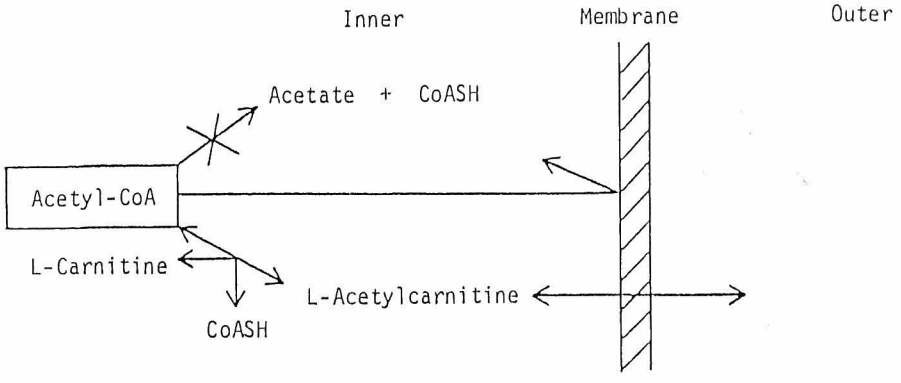
to be utilized by citrate synthase in mitochondria must be supplied from peroxisomes. Since acetyl-CoA itself is not permeable across the organelle membranes, a certain carrier of acetyl-unit must be substantiated to explain the function of glyoxylate cycle by the cooperation between peroxisomes and mitochondria.

The role of carnitine acetyltransferase

Carnitine, β-hydroxy-γ-(trimethylamino)butyric acid,[57-62] isolated from meat extracts as early as 1905 was identified as vitamin B_T which was one of growth factors.[63] The role of carnitine was first noticed from its requirement for the transport of fatty acyl residues across the physical barrier to permeability.[65] The activation of long chain fatty acids to their CoA esters occurs primarily outside mitochondria,[66,67] but acyl-CoA so formed is not directly available for β-oxidation by intact mitochondria. Mitochondrial membranes form a barrier which limits the migration of acyl-CoA and CoA-SH into the matrix,[65] where the enzymes of fatty acid oxidation and TCA cycle are located.[68] The necessity of the conversion from acyl-CoA into acylcarnitine was demonstrated in transporting of fatty acyl residues across the barrier to the site of β-oxidation.[65,69] Carnitine, which has been found in many eukaryotic cells,[70] acts as an acceptor of fatty acyl groups

from fatty acyl-CoAs[64,65,71-76] in the following reversible reaction. (Fig. 5)

L-carnitine + acyl-CoA $\rightleftarrows$ L-acylcarnitine + CoA-SH



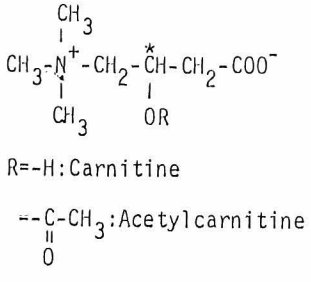


Fig. 5. Possible mechanism of acetyl-unit transport from yeast peroxisomes.

The enzymes to use carnitine as a substrate, carnitine acetyltransferase[77,78] [EC 2.3.1.7], carnitine palmitoyltransferase[79,80] [EC 2.3.1.23], and carnitine octanoyltransferase[81-83] have been reported. Carnitine palmitoyltransferase found in the inner membrane of rat and beef liver mitochondria has two types. Carnitine palmitoyltransferase I is located on the outer aspect of the inner mitochondrial membrane and is the malonyl-CoA-sensitive enzyme, representing a primary control site in the physiological regulation of hepatic fatty acid oxidation and ketogenesis in mammalian cells.[84] Another type, carnitine palmitoyltransferase II is located on the inner aspect of the inner mitochondrial membrane and is the malonyl-CoA-insensitive enzyme.[84] Carnitine octanoyltransferase which is regarded to play a role in transport of medium fatty acids has not been fully characterized. On the other hand, it has been recently established that carnitine acetyltransferase is present in peroxisomes and endoplasmic reticulum (microsome) as well as in mitochondria of liver and kidney of rat and pig.[78,85-87] Different from carnitine palmitoyltransferase, carnitine acetyltransferase has only one type even in mitochondria.[79] The enzyme in mitochondria is demonstrated to have a substrate specificity for short chain fatty acids. At present, we have no information about peroxisomal carnitine acetyltransferase and its function.

It is still unclear whether or not mitochondrial and peroxisomal carnitine acetyltransferases are identical, although they are very similar.[57]

Recently, β-oxidation system for long chain fatty acid has been proved to be localized not only in mitochondria but also in peroxisomes,[29,88] and cooperation between subcellular organelles, especially between peroxisomes and mitochondria, in fatty acid metabolism of eukaryotic cells, has been suggested to have an essential meaning for the maintenance of homeostasis. Moreover, it has been reported that mitochondrial β-oxidation system is dependent on long chain fatty acylcarnitine and peroxisomal β-oxidation is not.[78] In peroxisomes, long chain fatty acid is considered to be incorporated as the form of free fatty acids, degraded to medium or short chain fatty acyl-CoA, and transported to cytoplasm and mitochondria.

In n-alkane-utilizing yeast Candida tropicalis, the fatty acid β-oxidation system, which participates in acetyl-CoA production from alkane substrates, has not been detected in mitochondria.[51] Since glyoxylate cycle has been demonstrated to function between peroxisomes and mitochondria,[49] acetyl-CoA, the final degradation product of fatty acid β-oxidation in peroxisomes, must be transported from peroxisomes to mitochondria. Carnitine acetyltransferase has been induced to the high level in the alkane-grown cells, and suggested to play

an important role in the complete operation of glyoxylate cycle localized between peroxisomes and mitochondria.[89]

Hitherto, carnitine acetyltransferase has been isolated from pigeon breast muscle[77] and rat liver mitochondria.[90] However, its existense in microorganisms has not been demonstrated and the microbial enzyme was first found in the n-alkane-grown yeast, Candida tropicalis by the author.[91] Since the activity of carnitine acetyltransferase has been recently reported to increase in peroxisomes of tumor cells,[33] interest in physiological significance with characteristic localization in several organelles has been increasing.

Biogenesis of peroxisomes and biosynthesis of peroxisomal and mitochondrial carnitine acetyltransferases

Recently, many reports have been published about the protein secretions across membranes and the protein insertion into membranes. In this connection, studies called "intracellular protein topogenesis"[92] concerning the transport of specific proteins into the subcellular organelles, such as nuclei, lysosomes, chloroplasts, mitochondria, glyoxysomes or peroxisomes, are actively carried out.[93] On the study of subcellular organelles, it would be necessary to clarify the mechanism of sorting, distribution and transport of specific

proteins into their own subcellular compartments.

These studies have been stimulated with the help of *in vitro* translation system with exogeneous mRNA.[93,94] Using this system, many studies have been carried out to elucidate the mechanisms of sorting and transport of proteins directly related to the biogenesis and development of subcellular organelles in eukaryotic cells, such as cytochromes[95] and urea cycle enzymes[96] in mammalian mitochondria, and glyoxylate cycle enzymes in glyoxysomes of *Neurospora crassa* and plant seeds.[97-101] Several mitochodrial proteins, which are nuclear gene products and transported post-translationally into the organelles, have the respective precursors of higher molecular weight, and these precursors have been suggested to be nascent proteins containing extra peptide chains like signal peptides. The processing from precursor or immature proteins to mature proteins has significant meanings on biogenesis of specific organelles in relation to organization of several enzymes specific for the biogenesis of peroxisomes by budding from the endoplasmic reticulum was claimed by the electronmicroscopical studies.[102,103] However, this model is not accepted at present and an alternative model of development of preexisting peroxisomes has been proposed.[104,105] Mitochondria has its own DNA, some proteins in mitochondria being encoded by its own genes and others by nuclear genes.[106] In contrast, peroxisomal enzymes

are encoded only by nuclear genes,[107-110] and present one of the models of protein transport system from the site of synthesis to a membrane-bounded compartment.[112]

Two distinct mechanisms have so far been disclosed for the transfer of proteins across intracellular membranes. In "cotranslational" transfer, passage of proteins across the membrane is tightly coupled to the translation and proceeds only during synthesis of the protein on membrane-bound ribosomes.[113,114] In "post-translational" transfer, passage of proteins through the membranes occurs after protein synthesis and is not mediated by a ribosome-membrane junction.[115] Catalase[116] and uricase,[112] specific peroxisomal enzymes in mammalian cells, have been demonstrated to be synthesized by free ribosomes but not by membrane-bound ribosomes, and to be transported post-translationally. Hitherto, there have been only a few reports suggesting the existence of precursors of peroxisomal proteins,[117,118] and many problems remain to be solved about the mechanism of the localization of the specific peroxisomal enzymes.

The transport from the site of synthesis to the site of function involves a higherly specific recognition mechanism by the organelle envelope which only accepts the organelle-destined proteins for segregation within the organelle. So, the investigation using isoenzyme system, which has a unique

localization in different organelles within the same cell, has been required because differences in these proteins or their precursors can be considered to be related, at least in part, to the pattern of distribution. Although aspartate aminotransferase[119] and malate dehydrogenase from rat liver (present in mitochondria and cytoplasm)[120] and malate dehydrogenase from cotyledons of watermelons (present in glyoxysome and mitochondria)[121] have been known as isoenzyme system, reports concerning such system are few. To acquire further information about the biogenesis and development of peroxisomes, it is necessary to study the molecular properties of peroxisomal and mitochondrial carnitine acetyltransferase isoenzymes, because the isoenzymes seem to be a clue to investigate the directed distribution of nascent proteins. Moreover, it would be very important to study whether or not a precursor-type protein of mature carnitine acetyltransferase is synthesized in the cells.

At the "International Conference on Peroxisomes and Glyoxysomes" held at New York in 1981,[122] it has been clarified that peroxisomes and glyoxysomes with a single bilayer membrane can be regarded as more suitable organelles rather than mitochondria, with a complicated two-membrane system, to study the mechanism of localization of specific proteins, which includes a serial process of recognition, sorting, distribution

and transportation. Accordingly, in both aspects of cell technology and fundamental biochemistry, in vitro maturing system of peroxisomes by the incorporation of precursor proteins into immature or preexisting peroxisomes will be much interested. The analysis of gene structure encoded specific proteins for peroxisomes will be a great useful tool to understand the recognition mechanism between polypeptides and biomembrane.

On the other hand, peroxisomes having various enzymatic activities will be applicable to produce useful materials as a kind of bioreactor.

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SYNOPSIS

Part I. Localization of carnitine acetyltransferase in peroxisomes and in mitochondria of n-alkane-grown Candida tropicalis

The profuse appearance of peroxisomes (microbodies) in n-alkane-grown Candida tropicalis has been demonstrated, and the localization of enzymes related to the n-alkane assimilation has been confirmed with the isolated peroxisomes in Professor Fukui's laboratory. Peroxisomes, one of subcellular organelles in eukatyotic cells, have been clarified to play a physiologically essential role in fatty acid metabolism.

Part I describes the existence of a shuttle system for the transport of acetyl-CoA, the final product of fatty acid β-oxidation, from peroxisomes to mitochondria. Peroxisomes isolated from C. tropicalis contain the key enzymes of glyoxylate cycle, isocitrate lyase and malate synthase, while the rest of the enzymes in the cycle, common to the TCA cycle, are located in mitochondria. Furthermore, the activity of fatty acid β-oxidation has been detected only in peroxisomes but not in mitochondria. These results indicate that a certain transport system of acetyl units from peroxisomes to mitochondria should operate in the n-alkane-utilizing yeast cells.

Several transport systems can be suggested: direct transport of acetyl-CoA; transport of acetate after hydrolysis of acetyl-CoA; and transport *via* the acetylcarnitine shuttle mediated by carnitine acetyltransferase. However, acetyl-CoA is known not to be permeable through the mitochondrial membrane. Furthermore, activity to hydrolyze acetyl-CoA could not be detected in yeast peroxisomes.

The author investigated the induction and subcellular localization of carnitine acetyltransferase. The results demonstrated that carnitine acetyltransferase was induced in the *n*-alkane-grown cells of *C. tropicalis* in connection with the development of peroxisomes, and was found to be localized among peroxisomes, mitochondria and cytoplasm in the yeast cells. An "acetylcarnitine shuttle" was strongly proposed as a transport system of acetyl units, derived from the alkane degradation, from peroxisomes to mitochondria and to cytoplasm. Thus, carnitine acetyltransferase is the key enzyme for the complete operation of glyoxylate cycle in the yeast with the concerted functions of peroxisomes and mitochondria, and the enzyme localized in the cytoplasm seems to be related to the *de novo* synthesis of fatty acids.

Part II. Peroxisomal and mitochondrial carnitine acetyltransferases in n-alkane-grown yeast *Candida tropicalis*

Microbial carnitine acetyltransferase was first found in the n-alkane-grown yeast, *Candida tropicalis*, and was demonstrated to be localized in peroxisomes and in mitochondria of the yeast. Cellular level of the enzyme was correlated to the profuse appearance of peroxisomes in this yeast cells grown on n-alkane.

Part II deals with the first successful separation of peroxisomal and mitochondrial carnitine acetyltransferases from the cell-free extracts of *C. tropicalis* by means of DEAE (diethylaminoethyl) -Sephacel column chromatography.

Each carnitine acetyltransferase was purified from the cell-free extract as follows: ammonium sulfate fractionation and column chromatography on Sephadex G-200, Sepharose 6B, DEAE-Sephacel and Blue-Sepharose CL-6B. On DEAE-Sephacel column chromatography, peroxisomal carnitine acetyltransferase was eluted below 0.15 M KCl concentration and mitochondrial carnitine acetyltransferase above 0.15 M KCl concentration. Except for the localization, little difference was observed in their kinetic properties (K_m, V_{max}), substrate specificity and so on. These two carnitine acetyltransferase preparations

were only specific to acetyl and propionyl groups, the substrate specificity not being so broad as that of carnitine acetyltransferase obtained from mammalian tissues. Their substrate specificity only for acetyl and propionyl groups has a physiological significance, because acetyl-CoA and propionyl-CoA are final products from the β-oxidation system in peroxisomes (acetyl-CoA from alkanes with even and odd number carbon chains; propionyl-CoA from alkanes with odd number carbon chains). These two carnitine acetyltransferase isoenzymes are responsible for the transport of not only acetyl but also propionyl group.

Part III. Induction snd subcellular localization of carnitine acetyltransferase in propionate-grown *Candida tropicalis*

Peroxisomal and mitochondrial carnitine acetyltransferases of *n*-alkane-grown *C. tropicalis* cells are induced by *n*-alkanes and are specific for acetyl-CoA and propionyl-CoA, both of which are the degradation products from *n*-alkanes. These facts gave the author an incentive to investigate the role of carnitine acetyltransferase under simple conditions where acetate or propionate is sole source of carbon and energy for growth of the yeast.

In Part III, the induction and subcellular localization of carnitine acetyltransferase in C. tropicalis were investigated. When the yeast was cultivated on acetate and propionate, carnitine acetyltransferase was induced moderately on acetate and extensively on propionate. The ratio of mitochondrial type enzyme (localized in mitochondria and cytoplasm) to the peroxisomal type enzyme (localized in peroxisomes) was about equal in acetate-grown cells, while the mitochondrial type enzyme was major in propionate-grown cells. These results are in contrast to the predominant localization of the enzyme in peroxisomes of n-alkane-grown cells. Catalase, a marker enzyme of peroxisomes, was also induced by propionate as well as by alkanes. Also, in this case, a large part of the enzyme was localized in cytoplasm in propionate-grown cells, the result being significantly different from the predominant localization in peroxisomes of n-alkane-grown cells.

In harmony with the subcellular localization of carnitine acetyltransferase and catalase, morphological difference in peroxisomes was observed between propionate-grown and n-alkane-grown cells. In propionate-grown cells, a few number of large peroxisomes, which were sometimes in contact with each other, were detected electronmicroscopically. On the other hand, conspicuous numbers of small peroxisomes were observed in n-alkane-grown cells. These results suggest that peroxisomes

in the propionate-grown cells might be immature. If cytoplasmic catalase is non-functional and is only the pooled protein, propionate-grown cells would be an excellent system to differentiate the induction of catalase from the development of peroxisomes. Such system seems to be very useful to investigate the maturing of the organelles. Judging from the higher level of carnitine acetyltransferase in mitochondria and in cytoplasm in propionate-grown cells, the enzyme in both mitochondria and cytoplasm seems to take an important part in the transport of propionyl-unit from cytoplasm to mitochondria in the metabolism of propionate.

Propionate-utilizing cells of C. tropicalis give an excellent system to study two problems; to investigate the development of peroxisomes, and to study the propionate metabolism in eukaryotic cells.

Part IV. Characterization of peroxisomal and mitochondrial carnitine acetyltransferases purified from n-alkane-grown Candida tropicalis

The author has demonstrated that peroxisomal and mitochondrial carnitine acetyltransferases have a significant role to function glyoxylate cycle between peroxisomes and mitochondria. In this connection, a variety of interesting problems

remain to be elucidated: Whether or not peroxisomal and mitochondrial carnitine acetyltransferases are products of same gene; how these two enzymes are distributed into two subcellular compartments if they are the same gene products.

In Part IV, the author compared the molecular properties of peroxisomal and mitochondrial carnitine acetyltransferase isolated from the same cells to confirm the molecular identity of both enzymes. The enzymes isolated and purified from n-alkane-grown yeast cells were hetero-oligomers even when purified in the presence of protease inhibitors. Molecular weight of the larger subunits was 64,000 in both cases. Molecular weight of the smaller subunit of the peroxisomal enzyme was 57,000, while that of the mitochondrial enzymes was 52,000. The subcellularly distinct enzymes gave a similar amino acids; glycine, valine, glutamic acid and aspartic acid. Their isoelectric points showed slight difference; 5.11 for the peroxisomal enzyme and 5.22 for the mitochondrial enzyme. However, both enzymes had the same amino-terminal residue (glutamic acid or glutamine) and the heat stability, and were indistinguishable immunochemically.

The results of the immunochemical study especially suggest that carnitine acetyltransferases located in peroxisomes and mitochondria might be originated from a common product of the same nuclear gene. Moreover, the difference in the

molecular weight of the smaller subunits of each enzyme strongly suggests distinct sorting mechanism of these two isoenzymes, and offers an excellent model for investigating where peroxisomal and mitochondrial carnitine acetyltransferases are biosynthesized and how the enzymes are sorted and distributed into each organelles.

Part V. Synthesis *in vitro* of precursor-type carnitine acetyltransferase with messenger RNA from *Candida tropicalis*

It has been demonstrated by the author that existence of peroxisomal and mitochondrial carnitine acetyltransferase isoenzymes serves as a clue to elucidate the mechanism of localization of specific proteins, peroxisomal and mitochondrial carnitine acetyltransferases, into subcellular organelles.

Part V describes the isolation of mRNA from *n*-alkane-grown cells or propionate-grown cells of *C. tropicalis*, in which carnitine acetyltransferase was induced markedly, and the *in vitro* synthesis of the nascent carnitine acetyltransferase in the mRNA-dependent reticulocyte lysate translation system. The *in vitro* translation products obtained by immunoprecipitation with the antibody against peroxisomal or mitochondrial carnitine acetyltransferase were found to be composed of two polypeptides with molecular weight (Mr 71,000 and 57,000) higher than the

corresponding mature polypeptide (Mr 64,000 and 57,000 for peroxisomal carnitine acetyltransferase; Mr 64,000 and 52,000 for mitochondrial carnitine acetyltransferase). These results indicate the presence of the precursors common to respective subunits of peroxisomal and mitochondrial carnitine acetyltransferases. In addition, presence of two kind of the nascent subunits was confirmed by the fact that the in vitro synthesis of these two polypeptides was directed by two different mRNAs isolated from the yeast cells by sucrose density gradient centrifugation.

From the analysis of molecular weight, the mechanism of processing of the precursor carnitine acetyltransferase to the respective isoenzymes is supposed to be characteritic to peroxisomes and mitochondria, respectively. Especially, the smaller polypeptide of the in vitro translation products is strongly suggested to be directly related to the sorting and transport mechanisms.

The identical translation products were obtained with mRNA from the propionate-grown cells as in the case of the n-alkane-grown cells, both carbon sources being effective for the induction of carnitine acetyltransferase. However, the propionate-grown cells are characteristic in having a small number of large peroxisomes and a high ratio of mitochondrial-type carnitine acetyltransferase, which are in contrast to

the n-alkane-grown-cells having a large number of small peroxisomes and a high ratio of the peroxisomal-type enzyme. These differences may be useful to understand the biogenesis and development of the organelles in relation to the distribution of these isoenzymes.

Part I. Localization of carnitine acetyltransferase in peroxisomes and in mitochondria of n-alkane-grown *Candida tropicalis*

INTRODUCTION

The profuse appearance of peroxisomes (microbodies) in the n-alkane-grown cells of various yeasts has been demonstrated.[1,2] Subsequent isolation of the peroxisomes from n-alkane-grown *Candida tropicalis* was accomplished and the localization of the following enzymes was confirmed in the isolated peroxisomes: Catalase, D-amino acid oxidase, isocitrate lyase, malate synthase, NADP-linked isocitrate dehydrogenase, uricase and fatty acid β-oxidation system.[3-5]

In spite of the fact that isocitrate lyase and malate synthase, the key enzymes of glyoxylate cycle essential for gluconeogenesis in alkane-utilizing yeasts,[6] were located in peroxisomes, the other glyoxylate cycle enzymes common to the TCA cycle, malate dehydrogenase, citrate synthase and aconitase, were detected exclusively in mitochondria.[3] Furthermore, the fatty acid β-oxidation system, which participates in acetyl-CoA production from alkane substrates, was essentially peroxisome-associated under the experimental conditions employed.[5] These results strongly indicate that cooperation of peroxisomes

with mitochondria is necessary for the complete operation of the glyoxylate cycle and that acetyl-CoA utilized by citrate synthase in mitochondria must be supplied from peroxisomes.

The purpose of this part is to provide evidence for the localization of carnitine acetyltransferase [EC 2.3.1.7, acetyl-CoA:carnitine O-acetyltransferase] (CAT) both in peroxisomes and in mitochondria of n-alkane-grown C. tropicalis. The possible existence of a shuttle system for the transport of acetyl units from peroxisomes to mitochondria is discussed.

MATERIALS AND METHODS

1. Cultivation of yeasts

The yeast used in this study was Candida tropicalis (Castellani) Berkhout strain pK 233 (ATCC 20336), which was maintained on an agar slant of a glucose medium. The organism was cultured as follows. Cells, precultured aerobically for 22 h in 1.65 % glucose medium, were inoculated in a flask containing an alkane medium, and incubated for 17 h at 30 °C with shaking (220 rev./min). The alkane medium was composed of 5.0 g of $NH_4H_2PO_4$, 2.5 g of KH_2PO_4, 1.0 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $FeCl_3 \cdot 6H_2O$, 1.0 ml of corn steep liquor, 0.5 ml of Tween 80 and 10 ml of n-alkane mixture per liter of tap water (pH 5.2). n-Alkane mixture used as carbon and energy source was "mixture

11", composed of C_{10} 16.4, C_{11} 50.4, C_{12} 32.5 and C_{13} 0.7 % by weight. The glucose medium contained 1.65 % (w/v) glucose in place of 1 % (v/v) n-alkane mixture.[4)]

2. Preparation of protoplasts and fractionation of subcellular organelles

Protoplasts from the cells of exponential growth phase were prepared in the lysis medium composed of 50 mM potassium phosphate buffer (pH 7.2), 0.8 M sorbitol, 0.1 M 2-mercapto-ethanol and 0.2 g/l of a bacterial lytic enzyme, "Zymolyase-5000" (Kirin Brewing Company, Tokyo).[3)] The protoplasts thus obtained were suspended in solution A (50 mM potassium phosphate buffer, pH 7.2, 0.6 M sorbitol and 0.5 mM EDTA), and homogenized for 10 min under cooling on ice with a Teflon homogenizer. The homogenate was centrifuged at 3,000 x g for 10 min to separate heavy particulate fraction (P_1) and supernatant fraction (S_1). The latter was further subjected to centrifugation at 20,000 x g for 15 min to obtain particulate fraction (P_2) and supernatant fraction (S_2).[3)] Intact peroxisomes, well separated from mitochondria, were isolated by a discontinuous sucrose density gradient centrifugation of P_2 fraction at 20,000 rev./min (g_{av} = 77,561) for 5 h using a Hitachi preparative ultracentrifuge model 65P with a RPS 25-3 rotor. S_2 fraction was further centrifuged at

44,000 rev./min (g_{av} = 139,000) for 2 h with a RPS 65T rotor to obtain particulate fraction (P_3) and supernatant fraction (S_3).

3. Enzyme assays

Catalase [EC 1.11.1.6] was assayed spectrophotometrically by the same method described by Roggenkamp et al.[7,8] Cytochrome oxidase [EC 1.9.3.1] was measured by the method of Polakis et al.[9] Protein was determined by Lowry's method.[10] Carnitine acetyltransferase (CAT) was assayed spectrophotometrically at 30 °C by following the release of CoA-SH from acetyl-CoA using the general thiol reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).[11] The reaction mixture contained 40 mM potassium phosphate buffer (pH 7.8), 0.05 mM acetyl-CoA (lithium salt, Sigma Chemical Co., St louis), 0.12 mM DTNB, 2.2 mM DL-carnitine (β-hydroxy-γ-trimethyl-ammonium butyrate) chloride (Wako Pure Chemical Industries, Osaka) and enzyme in final vol. 1.5 ml. The reaction was initiated by adding the enzyme and the increase in absorbance was followed at 412 nm. Carnitine-independent release of CoA-SH from acetyl-CoA was not observed under the conditions employed. When 1.1 mM L-carnitine chloride (P-L Biochemicals, Milwaukee), which is known to be the active isomer for CAT activity[11] was used in place of 2.2 mM DL-carnitine chloride in the reaction mixture, the

activity showed almost the same value.

RESULTS

The level of carnitine acetyltransferase activity in the alkane-grown cells (7430 $nmol.min^{-1}.mg\ protein^{-1}$) was about twenty times higher than that in the glucose-grown cells (376 $nmol.min^{-1}.mg\ protein^{-1}$). These results revealed that carnitine acetyltransferase activity was induced when alkanes were metabolized in the cells. (Table 1)

Table 1. Level of carnitine acetyltransferase activity in Candida tropicalis.

Cells	Enzyme activity
	$nmol.min^{-1}.mg\ protein^{-1}$
Glucose-grown	376
Alkane-grown	7,430

Enzyme activity was measured with cell-free extracts.

In the assay of carnitine acetyltransferase, reaction mixture is composed of acetyl-CoA, L-carnitine, DTNB and enzyme. Each component was found to be necessary for this assay as shown in Table 2. It was demonstrated that only L-isomer of

carnitine was active for the enzyme (Table 3).

Table 2. Effect of omission of mixture components on carnitine acetyltransferase activity of alkane-grown Candida tropicalis.

Omission	Relative activity
None	100 (%)
Acetyl-CoA	0
L-Carnitine	0
DTNB	0
Enzyme	0

Table 3. Effect of carnitine isomers on carnitine acetyltransferase of alkane-grown Candida tropicalis.

Carnitine	Relative activity
L-Carnitine	100 (%)
D-Carnitine	0
DL-Carnitine	76

As shown in Table 4 and Fig. 1, in n-alkane-grown cells of C. tropicalis, carnitine acetyltransferase was found mainly

in the particulate fraction P_2 (20,000 x g pellets) composed of peroxisomes and mitochondria.[3)]

Table 4. Subcellular localization of enzymes in alkane-grown *C. tropicalis* cells.

	Fraction		
	S_2	P_2	S_3
Protein (mg)	77	27	37
Cytochrome oxidase			
rel. act. (%)	100	96	0.5
spec. act. (nmol/min x mg protein)	86	240	0.9
Catalase			
rel. act. (%)	100	79	41
spec. act. (μmol/min x mg protein)	364	838	315
Carnitine acetyltransferase			
rel. act. (%)	100	53	22
spec. act. (nmol/min x mg protein)	1910	2910	877

The experimental procedures were the same as in 'MATERIALS AND METHODS'. S_1, 3,000 x g supernatant of protoplast homogenate; P_2, 20,000 x g pellets of S_1; S_2, 20,000 x g supernatant of S_1. Activities in S_1 were expressed as 100 %.

Further fractionation of P_2 by means of sucrose density gradient centrifugation showed that carnitine acetyltransferase in P_2 was detected both in fraction 2 (30-40 % sucrose fraction)

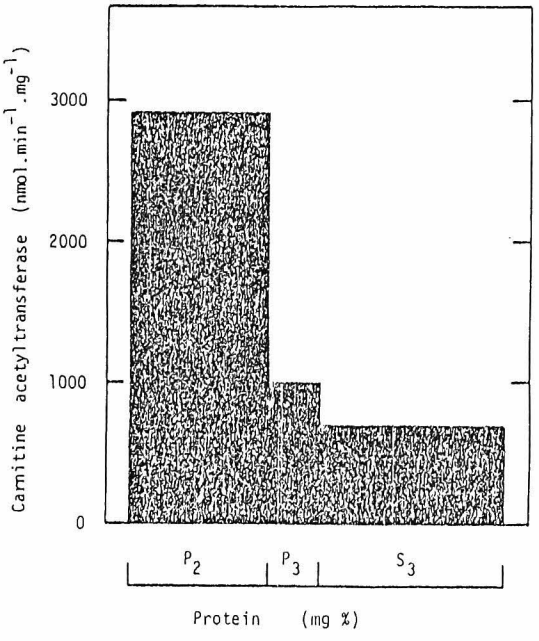


Fig. 1. Subcellular localization of carnitine acetyltransferase in Candida tropicalis.

P_2, 20,000 x g pellets (peroxisomes and mitochondria); P_3, 139,000 x g pellets (microsomes); S_3, 139,000 x g supernatant (cytoplasm).

and in fraction 5 (42.5 - 50 % sucrose fraction) (Fig. 2). Cytochrome oxidase, a marker enzyme of mitochondria, was detected mainly in fraction 2, and catalase, a marker enzyme of of peroxisomes, predominantly in fraction 5. The results indicate that CAT is located both in peroxisomes and in mitochondria of the yeast.

S_2 (20,000 x g supernatant) was further centrifuged at a higher speed (139,000 x g) to obtain the microsomal fraction (P_3) and the cytoplasmic fraction (S_3). Most of the carnitine acetyltransferase activity in S_2 was recovered in S_3 (Fig. 1), suggesting the localization of the enzyme in cutoplasm. However, the possibility cannot be excluded that this may be

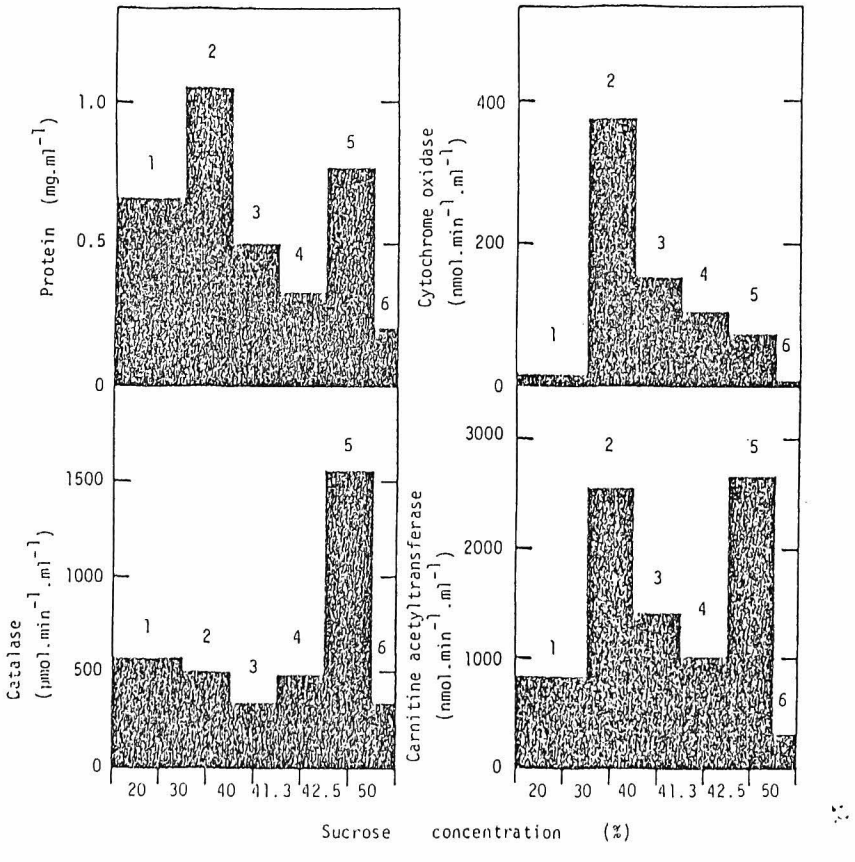


Fig. 2. Particulate localization of enzymes in P_2.

The experimental procedures were as 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.

due, at least in part, to disruption of the organelles during the preparation steps; carnitine acetyltransferase does not appear to occur in the cytoplasm of animal cells.
The localization of carnitine acetyltransferase in the microsomal materials is not conclusive.

DISCUSSION

Yeast peroxisomes in Candida tropicalis contain the key enzymes of glyoxylate cycle, isocitrate lyase and malate synthase, while the rest of the enzymes in the cycle, common to the TCA cycle also, are located in mitochondria.[3] Furthermore, the activity of fatty acid β-oxidation has been detected only in peroxisomes but not in mitochondria.[5] Although the possibility of presence of a different β-oxidation system in mitochondria would not be excluded as the case of rat liver,[12] these results strongly suggested that a certain transport system of acetyl units from peroxisomes to mitochondria should operate in the alkane-utilizing yeast cells.

Carnitine acyltransferase, carnitine acetyltransferase and carnitine palmitoyltransferase [EC 2.3.1.23, acyl-CoA:carnitine acyltransferase] (CPT), especially the latter enzyme, have been shown to be essential in the transport of fatty acyl residues from cytoplasmic acyl-CoA into the mitochondrial

matrix.[13] Recently, carnitine acyltransferases have been detected not only in mitochondria but also in the peroxisomes of mammalian cells.[14-16] The results presented in this paper suggest that an 'acetylcarnitine shuttle' may have an important role in the transfer of acetyl units formed in peroxisomes by β-oxidation to the mitochondria for the complete operation of the glyoxylate cycle in Candida.

In addition, when C. tropicalis is grown on relatively short chain alkanes, such as undecane (C_{11}), the de novo synthesis pathway for fatty acids is operative.[17] Fatty acids derived from alkanes are activated to acyl-CoA in peroxisomes[18] and are degraded to acetyl-CoA in peroxisomes.[5] Carnitine acetyltransferase in peroxisomes may also participate in the transfer of acetyl units from peroxisomes to cytoplasm, and the enzyme in cytoplasm may convert acetylcarnitine to acetyl-CoA, which can be utilized for the fatty acid synthesis. A possible role of peroxisomes and mitochondria, and also that of carnitine acetyltransferase in alkane-assimilating yeasts are illustrated in Fig. 3.

In Candida, it has been established that several enzymes concerning lipid metabolism are located in peroxisomes: acyl-CoA synthetase II,[18] the fatty acid β-oxidation system,[5,18] carnitine acetyltransferase and NAD-linked glycerophosphate dehydrogenase. The peroxisomes seem to be one of the major

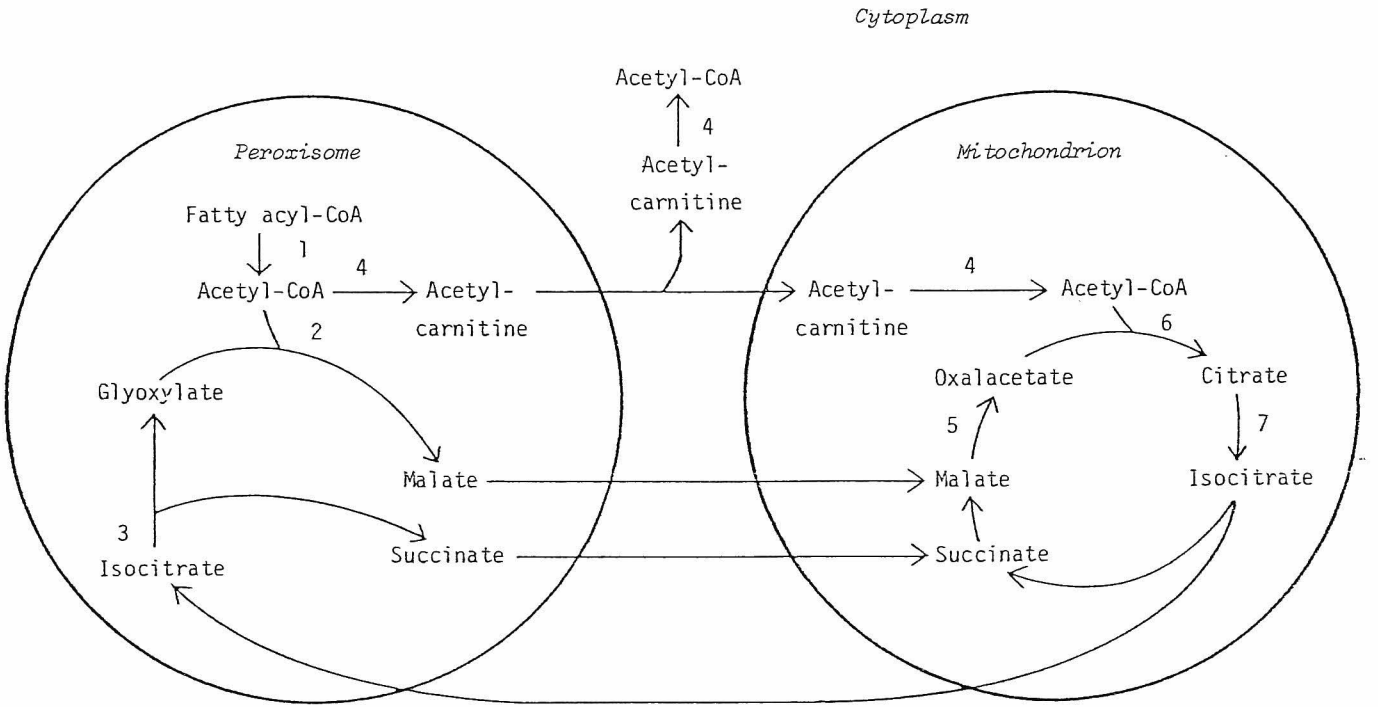


Fig. 3. Possible role of peroxisome and mitochondrion in alkane assimilation by yeasts.

1, β-oxidation system; 2, malate synthase; 3, isocitrate lyase; 4, carnitine acetyltransferase (CAT); 5, malate dehydrogenase; 6, citrate synthase; 7, aconitase.

site of lipid metabolism in Candida utilizing n-alkanes and higher fatty acids.

SUMMARY

In connection with the development of peroxisomes (microbodies), carnitine acetyltransferase (CAT) was induced in the alkane-grown cells of Candida tropicalis. Carnitine acetyltransferase was found to be distributed among peroxisomes, mitochondria and cytoplasm in the yeast cells. An "acetylcarnitine shuttle" is proposed as a transport system of acetyl units, derived from the alkane degradation, from peroxisomes to mitochondria and to cytoplasm, based on the subcellular localization of carnitine acetyltransferase. Thus, carnitine acetyltransferase seems to be the key enzyme for the complete operation of glyoxylate cycle in the yeast with the concerted functions of peroxisomes ans mitochondria. Carnitine acetyltransferase localized in cytoplasm was supposed to be related to the de novo synthesis of fatty acids.

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Part II. Peroxisomal and mitochondrial carnitine acetyltransferases in n-alkane-grown yeast *Candida tropicalis*

INTRODUCTION

In mammalian cells it has been found that type I carnitine acyltransferase is localized outside the mitochondrial inner membrane and type II enzyme inside the mitochondrial inner membrane, the former being regulated by malonyl-CoA.[1] Carnitine acetyltransferases have also been detected in peroxisomes and microsomes as well as in mitochondria of mammalian cells, and their important roles in fatty acid metabolism have been reported.[2-5] Recently its activity was reported to increase in peroxisomes of tumor cells.[6] These results indicate an interesting physiological significance of the enzyme.

Hitherto, carnitine acetyltransferase has been isolated from pigeon breast muscle[7] and rat liver mitochondria.[8] However, its existence in microorganisms has not been demonstrated. Carnitine acetyltransferase of a microorganism was first found in the n-alkane-grown yeast, *Candida tropicalis* pK 233.[9] As described in Part I, this enzyme was localized in peroxisomes and in mitochondria of alkane-grown cells of

C. tropicalis.[9] Profuse appearance of peroxisomes was first demonstrated by Professor Fukui's group in yeast cells grown on alkanes, higher fatty acids and methanol.[10] Peroxisomal and mitochondrial carnitine acetyltransferases in alkane-grown yeast cells seem to constitute an 'acetylcarnitine shuttle' system between these organelles, and play an important role in the complete operation of the glyoxylate cycle localized between peroxisomes and mitochondria.[10,11]

Part II describes the isolation and purification of peroxisomal and mitochondrial carnitine acetyltransferases, and their characteristics. Their function in alkane metabolism is also discussed.

MATERIALS AND METHODS

1. Cultivation of yeast

Cultivation of Candida tropicalis Berkhout strain pK 233 (ATCC 20336) were carried out as in Part I.[12]

2. Subcellular Fractions

Subcellular fractionation of C. tropicalis cells was performed as in Part I. Peroxisomal, mitochondrial and fraction P_2 so obtained were suspended in 10 mM Tris/HCl buffer (pH 8.0) containing 1 % Triton X-100 and 1 mM dithiothreitol, sonicated

for 5 min and centrifuged at 139,000 x g for 2 h. Respective supernatants were used as the sources of peroxisomal, mitochondrial and both carnitine acetyltransferases.

3. Preparation of cell-free extract and solubilization method

C. tropicalis pK 233 was cultivated for 24 h at 30 °C. Cells harvested by centrifugation (160 g wet cells) were suspended in 250 ml 0.1 M Tris/HCl buffer (pH 8.0) containing 5 mM EDTA, 1 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride, and homogenized using a Braun homogenizer (for 150 s per 10 g wet cells). The homogenized cell suspension was centrifuged at 10,000 x g for 15 min. The precipitate was discarded and the supernatant was used as the cell-free extract. This extract was treated to solubilize the enzyme with 1 % Triton X-100 and 50 mM KCl at 4 °C for 18 h with stirring.

4. Enzyme assay

Carnitine acetyltransferase activity was assayed at 30 °C by the 5,5'-dithiobis(2-nitrobenzoic acid) method[13] or by following the increase in the absorbance of acetyl-CoA at 233 nm,[14] as described in Part I. Protein was quantified by the modified Lowry method.[15]

5. Electrophoresis

Polyacrylamide gel electrophoresis in the absence and presence (0.1 % by weight) of sodium dodecylsulfate was performed by the method of Ornstein[16] and Davis,[17] using 7 % cross-linked gel, and by the method of Weber and Osborn,[18] using 10 % cross-linked gel, respectively. The enzyme proteins were treated with 1.0 % (by weight) sodium dodecylsulfate before gel electrophoresis.

6. Chemicals

L-Acetylcarnitine chloride and L-carnitine chloride were kindly donated by Otsuka Pharmaceutical Factory, Naruto, Tokushima, Japan. Other chemicals were obtained from commercial sources.

RESULTS

1. Separation of peroxisomal and mitochondrial carnitine acetyltransferases

Respective preparations solubilized from the particulate fraction (P_2), peroxisomes and mitochondria were applied to DEAE-Sephacel columns (1.5 x 23 cm) equilibrated with 10 mM Tris/HCl buffer (pH 8.0) containing 1 mM dithiothreitol (buffer A). Elution was carried out with a linear concentration

gradient prepared from 200 ml of buffer A and the same volume of buffer A containing 0.35 M KCl. Fig. 1 shows elution patterns of peroxisomal and mitochondrial carnitine acetyltransferases on DEAE-Sephacel columns: Fig. 1A indicates that fraction P_2, consisting of peroxisomes and mitochondria, contains two distinct enzyme activities; carnitine acetyltransferase from the peroxisome-containing fraction was found to be eluted below 0.15 M KCl (Fig. 1B), whereas carnitine acetyltransferase from the mitochondrion-containing fraction was eluted above 0.15 M KCl (Fig. 1C). Thus, peroxisomal carnitine acetyltransferase was apparently different from mitochondrial carnitine acetyltransferase. This difference made it possible to separate easily each carnitine acetyltransferase in the cell-free extract obtained from disintegrated whole cells.

2. Comparison of the levels of peroxisomal and mitochondrial carnitine acetyltransferases at different cultivation phases

The levels of peroxisomal and mitochondrial carnitine acetyltransferases were compared at different phases of growth on alkanes by means of DEAE-Sephacel chromatography, mentioned above. The ratio of the level of the peroxisomal enzyme to that of the mitochondrial enzyme was about 3-5 at each growth

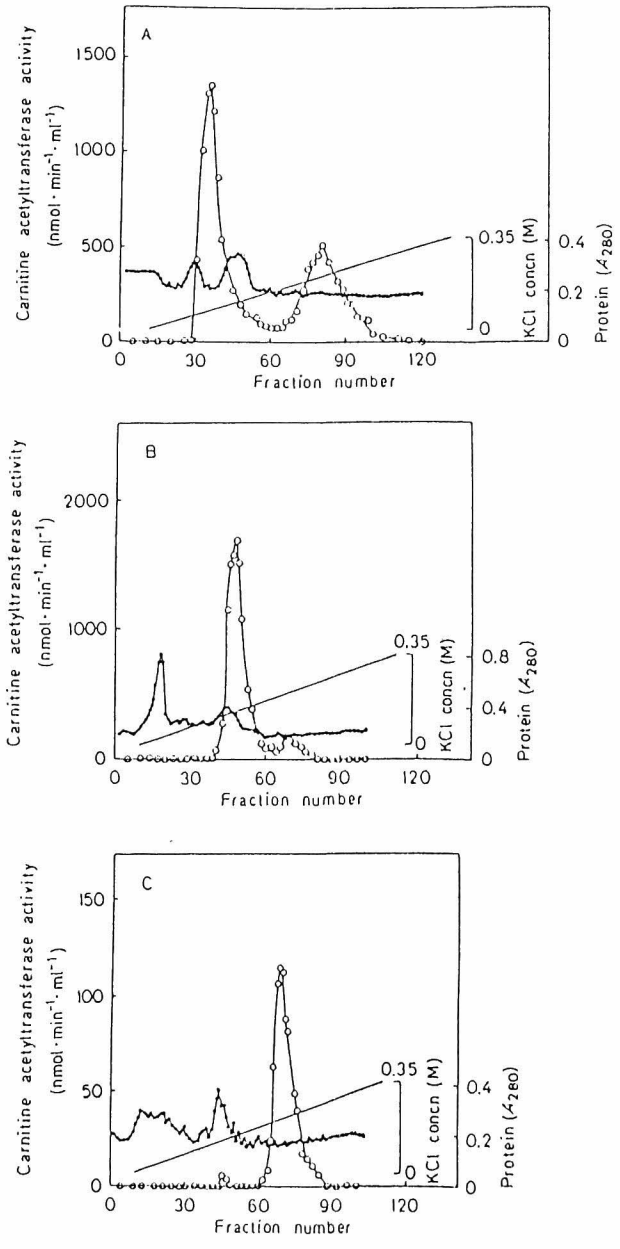


Fig. 1. Elution patterns of peroxisomal and mitochondrial carnitine acetyltransferase activities from DEAE-Sephacel columns.

(A) Preparation solubilized from fraction P_2 containing both

peroxisomes and mitochondria. (B) Preparation solubilized from peroxisomes. (C) Preparation solubilized from mitochondria. To prevent contamination of peroxisomes, mitochondria obtained by sucrose density gradient centrifugation were purified again by the same method. The volume of each fraction was 4 ml. (○) Enzyme activity; (●) protein; (—) KCl concentration.

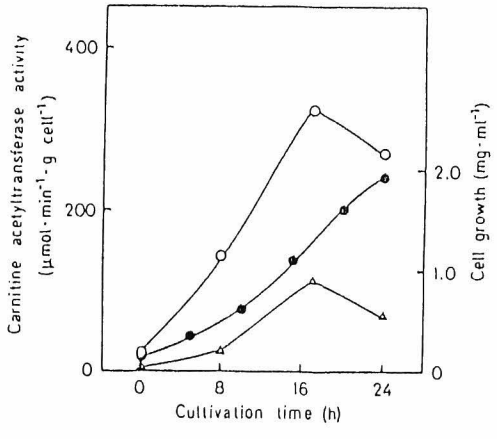


Fig. 2. Time course of changes in peroxisomal and mitochondrial carnitine acetyltransferase activities.

Glucose-grown cells were transferred into alkane medium and cultivated aerobically. (○) Peroxisomal carnitine acetyltransferase activity; (●) mitochondrial carnitine acetyltransferase activity; (△) cell growth.

phase examined when the cells of Candida tropicalis precultured in a glucose medium were transferred to an alkane medium and cultivated aerobically. Fig. 2 shows that the activity of each carnitine acetyltransferase began to increase at the early exponential growth phase (8 h) and reached the maximum at the middle exponential growth phase (17 h).

3. Purification of carnitine acetyltransferases

As described above, peroxisomal and mitochondrial carnitine acetyltransferases could be separated from each other by DEAE-Sephacel chromatography. Therefore, in the purification of each carnitine acetyltransferase, the cell-free extract obtained from whole cells was used as the source of each carnitine acetyltransferase. After solubilization, this cell-free extract was fractionated with ammonium sulfate between 30 % and 65 % saturation. The precipitate obtained was dissolved in buffer A containing 0.3 M KCl and applied to a Sephadex G-200 column (2.5 x 77 cm) equilibrated with buffer A containing 0.3 M KCl. Elution was carried out with buffer A containing 0.3 M KCl. (Fig. 3) Fractions showing the enzyme activity were collected and concentrated using a Diaflo membrane filter PM-10 (Amicon Far East, Tokyo, Japan). The concentrated enzyme solution was chromatographed twice on Sepharose 6B columns (2.3 x 73 cm) equilibrated with buffer A.

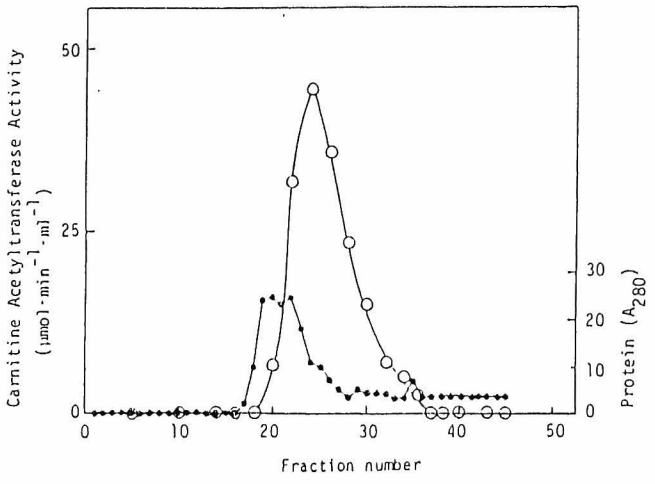


Fig. 3. Sephadex G-200 column chromatography of carnitine acetyltransferase from the cell-free extract.

The volume of each fraction was 6 ml. (O) Enzyme activity; (●) protein.

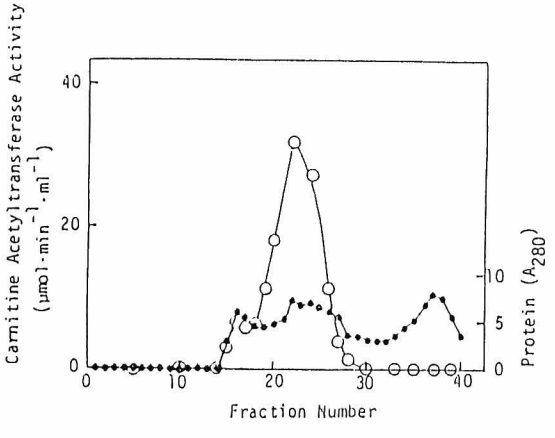


Fig. 4. Second Sepharose 6B column chromatography of carnitine acetyltransferase.

The volume of each fraction was 6 ml. (O) Enzyme activity; (●) protein.

(Fig. 4) The enzyme solution, concentrated by the same method, was applied to a DEAE-Sephacel column (1.5 x 26 cm) equilibrated previously with buffer A. The column was washed with 500 ml buffer A and then eluted with a linear concentration gradient prepared from 350 ml buffer A containing 0.05 M KCl and the same volume of buffer A containing 0.4 M KCl. Carnitine acetyltransferases were eluted from the column in two peaks (Fig. 5). The enzyme eluted below 0.15 M KCl was regarded as peroxisomal carnitine acetyltransferase (Ps type), judging from the elution patterns from DEAE-Sephacel described above. Similarly, carnitine acetyltransferase eluted above 0.15 M KCl was regarded as the mitochondrial enzyme (Mt type). Fractions containing the peroxisomal and mitochondrial enzymes were separately collected, concentrated with the Diaflo membrane filter and desalted using Sephadex G-50 columns (2 x 50 cm) equilibrated with buffer A. Respective fractions containing each enzyme were collected and applied to Blue-Sepharose CL-6B columns (2 x 15 cm) equilibrated with buffer A. After the column was washed with 200 ml buffer A, elution was carried out with buffer A containing 0.35 M KCl (Fig. 6). Each enzyme activity was eluted in a single peak. The fractions exhibiting the enzyme activity were concentrated and desalted by the same method mentioned above, and used as purified enzyme preparations. All these purification procedures were carried out at 0-4 °C.

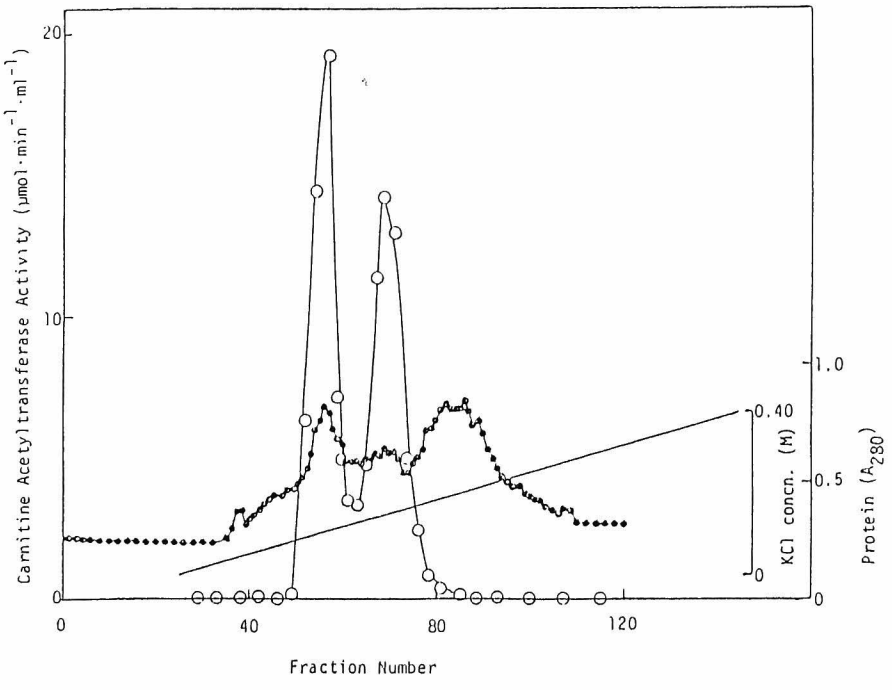


Fig. 5. DEAE-Sephacel column chromatography of carnitine acetyltransferases obtained from cell-free extract.

The volume of each fraction was 6 ml. (O) Enzyme activity; (●) protein; (—) KCl concentration.

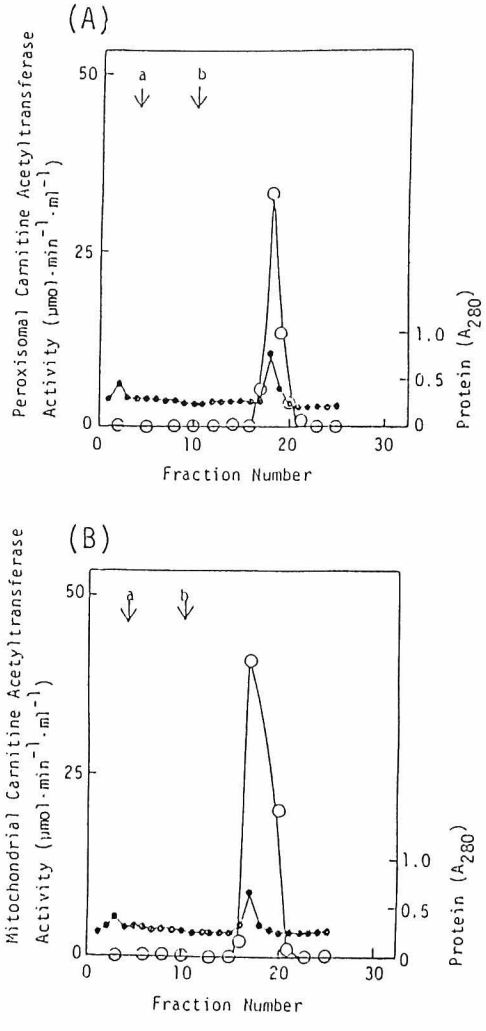


Fig. 6. Blue-Sepharose CL-6B column chromatography of peroxisomal (A) and mitochondrial (B) carnitine acetyltransferases.

Buffer A was applied at arrow a, and buffer A containing 0.35 M KCl at arrow b. The volume of each fraction was 6 ml. (○) Enzyme activity; (●) protein.

Table 1. Purification of peroxisomal and mitochondrial carnitine acetyltransferases.

Purification step	Total protein (mg)	Total activity ($\mu mol.min^{-1}$)	Yield (%)	Specific activity ($\mu mol.min^{-1}.mg\ protein^{-1}$)	Fold
Cell free extract (Detergent treated)	3170	1610	100	0.508	1
Ammonium sulfate (30-65 % saturation)	2330	1600	99.4	0.686	1.35
Sephadex G-200	407	1350	83.9	3.32	6.54
1st Sepharose 6B	223	917	57.0	4.11	8.09
2nd Sepharose 6B	168	922	57.3	5.49	10.8
DEAE-Sephacel					
Ps-type	15.1	253	15.7	16.8	33.1
Mt-type	11.3	201	12.5	17.8	35.0
Blue-Sepharose CL-6B					
Ps-type	4.80	425	26.4	88.5	174
Mt-type	2.79	315	19.6	113	222

Enzyme activity was measured by the ultraviolet method.

Ps, peroxisomal; Mt, mitochondrial.

The results of the purification of peroxisomal and mitochondrial carnitine acetyltransferases from the cell-free extract are summarized in Table 1. The overall purification for the peroxisomal enzyme was 174-fold with a yield of 26.4 % and for the mitochondrial enzyme 222-fold with a yield of 19.6 %. The specific activity of purified peroxisomal carnitine acetyltransferase was 88.5 $\mu mol.min^{-1}.mg\ protein^{-1}$ and that of the mitochondrial enzyme was 113 $\mu mol.min^{-1}.mg\ protein^{-1}$. These enzymes were stored at 4 °C, because they were inactivated under the conditions of freeze/thaw.

The final preparations of peroxisomal and mitochondrial carnitine acetyltransferases seemed homogeneous based on the criteria of their behaviour in column chromatography. Nevertheless, each final preparation gave similar three protein bands on disc gel electrophoresis. When these three bands were sliced out and each protein was extracted with buffer A, the carnitine acetyltransferase activity was detected in all these three bands. This apparent multiplicity on electrophoresis might be ascribable to the different aggregated forms of the enzymes. On sodium dodecylsulfate/polyacrylamide gel electrophoresis each preparation was found to consist of two kinds of subunits of different molecular weight (about 70,000 and 60,000 for the respective carnitine acetyltransferases) (Fig. 7).

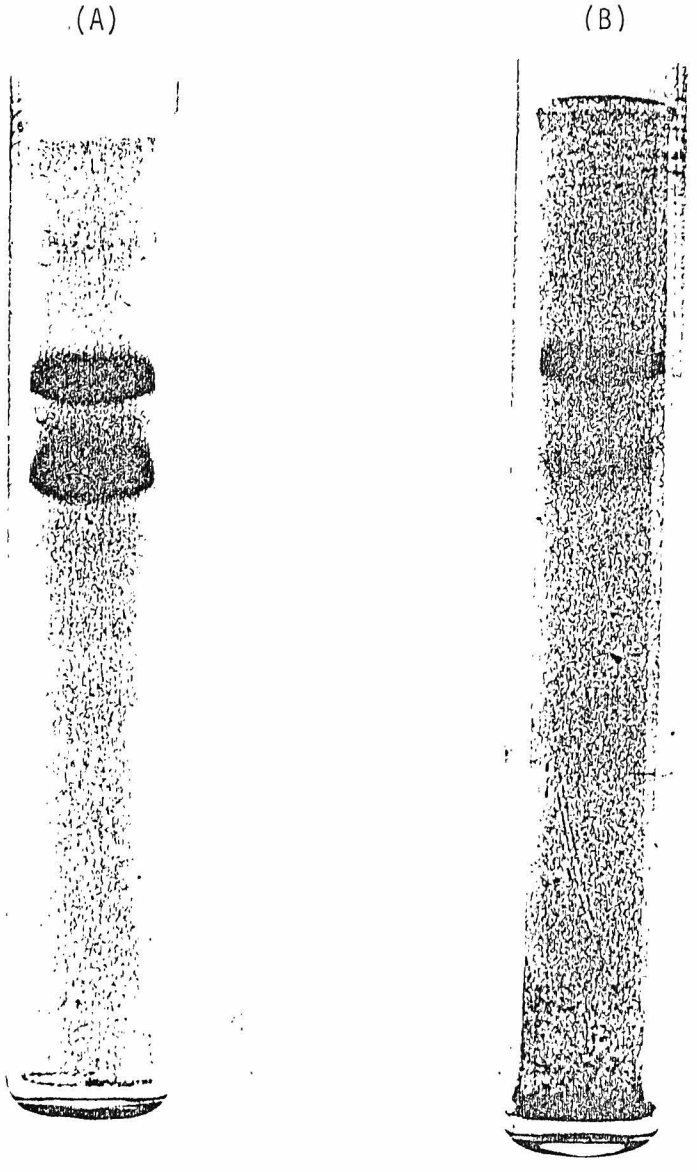


Fig. 7. Sodium dodecylsulfate/polyacrylamide gel (10 %) electrophoresis of purified carnitine acetyltransferase.

(A) Peroxisomal carnitine acetyltransferase; (B) mitochondrial carnitine acetyltransferase.

4. Properties of peroxisomal and mitochondrial carnitine acetyltransferases

Table 2 shows the properties of peroxisomal (Ps type) and mitochondrial (Mt type) carnitine acetyltransferases. Except for their cellular localization and elution pattern, there was no great difference in their properties. The optimum pH for both enzyme activities was 8.0. Both enzymes were inhibited by divalent cations (Ca^{2+}, Mg^{2+} *etc.*) and by an -SH reagent, *p*-chloromercuribenzoic acid. The substrate specificity of these carnitine acetyltransferases was very characteristic. Compared with carnitine acetyltransferases from mammalian tissues, these enzymes from the yeast were specific only to acetyl and propionyl groups, and showed no activity on medium-chain and long-chain acyl groups.

Peroxisomal and mitochondrial carnitine acetyltransferase activities were assayed at various concentrations of each substrate. The K_m and V values obtained with each substrate are shown in Table 3. Although these two enzyme preparations showed slight differences in these kinetic values with propionyl-CoA and acetylcarnitine, no remarkable differences in the K_m and V values with the substrates examined were observed between the peroxisomal and mitochondrial enzymes. As a result, peroxisomal and mitochondrial carnitine acetyltransferases are very similar isoenzymes in spite of their

Table 2. Properties of peroxisomal and mitochondrial carnitine acetyltransferases.

	Ps-type	Mt-type
Localization	Peroxisomes	Mitochondria
KCl concentration of DEAE-Sephacel elution (M)	< 0.15	> 0.15
Specific activity ($\mu mol.min^{-1}.mg\ protein^{-1}$)	88.5	113
Optimum pH	8.0	8.0
Inhibitors	Mg^{2+}, Ca^{2+} etc. p-CMB	Mg^{2+}, Ca^{2+} etc. p-CMB
Substrate specificity	Acetyl-, propionyl-	Acetyl-, propionyl-

Table 3. Kinetic properties of peroxisomal and mitochondrial carnitine acetyltransferases

Substrate	Ps-type		Mt-type	
	K_m (μM)	V_{max} ($\mu mol.min^{-1}.mg\ protein^{-1}$)	K_m (μM)	V_{max} ($\mu mol.min^{-1}.mg\ protein^{-1}$)
Acetyl-CoA	42	1200	36	1380
Propionyl-CoA	100	221	162	417
CoA	304	210	257	242
Acetylcarnitine	417	162	639	219
L-Carnitine (Acetyl-CoA, 50 μM)	719	921	622	1150
L-Carnitine (Propionyl-CoA, 50 μM)	655	112	774	160

distinct localization in the cells.

DISCUSSION

Yeasts assimilating n-alkanes or higher fatty acids as the sole source of carbon and energy seem to be an excellent model with which to investigate the fatty acid metabolism in mammalian tissues. Candida yeast is known as a typical alkane-utilizing yeast, and the mechanism of its fatty acid metabolism has been partly investigated.[19-22] In addition, when C. tropicalis was grown on alkanes, conspicuous numbers of peroxisomes (microbodies) appeared in the yeast cells.[23,24] These peroxisomes have been proved to contain various enzymes, such as catalase, the fatty acid β-oxidation system, acyl-CoA synthetase and so on. The key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, are also found to be located in peroxisomes. On the other hand, malate dehydrogenase, citrate synthase and aconitase, which are common to the tricarboxylic acid and glyoxylate cycles, are localized in mitochondria.[25] The activity of the fatty acid β-oxidation system has been detected exclusively in peroxisomes but not in mitochondria under the conditions examined.[26] These results strongly suggested that acetyl-CoA necessary for the citrate synthase reaction in mitochondria could be supplied

from peroxisomes by a certain transport system of acetyl units. Several transport systems can be suggested: direct transport of acetyl-CoA; transport of acetate after hydrolysis of acetyl-CoA; and transport *via* the acetylcarnitine shuttle mediated by carnitine acetyltransferase. However, acetyl-CoA is known not to permeate the mitochondrial membrane. Furthermore, activity to hydrolyze acetyl-CoA could not be detected in yeast peroxisomes. On the other hand, the level of carnitine acetyltransferase in the cells was greatly enhanced when the yeast was grown on alkanes. These results suggest that an 'acetylcarnitine shuttle' may play an indispensable role in the transport of acetyl units. This assumption seems to be true because carnitine acetyltransferases are localized in both peroxisomes and mitochondria isolated from alkane-grown cells.[9)]

Carnitine acetyltransferases, first isolated from alkane-grown yeast *Candida tropicalis*, were very different from those isolated from mammalian tissues. As shown in Fig. 1, these carnitine acetyltransferases could be easily separated by DEAE-Sephacel column chromatography. This results indicates that peroxisomal carnitine acetyltransferase is different from the mitochondrial enzyme, these being regarded as isoenzymes. The existence of carnitine acetyltransferase isoenzymes strongly supports a transport system, an 'acetylcarnitine

shuttle', between peroxisomes and mitochondria. Except for their localization and elution behaviour on DEAE-Sephacel column chromatography, there was little difference in properties between these isoenzymes. Their substrate specificity only for acetyl and propionyl groups has a physiological significance, because acetyl-CoA and propionyl-CoA are final products from the β-oxidation system in peroxisomes when C. tropicalis is grown on alkanes with odd-number carbon chains. The fact that peroxisomal and mitochondrial carnitine acetyltransferases have activity for acetyl and propionyl groups shows that two carnitine acetyltransferases are responsible for the transport of not only acetyl group but also propionyl group. This substrate specificity of the yeast enzymes is characteristic compared with that of the enzymes from mammalian tissues (Table 4).

Acetyl-CoA regenerated in mitochondria is condensed with oxaloacetate by citrate synthase in mitochondria and, similarly regenerated propionyl-CoA is condensed with oxaloacetate by methylcitrate synthase localized in mitochondria. That is, acetyl-CoA is incorporated into the glyoxylate cycle and propionyl-CoA into the methylcitrate cycle, which has been proved to be operating in Candida lipolytica,[29,30] as illustrated in Fig. 8.

In connection with the development of peroxisomes, it is

Table 4. Substrate specificity of carnitine acetyltransferases from different sources for acyl-CoA derivatives.

Acyl-CoA derivatives	Candida tropicalis[a]			Rat liver peroxisomes[b]	Rat liver microsomes[b]	Pigeon breast muscle[c]	Pig heart[d]
	Crude enzyme	Ps-type	Mt-type				
	(%)						
Acetyl-	100	100	100	100	100	100	100
Propionyl-	14	9.4	15	110	112	77	123
Butyryl-	1.5	0.12	0.26	82	75	41	100
Pentanoyl-	0	0	0	—	—	—	—
Hexanoyl-	0	0	0	14	17	13	23
Decanoyl-	0	0	0	0	0	4	13
Palmitoyl-	0	0	0	0	0	0	0

Enzyme activity, measured by the 5,5'-dithiobis(2-nitrobenzoic acid) method with acetyl-CoA as substrate, was expressed as 100 %.

a: This study.

b, c, d: Reported values.[4,27,28]

Ps, peroxisomal; Mt, mitochondrial.

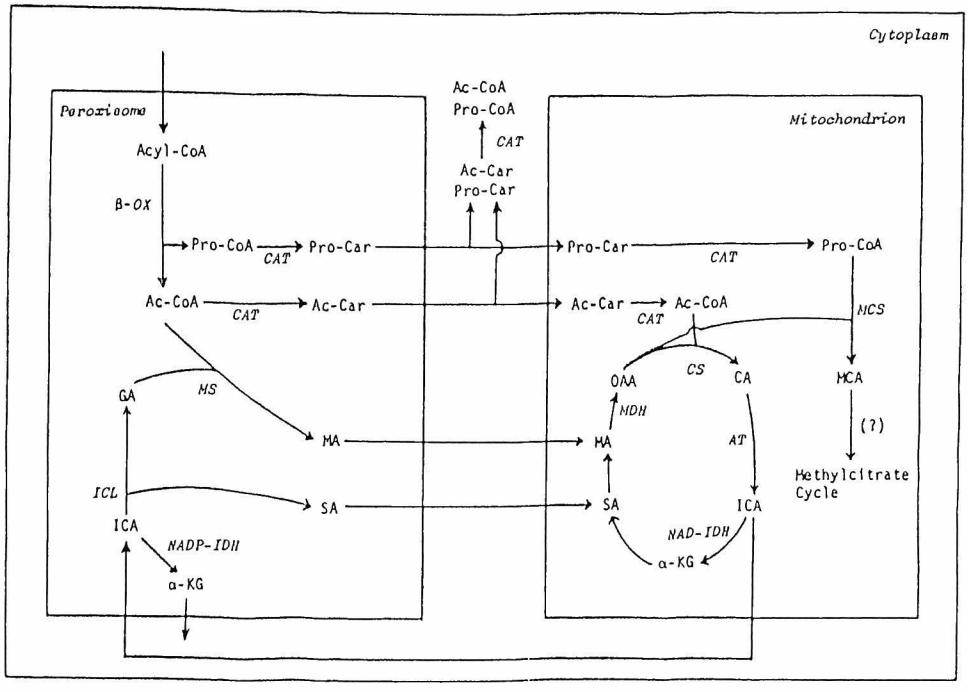


Fig. 8. Presumptive roles of peroxisomes and mitochondria in alkane-assimilating yeast.

Abbreviation used: *AT*, aconitase; *CAT*, carnitine acetyltransferase; *CS*, citrate synthase; *ICL*, isocitrate lyase; *NAD-IDH*, NAD-dependent isocitrate dehydrogenase; *NADP-IDH*, NADP-dependent isocitrate dehydrogenase; *MCS*, methlcitrate synthase; *MDH*, malate dehydrogenase; *MS*, malate synthase; β-*OX*, β-oxidation system. Ac-Car, acetylcarnitine; Ac-CoA, acetyl-CoA; CA, citrate; GA, glyoxylate; ICA, isocitrate; α-KG, α-ketoglutarate; MA, malate; MCA, methylcitrate; OAA, oxaloacetate; Pro-Car, propionylcarnitine; Pro-CoA, propionyl-CoA; SA, succinate.

of interest that peroxisomes, which are induced by alkanes, and mitochondria, which are inherent, have isoenzymes with the same functions, and that the glyoxylate cycle is functioning between these two organelles. Accordingly, carnitine acetyltransferases, which are localized in peroxisomes and mitochondria of alkane-grown C. tropicalis, would be good target enzymes to study the development of peroxisomes.

SUMMARY

Two types of carnitine acetyltransferase [EC 2.3.1.7] were first isolated from a microorganism, alkane-grown yeast Candida tropicalis. Carnitine acetyltransferase activity was induced in the alkane-grown cells, reaching about twenty times higher than that in the glucose-grown cells. Localization of the enzyme activity was demonstrated, at least, in peroxisomes (microbodies), profusely occurred in the alkane-grown cells, and in mitochondria. Peroxisomal and mitochondrial carnitine acetyltransferases could be separated using the method of DEAE-Sephacel column chromatography and both types were found to exist in the alkane-grown cells of C. tropicalis.

Each carnitine acetyltransferase was purified using Sephadex G-200, Sepharose 6B, DEAE-Sephacel and Blue-Sepharose CL-6B. In DEAE-Sephacel chromatography, peroxisomal carnitine

acetyltransferase was eluted below 0.15 M KCl concentration and mitochondrial carnitine acetyltransferase above 0.15 M KCl concentration. Except for the localization, little difference was observed in their kinetic properties, substrate specificity and so on. These two carnitine acetyltransferase preparations were only specific to acetyl and propionyl groups, the substrate specificity not being so broad as that of carnitine acetyltransferase obtained from mammalian tissues.

Roles of these carnitine acetyltransferases in alkane metabolism in yeast were also discussed.

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Part III. Induction and subcellular localization of carnitine acetyltransferase in propionate-grown *Candida tropicalis*

INTRODUCTION

Yeast cells assimilating *n*-alkanes or higher fatty acids as sole source of carbon and energy are an excellent model to investigate the fatty acid metabolism in mammalian cells. When *Candida tropicalis* was grown on alkanes, conspicuous numbers of peroxisomes (microbodies) appeared in the yeast cells. These peroxisomes have been proved to play an indispensable role in the fatty acid metabolism.[1-4]

The author[5, 6] has reported that peroxisomal and mitochondrial carnitine acetyltransferases [EC 2.3.1.7] constitute "acetylcarnitine shuttle" system for transportation of acetyl-group between peroxisomes and mitochondria, which is essential for the operation of the glyoxylate cycle by cooperation of these two organelles. Carnitine-dependent transferase specific for medium- and long-chain fatty acids was not detected in the crude extract and purified preparations from *C. tropicalis*. Both carnitine acetyltransferases are absolutely specific for acetyl- and propionyl- groups. This fact seems reasonable because the final products of peroxisomal β-oxidation of fatty

acids with even and odd carbon chains are acetyl-CoA and ropionyl-CoA. Acetyl-CoA is metabolized *via* tricarboxylic acid cycle and glyoxylate cycle, while propionyl-CoA is supposed to be metabolized *via* methylcitric acid cycle.[7)]

However, many questions remain to be solved about the metabolic fate of propionyl-CoA, the final degradation product of fatty acids with odd carbon chain, in eukaryotic cells.[8)] One of the interesting problems is to investigate how propionate is transferred to the site of metabolism compared with the case of acetate. Propionate-grown *C. tropicalis* cells having subcellularly distinct compartmentation will be a potent tool to get information on this matter.

Part III describes the induction and subcellular localization of carnitine acetyltransferase in propionate-grown *C. tropicalis* cells having a different type of peroxisomes. A possible metabolic pathway of propionate, comparing with that of acetate, is also discussed.

MATERIALS AND METHODS

1. Cultivation of yeast

Candida tropicalis Berkhout strain pK 233 (ATCC 20336) was cultivated aerobically at 30 °C in a medium of an indicated initial pH. The basic composition of the medium is; $NH_4H_2PO_4$

5.0 g/l, KH_2PO_4 2.5 g/l, $MgSO_4 \cdot 7H_2O$ 1.0 g/l, $FeCl_3 \cdot 6H_2O$ 0.02 g/l, corn steep liquor 1.0 ml/l and a carbon source (glucose 16.5 g/l, sodium acetate 13.6 g/l, sodium propionate 10 g/l or n-alkane mixture (C_{10}-C_{13}) 10 ml/l). In the case of alkane mixture, 0.5 ml/l of Tween 80 was added to the medium.[9] Initial pH of the medium was adjusted with 1 N NaOH before sterilization.

2. Preparation of enzyme sources

(a) Cells harvested by centrifugation at 1,000 x g for 5 min were washed twice with deionized water, and suspended into 50 mM potassium phosphate buffer (pH 7.2) to give a concentration of 2 g dry cell/l. An aliquot of the cell suspension (10 ml) was subjected to ultrasonic disintegration (20 KHz, 5 min) at 0 °C. The cell homogenate was centrifuged at 10,000 x g for 15 min, and the resulting supernatant was used as a cell-free extract.

(b) Cells harvested by centrifugation were washed twice with deionized water and once with 100 mM Tris/HCl buffer (pH 8.0) containing 1 mM dithiothreitol (buffer A). Washed cells were suspended in the respective buffer solution and homogenized with a Braun homogenizer (for 150 s per 1 g dry cells). The homogenized cell suspension was centrifuged twice at 10,000 x g for 15 min and ultracentrifuged once at 139,000 x g for 2 h. The final supernatant was diluted with an equal

volume of buffer A used as the sample solution applied to DEAE-Sephacel column.

3. Subcellular fractionation

Preparation of yeast protoplasts[5,10] was carried out as described in Part I. Fractions obtained were as follows: P_2 fraction (20,000 x g pellets), mitochondria and peroxisomes; P_3 fraction (139,000 x g pellets), microsomes; and S_3 fraction (139,000 x g supernatant), cytoplasm.

4. Enzyme assay

Catalase [EC 1.11.1.6],[11] carnitine acetyltransferase activity[12] and protein[13] was measured as described in Part I.

5. Electronmicroscopy

Cells were pre-fixed with 5 % glutaraldehyde, and post-fixed with potassium permanganate and peroxisomes were pre-fixed with 1 % glutaraldehyde, and post-fixed with OsO_4 as described previously.[14]

6. Chemicals

ATP was obtained from P-L Biochemicals, Inc., Milwaukee, USA; CoA, acetyl-CoA and propionyl-CoA from Sigma, St. Louis, USA; glutathione reduced form from Wako Pure Chemicals, Osaka,

Japan; and DEAE-Sephacel from Pharmacia, Uppsala, Sweden. L-Acetylcarnitine chloride and L-carnitine chloride were kindly donated by Otsuka Pharmaceutical Factory, Naruto, Tokushima, Japan. Other chemicals were purchased from commercial sources.

RESULTS

1. Effect of initial medium pH on growth of Candida tropicalis in propionate medium

Candida tropicalis was usually grown on glucose, acetate or alkanes at initial pH of 5.2. However, the yeast could not grow on sodium propionate at pH 5.2. When initial pH of the medium was varied from 5.2 to 6.4, yeast growth occurred in the higher pH region (Fig. 1). Such the effect of medium pH was not observed with the cells utilizing glucose, acetate or alkanes. From these results, medium pH of 6.0 was adopted for the carbon sources employed.

2. Activities of catalase and carnitine acetyltransferase in cells grown on different carbon sources

The activities of catalase and carnitine acetyltransferase in the cell-free extracts were assayed and compared using the cells of the middle exponential growth phase on

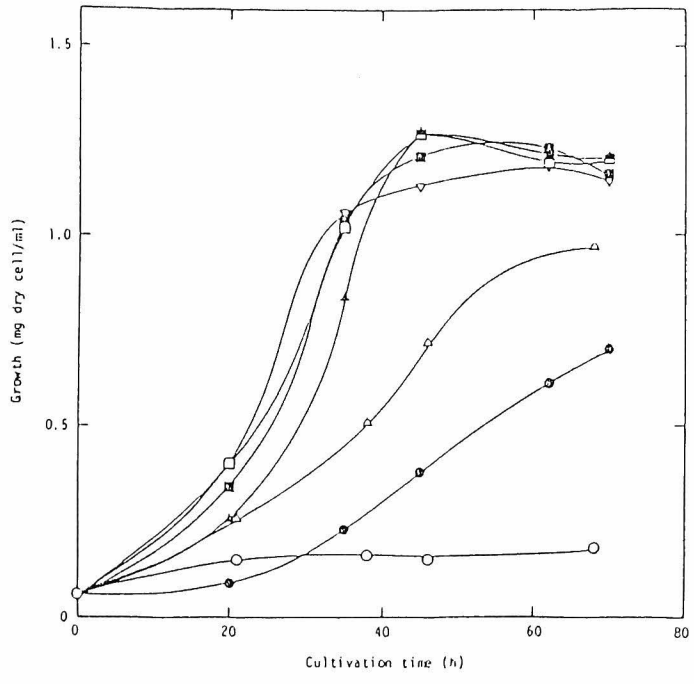


Fig. 1. Effect of initial medium pH on growth of C. tropicalis in propionate medium.

The yeast was cultivated aerobically at 30 °C in a medium with initial pH of 5.2 (○), 5.4 (●), 5.6 (△), 5.8 (▲), 6.0 (□), 6.2 (■) or 6.4 (▽).

different carbon sources (Fig. 2). Significantly high levels of both enzyme activities were observed in propionate-grown cells compared with those in glucose-grown and acetate-grown cells. The level of carnitine acetyltransferase activity was almost the same as that in alkane-grown cells, and the level

of catalase activity was about a half of that observed in alkane-grown cells. This increase in both enzyme activities was proved immunochemically to be arised from the increase in the amount of these enzyme proteins but not from the modification of the proteins themselves.

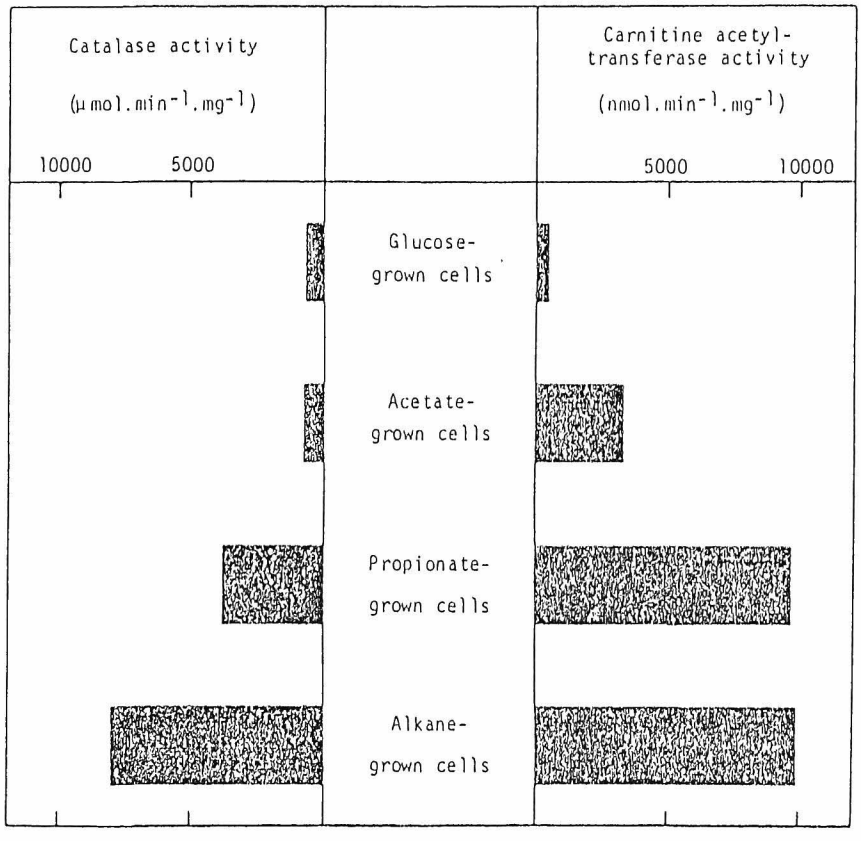
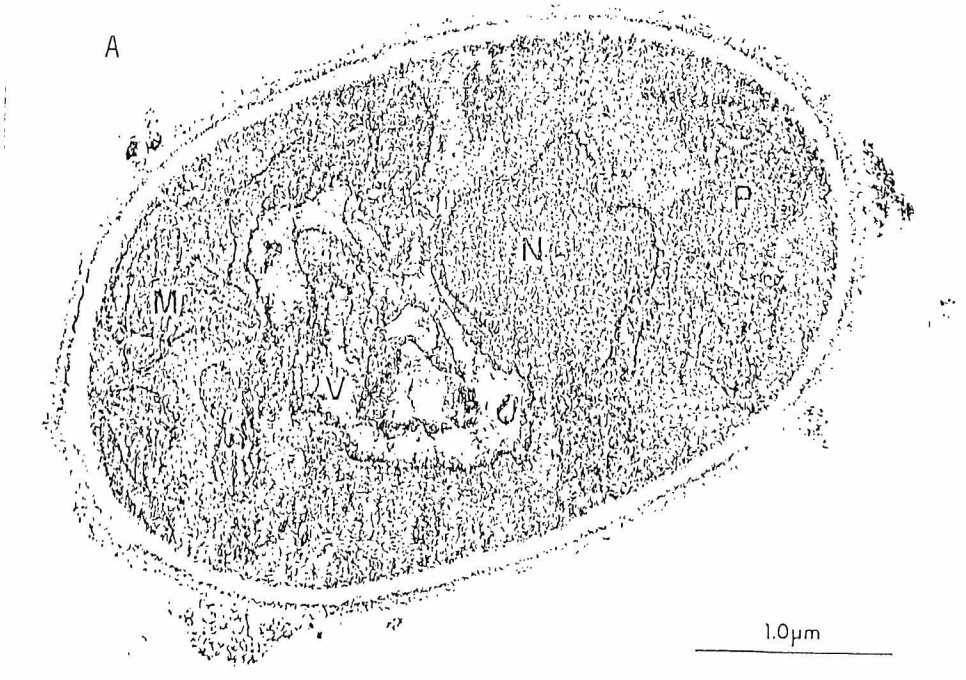


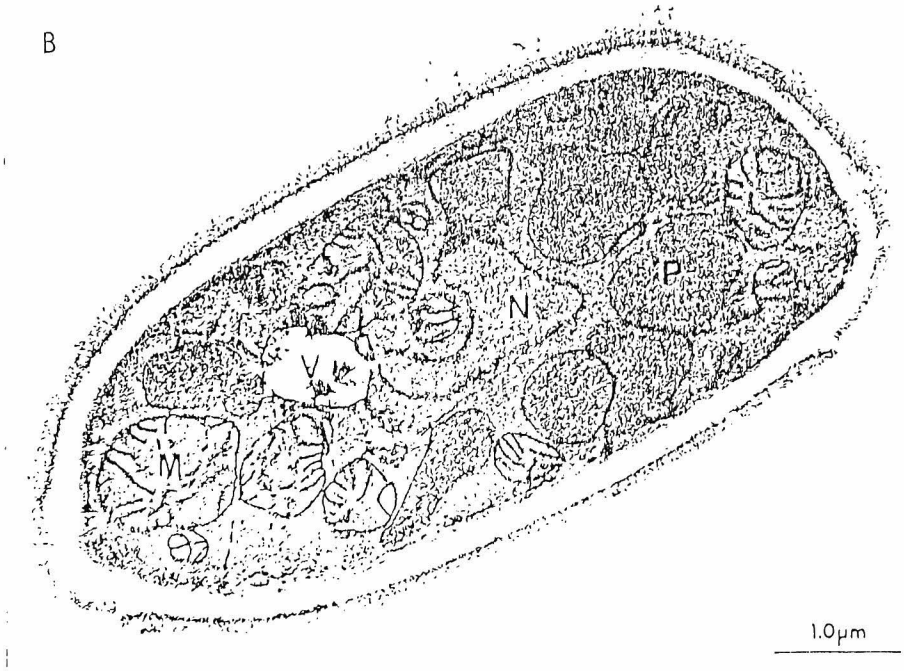
Fig. 2. Comparison of catalase and carnitine acetyltransferase activities in C. tropicalis grown on different carbon sources.

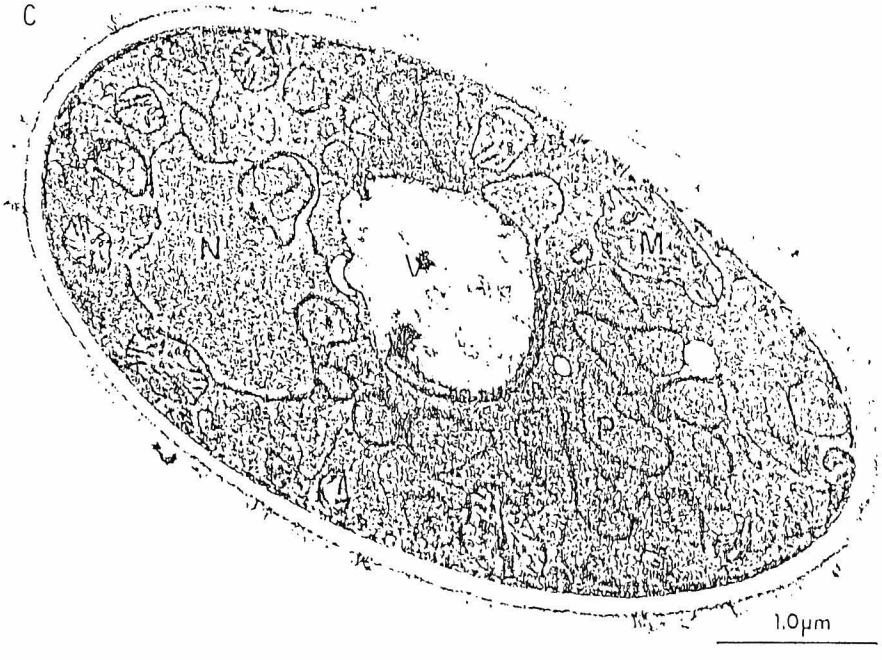
Cell-free extracts were prepared from cells harvested at exponential growth phase.

3. Electronmicroscopical observation

Judging from the increase in the activities of catalase and carnitine acetyltransferase, appearance of peroxisomes was expected in propionate-growth cells. Electronmicrographs of acetate-. propionate-, glucose- and alkane-grown cells are shown in Fig. 3 to compare morphological differences of peroxisomes. Acetate-grown cells have few peroxisomes, in contrast with alkane-grown cells which contain conspicuous numbers of the organelles. Peroxisomes are rarely observed in glucose-grown cells. Propionate-grown cells have large but a few number of peroxisomes morphologically different from those in alkane-grown cells. Such the large peroxisomes were detected at different growth phases examined (Fig. 4). In addition, development of mitochondria was also observed in propionate-grown cells.




B
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N
V
M
1.0μm


C
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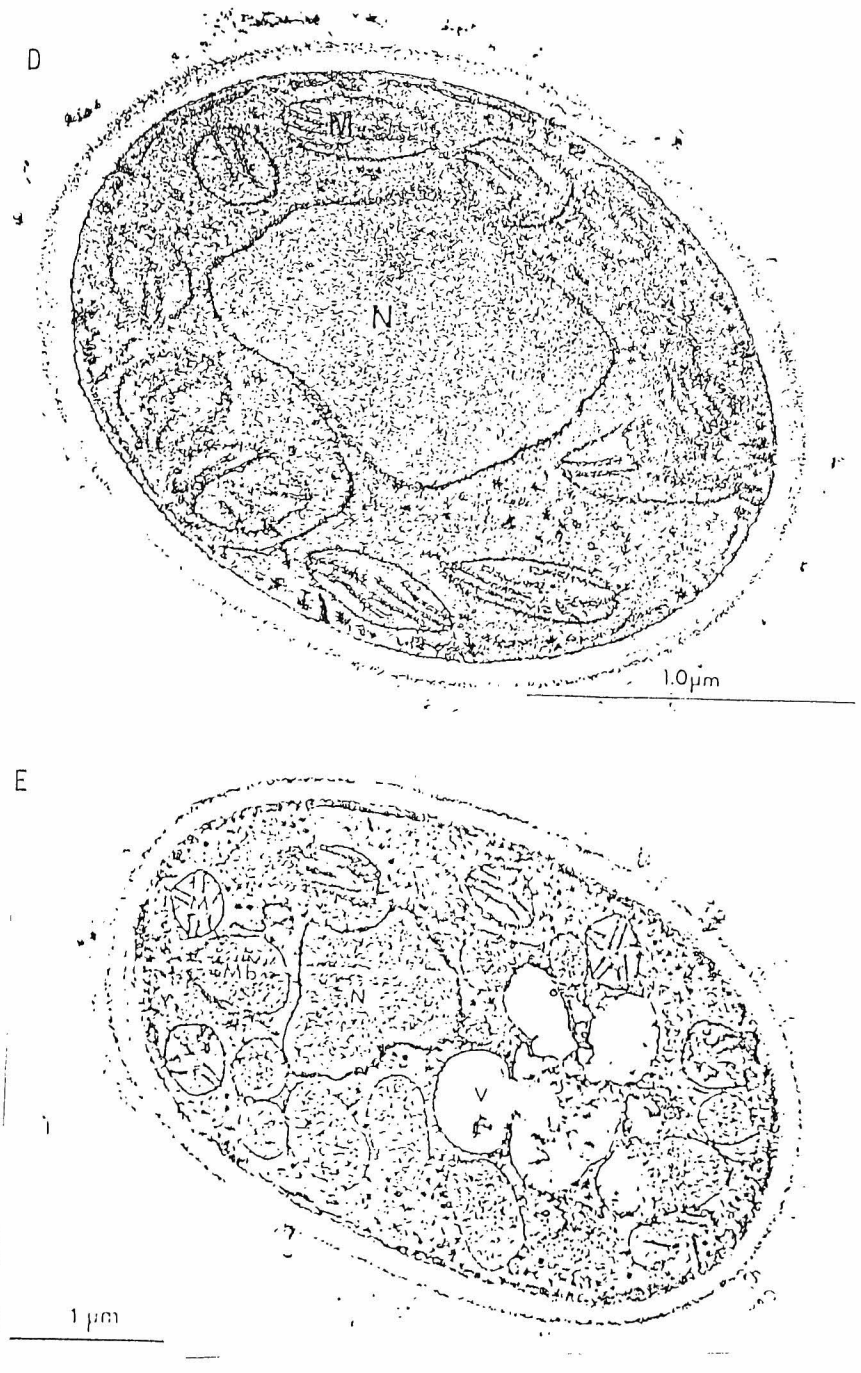


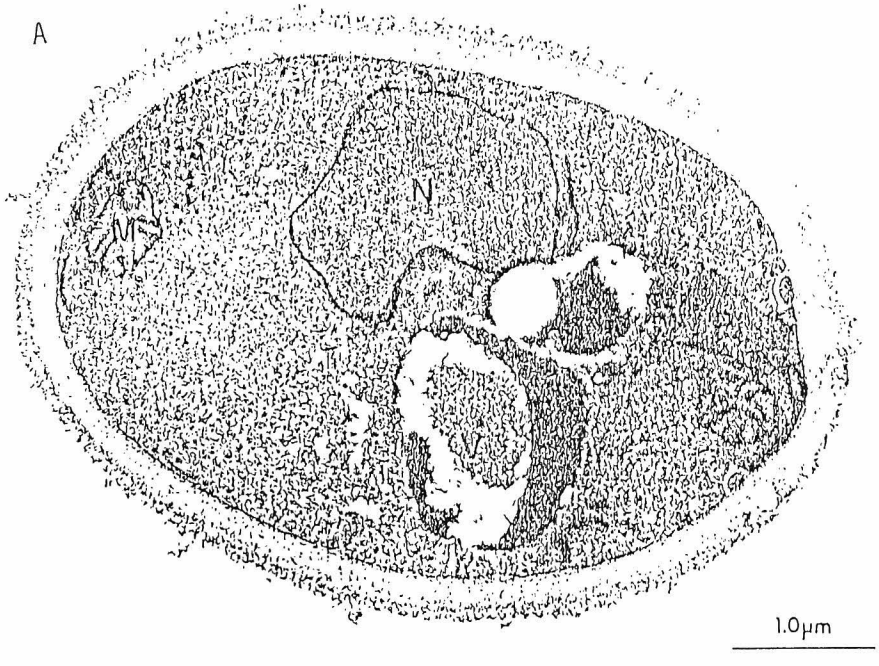
Fig. 3. Electronmicrographs of C. tropicalis cells harvested at exponential growth phase.

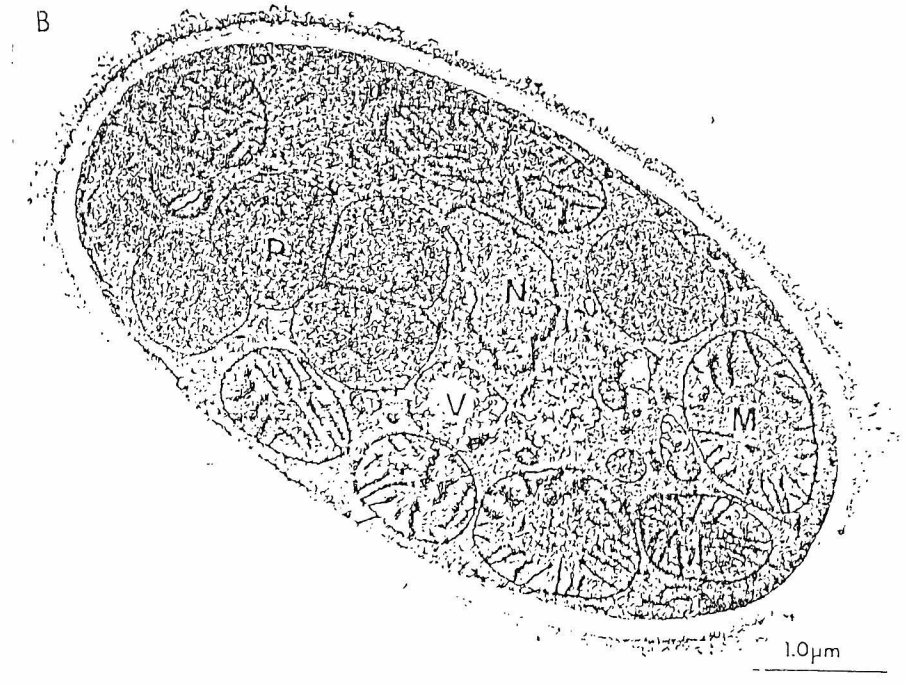
A, acetate-grown cell cultivated for 10 h;

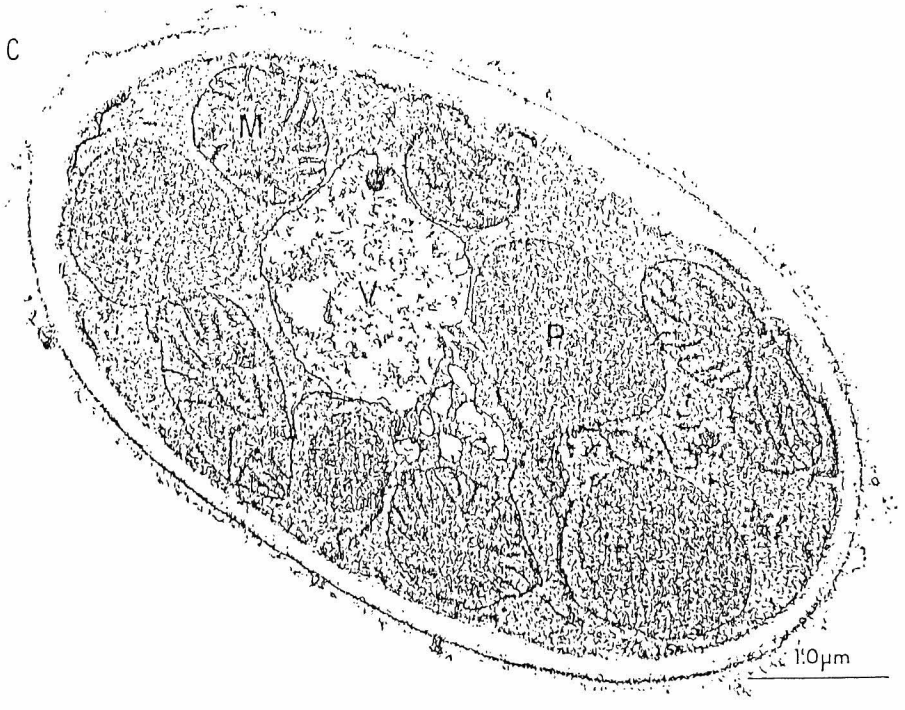
B, propionate-grown cell cultivated for 12 h;

C, alkane-grown cell cultivated for 12 h;

D, glucose-grown cell cultivated for 10 h;
E, butyrate-grown cell cultivated for 48 h.
M, mitochondrion; N, nucleus; P or Mb, peroxisome;
V, vacuole. Bar, 1 μm.




B
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N
V
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1.0μm


C
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1.0μm

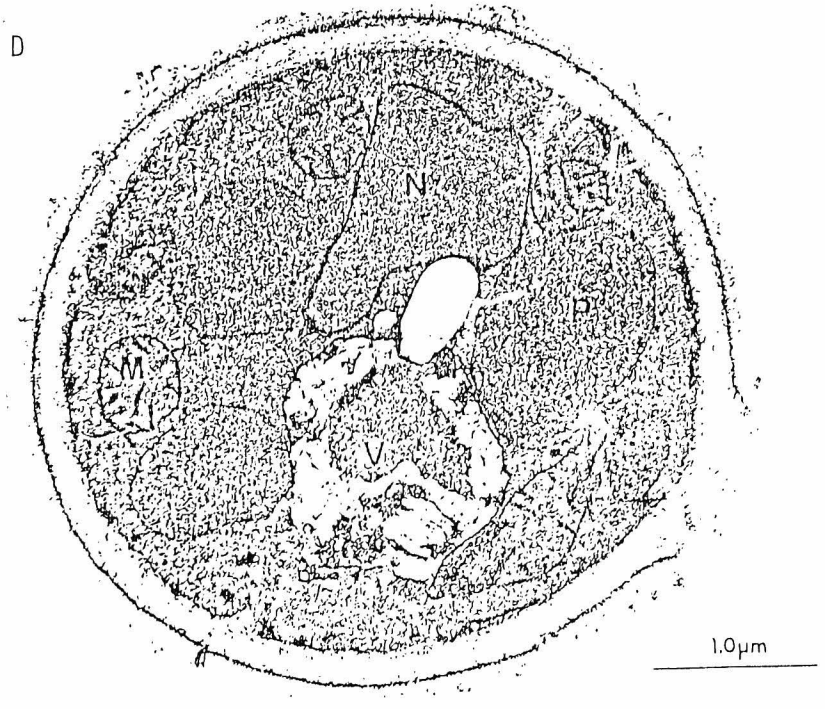


Fig. 4. Development of peroxisomes in propionate-assimilating cells of C. tropicalis.

Glucose-grown cells (A) were cultivated for 12 h (B), 24 h (C) and 36 h (D) in a propionate medium. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar, 1 µm.

4. Subcellular localization of catalase and carnitine acetyltransferase

As described above, peroxisome-associated enzymes, such as catalase and carnitine acetyltransferase, were induced in propionate-grown cells which contained a few numbers of large peroxisomes. Hence, it seemed interesting and important to clarify the subcellular localization of these enzymes. Catalase was mainly detected in P_2 fraction (mitochondria + peroxisomes) of alkane-grown, acetate-grown and glucose-grown cells, whereas the enzyme was found in S_3 fraction (cytoplasm) of propionate-grown cells as well as in P_2 fraction (Fig. 5). Carnitine acetyltransferase, localized in P_2 fraction of alkane-grown and propionate-grown cells (Fig. 6).

There is a fear that such the high levels of catalase and carnitine acetyltransferase in S_3 fraction of propionate-grown cells might be arised from the disintegration of peroxisomes during the fractionation process. However, the distribution pattern of both enzymes between P_2 and S_3 fractions did not change when the protoplasts from propionate-grown cells were homogenized for different periods (Fig. 7), while the ratios of these enzymes in P_2 fraction was low under hypotonic conditions. Furthermore, an electromicrograph clearly indicates the presence of intact peroxisomes in P_2fraction (Fig. 8). As a result, catalase and carnitine acetyltransferase in S_3

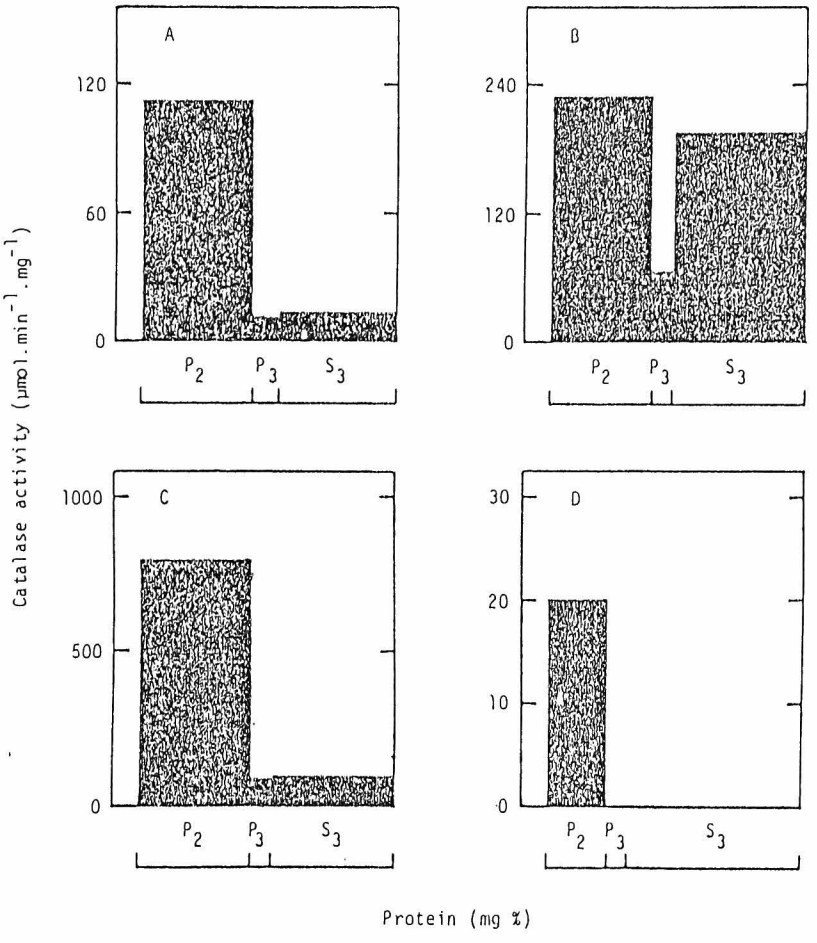


Fig. 5. Subcellular localization of catalase in C. tropicalis cells grown on different carbon sources.

Cells were harvested at exponential growth phase. A, acetate-grown cells; B, propionate-grown cells; C, alkane-grown cells; D, glucose-grown cells. P_2, 20,000 x g pellets (mitochondria + peroxisomes); P_3, 139,000 x g pellets (microsomes); S_3, 139,000 x g supernatant (cytoplasm).

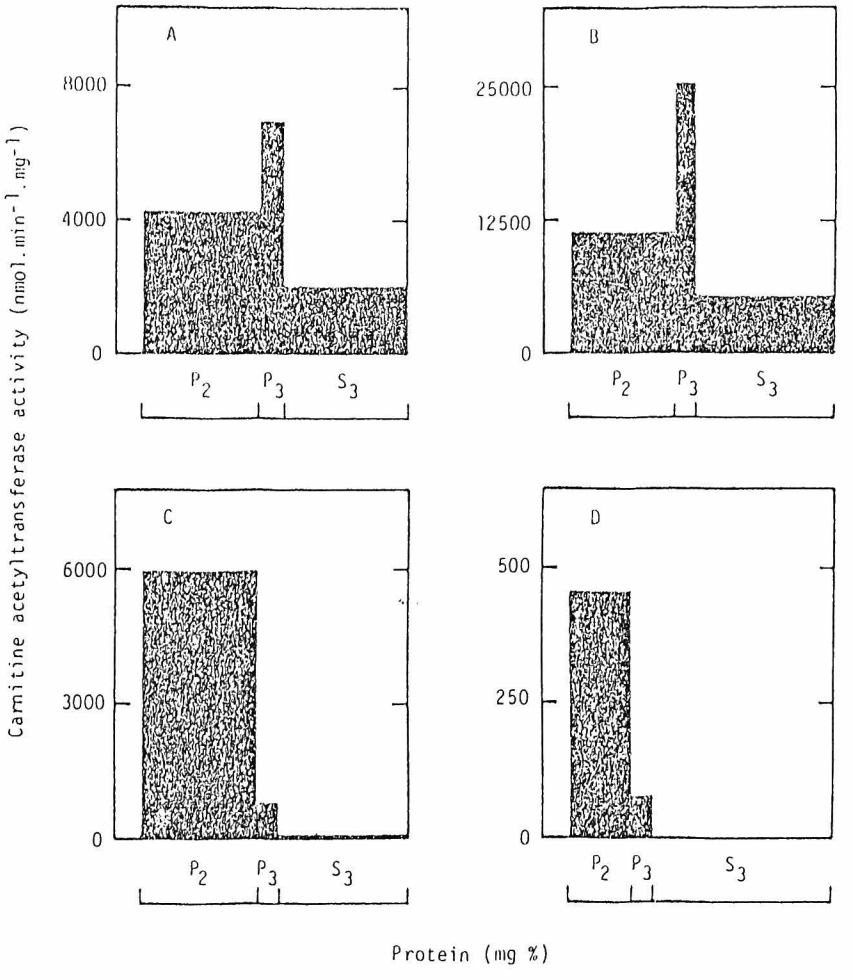


Fig. 6. Subcellilar localization of carnitine acetyltransferase in C. tropicalis grown on different carbon sources.

Experimental conditions are the same as those in Fig. 5.
A, acetate-grown cells; B, propionate-grown cells; C, alkane-grown cells; D, glucose-grown cells.

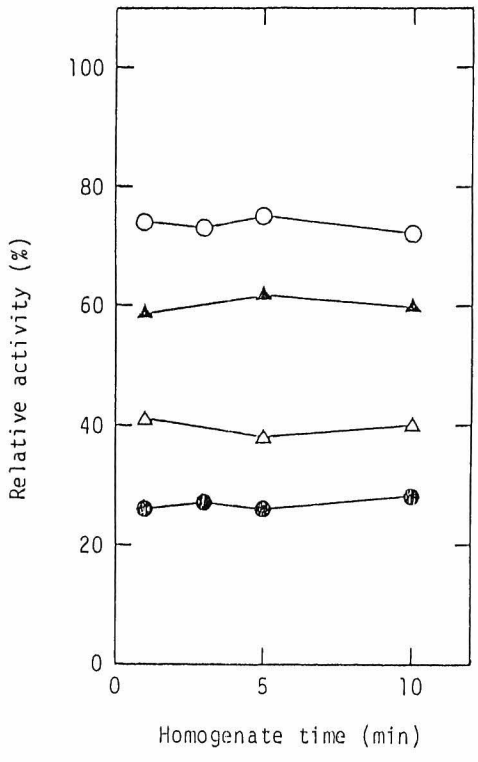


Fig. 7. Effect of homogenization period on subcellular distribution of catalase and carnitine acetyltransferase.

Protoplasts from propionate-grown cells of C. tropicalis were homogenized for indicated period with a teflon homogenizer to obtain subcellular fractions. P_2 (20,000 x g pellets) and S_2 (20,000 x g supernatant) were obtained by centrifugation of S_1 (2,000 x g supernatant). Enzyme activity in S_1 was expressed as 100 %. (○), Catalase activity in S_2; (●), catalase activity in P_2; (△), carnitine acetyltransferase activity in S_2; (▲), carnitine acetyltransferase activity in P_2.

fraction were considered to be cytoplasmic enzymes. Such cytoplasmic catalase and carnitine acetyltransferase were immunochemically indistinguishable from the respective enzymes obtained from peroxisomes of alkane-grown cells.

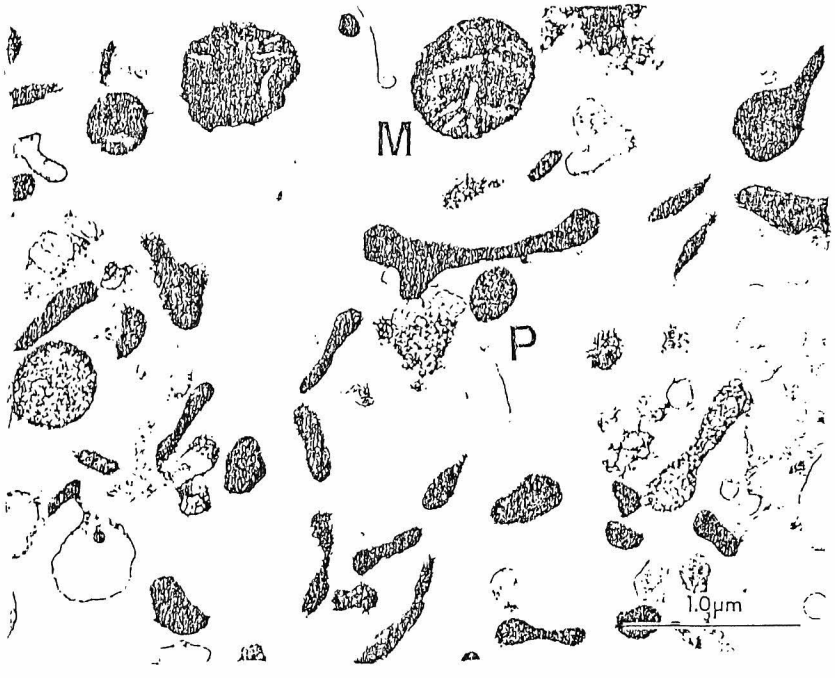


Fig. 8. Electromicrograph of P_2 fraction (20,000 x g pellets). M, mitochondrion; P, peroxisome. Bar, 1 µm.

5. Ratio of peroxisomal and mitochondrial carnitine acetyltransferases in cells grown on different carbon sources

Crude enzyme solutions obtained from the cells grown to the exponential growth phase on different carbon sources were applied to DEAE-Sephacel columns (1.5 x 26.5 cm) equilibrated with beffer A. Each column was washed with buffer A and then proteins were eluted with a linear concentration gradient of KCl prepared from 200 ml of buffer A and the same volume of buffer A containing 0.35 M KCl. The enzyme activity eluted with lower ionic strength corresponded to peroxisomal carnitine acetyltransferase and that with higher ionic strength to mitochondrial enzyme (Fig. 9), similarly to those of alkane-grown cells as described in the previous part.[6] In spite of no difference in the total enzyme activity between alkane-grown cells and propionate-grown cells, the ratio of peroxisomal and mitochondrial carnitine acetyltransferases was quite different. Most part of the enzyme induced in propionate-grown cells was found to be the mitochondrial type, while the peroxisomal enzyme was predominant in alkane-grown cells. In acetate-grown cells, the activity of the peroxisomal enzyme was comparable to that of the mitochondrial enzyme.

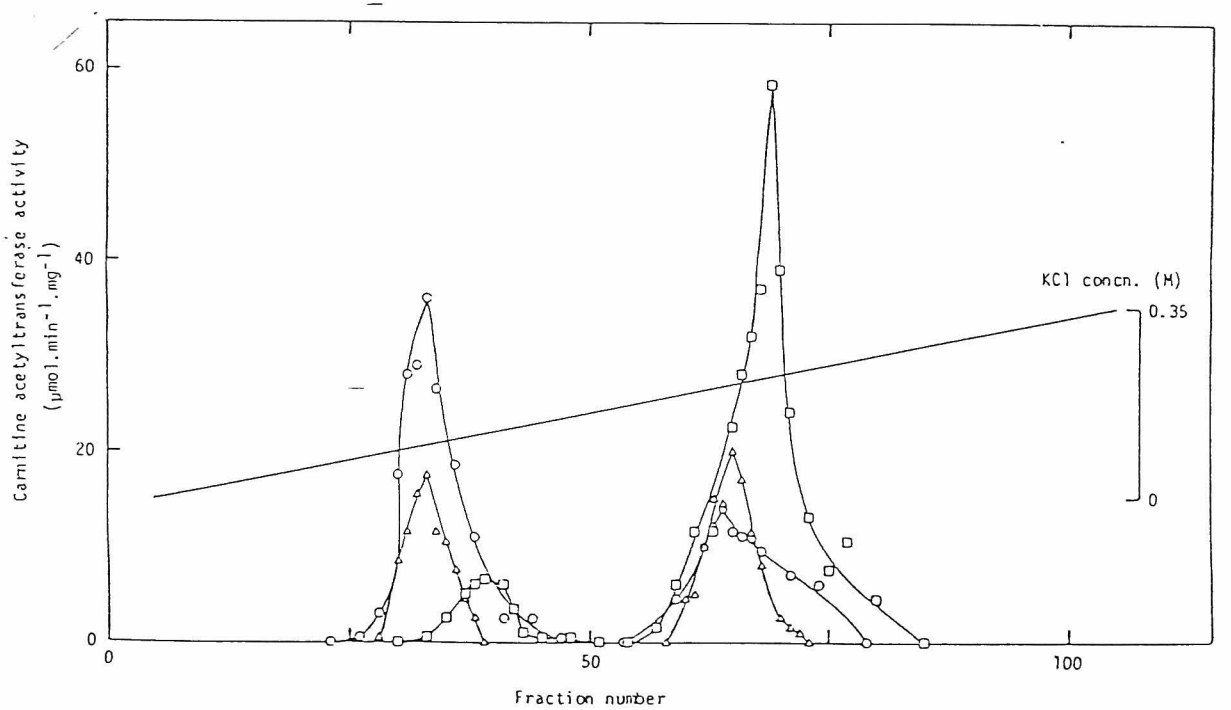


Fig. 9. Comparison of ratio of peroxisomal and mitochondrial carnitine acetyltransferases in C. tropicalis grown on different carbon sources.

Crude enzyme solutions from alkane-grown cells (○), acetate-grown cells (△) and propionate-grown cells (□) were prepared as described in the text, and applied to DEAE-Sephacel columns.

DISCUSSION

Recently, lively discussion has been made on the biogenesis and development of peroxisomes and glyoxysomes in various eukaryotic cells.[15] In medical field, increasing interest has been directed toward studies treating of a relationship between tumors and peroxisomes.[16] Although chemicals, such as clofibrate, di(2-ethylhexyl)phthalate *etc.*, have been used to proliferate peroxisomes in mammalian cells,[15] it appears very difficult to investigate the proliferating mechanism of peroxisomes with these reagents because these systems involve various factors to be examined. A simple system, in which peroxisomes develop well, is required to elucidate the mechanism of biogenesis and development of the organelles. In this sense, unicellular organisms, such as yeasts, seem to be very promising. When *Candida tropicalis* is grown on alkanes, conspicuous numbers of peroxisomes (microbodies) appear in the yeast cells.[14,17] These peroxisomes have been proved to contain catalase, the fatty acid β-oxidation system, acyl-CoA synthetase, key enzymes of glyoxylate cycle (isocitrate lyase and malate synthase), carnitine acetyltransferase *etc.*[1-4] Two types of carnitine acetyltransferases, peroxisomal and mitochondrial enzymes, are included in the transport of C_2- and C_3-units formed in peroxisomes to mitochondria, both organelles cooperating to complete

the glyoxylate cycle.[5,6]

As described previously,[6] peroxisomal and mitochondrial carnitine acetyltransferases of alkane-grown C. tropicalis cells are induced by alkanes and are specific to acetyl-CoA and propionyl-CoA, both of which are the degradation products from alkanes. These facts gave us incentive to investigate the role of carnitine acetyltransferase under simple conditions where acetate or propionate is sole source of carbon and energy for growth of the yeast. When the yeast was cultivated on these acids, carnitine acetyltransferase was induced moderately on acetate and extensively on propionate. The ratio of the mitochondrial type enzyme (localized in mitochondria and cytoplasm) to the peroxisomal type enzyme was almost equal in acetate-grown cells, while the mitochondrial type enzyme was major in propionate-grown cells. These results are in contrast to the predominant localization of the enzyme in peroxisomes of alkane-grown cells.

Catalase, which is known as a marker enzyme of peroxisomes, was also induced by propionate as well as alkanes. In this case also, a large part of the enzyme was localized in cytoplasm in propionate-grown cells, the result being significantly different from the predominant localization in peroxisomes of alkane-grown cells. In harmony with the subcellular localization of carnitine acetyltransferase and catalase, morphological

difference in peroxisomes was observed between propionate-grown and alkane-grown cells. In propionate-grown cells, a few number of large peroxisomes, which were sometimes in contact with each other like the organelles in methanol-grown Kloeckera sp. cells,[18] were detected electronmicroscopically. On the other hand, conspicuous numbers of small peroxisomes were observed in alkane-grown cells. These results suggest that peroxisomes in propionate-grown cells might be immature. At present, it is impossible to conclude whether cytoplasmic catalase has a metabolic role in propionate-utilizing cells or not. If the cytoplasmic enzyme is non-functional and is only the pooled protein, propionate-grown cells would be an excellent system to differentiate the induction of catalase from development of peroxisomes. Such system seems to be very useful to investigate the maturing of the organelles.

It is interesting that the level of carnitine acetyltransferase in mitochondria and in cytoplasm was much higher than in peroxisomes in propionate-grown cells. In the metabolism of propionate, the enzyme in both mitochondria and cytoplasm seems to take an important part in the transport of propionyl-unit from cytoplasm to mitochondria. Mishina et al.[19,20] reported the presence of two distinct long-chain fatty acyl-CoA synthetases in Candida lipolytica cells grown on alkanes or long-chain fatty acids. Acyl-CoA synthetase I localized in mitochondria

and microsomes plays a role in lipid synthesis, while scyl-CoA synthetase II detected exclusively in peroxisomes participates in fatty acid oxidation. These enzymes are distinguishable each other by the immunochemical property and the requirement of phosphatidylcholine. Although existence of such different types of acyl-CoA synthetases have not been recognized in C. tropicalis cells grown on alkanes, the enzyme was also localized in peroxisomes, mitochondria and microsomes, having distinct metabolic functions according to its distinct localization.[21] In addition to acyl-CoA synthetase specific for long-chain fatty acids, enzymes active on short-chain fatty acids were found in the cells of C. tropicalis. An enzyme most active on acetate was found in both acetate-grown and propionate-grown cells.

In acetate-utilizing cells of C. tropicalis, acetate is supposed to be activated to acetyl-CoA by acetyl-CoA synthetase and then converted to acetylcarnitine by carnitine acetyltransferase in cytoplasm. The carnitine ester seems to be transported into mitochondria, and again transformed to acetyl-CoA by mitochondrial carnitine acetyltransferase, the CoA ester being metabolized via TCA cycle in mitochondria and/or via glyoxylate cycle cooperating between mitochondria and cytoplasm.[22] In the meantime, propionate activated by the propionate-activating enzyme in cytoplasm is considered to be transported

similarly to mitochondria as the form of the carnitine derivative, and metabolized through methylcitric acid cycle whose operation has been demonstrated in Yarrowia lipolytica grown on alkanes,[7] although the operation and subcellular localization of methylcitric acid cycle in propionate-grown cells of C. tropicalis have not been confirmed.

As discussed above, propionate-utilizing cells of C. tropicalis give an excellent system to study two problems: One is the appearance of characteristic peroxisomes which will be very useful to investigate the development of the organelles, and the other is the propionate metabolism in eukaryotic cells.

SUMMARY

Candida tropicalis, a representative alkane- and higher fatty acid-utilizing yeast, can grow on propionate used as sole carbon and energy source. Initial pH of the medium markedly affected the growth of the yeast on propionate. In propionate-grown cells, several enzymes associated with peroxisomes were induced in connection with the appearance of the characteristic peroxisomes. Acetate-grown cells of this yeast had only few peroxisomes, while alkane-grown cells contained conspicuous numbers of the organelles. As compared with alkane-grown cells, some specific features were observed in peroxisomes and enzymes

associated with the organelles of propionate-grown cells: The shape of peroxisomes was large but the number was small; unlike localization of catalase in peroxisomes of alkane-grown cells, the enzyme of propionate-grown cells was mainly localized in cytoplasm; as for carnitine acetyltransferase localized almost equally in peroxisomes and mitochondria in alkane-grown cells, propionate-grown cells contained mainly the mitochondrial type enzyme. The role of carnitine acetyltransferase in propionate metabolism is discussed in comparison with the role of carnitine acetyltransferase in acetate metabolism.

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Part IV. Characterization of peroxisomal and mitochondrial carnitine acetyltransferases purified from n-alkane-grown Candida tropicalis

INTRODUCTION

Carnitine acetyltransferase, which catalyzes the reversible transfer of acetyl group between CoA and L-carnitine, has an important role in the metabolism of fatty acids in eukaryotic cells, because acetyl-CoA, a final product of β-oxidation of fatty acids, is not permeable through the various organelle membranes.[1-4] This enzyme has been detected in peroxisomes and microsomes as well as in mitochondria of mammalian cells, and isolated from pigeon breast muscle and rat liver mitochondria.[1-6] However, there is no information about the molecular identity of peroxisomal and mitochondrial carnitine acetyltransferases isolated from the same cells.

In this connection, a variety of interesting problems remain to be elucidated: whether or not peroxisomal and mitochondrial carnitine acetyltransferases are products of same gene; how these two enzymes are distributed into two subcellular compartments if they are the same gene products; what relationships exist between these two enzymes and the biogenesis of organelles, especially peroxisomes and so on.

To get useful information on these matters, it is necessary to compare the molecular properties of peroxisomal and mitochondrial carnitine acetyltransferases.

In Part I,[7] we have demonstrated that carnitine acetyltransferases induced in alkane-grown cells of Candida tropicalis are localized in peroxisomes and mitochondria and that both enzymes take an essential part in the transfer of acetyl units from peroxisomes to mitochondria for the complete operation of the glyoxylate cycle. These enzymes in both organelles were separated by DEAE-Sephacel column chromatography.[8]

This part deals with some molecular properties of these two enzymes purified from n-alkane-grown C. tropicalis cells.

MATERIALS AND METHODS

1. Purification and assay of carnitine acetyltransferases

Peroxisomal and mitochondrial carnitine acetyltransferases were purified from n-alkane-grown cells of Candida tropicalis, and the assay of the enzymes was carried out as described in Part II.[8]

2. Estimation of molecular weight

Electrophoresis on discontinuous sodium dodecylsulfate/polyacrylamide slab gel containing 10 % acrylamide was performed

as described by King and Laemmli[9] except that both the stacking and the separating gels contained 0.25 mM EDTA. Phosphorylase b, bovine serum albumin, ovalbumin, and lactate dehydrogenase were used as standards. After electrophoresis at room temperature, gels were stained for protein with 0.1 % Coomassie brilliant blue dissolved in 2-propanol/acetic acid/water (25/10/65, by vol.) and were then destained with 2-propanol/acetic acid/water (10/10/80, by vol.).

Determination of the molecular weight by gel filtration chromatography was performed under the following conditions. The enzyme dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM dithiothreitol and 1 M KCl was applied to a Sepharose 6B column (1.5 x 65 cm) equilibrated with the above buffer, and eluted under the conditions to be described later. Blue-dextran, thyroglobulin, catalase, aldolase, bovine serum albumin, ovalbumin, cytochrome c, and DNP-alanine were used as markers of molecular weight.

The $\underline{s}_{20,w}$ values and the molecular weight of both native enzymes were also determined by analytical centrifugation.

3. Analyses of amino acid composition and amino terminus

The purified preparations of both enzymes were extensively dialyzed against distilled water, and then lyophilized. The lyophilized samples were hydrolyzed at 110 °C for 20 h with

6 N HCl in sealed tubes *in vacuo*. Amino acids in the hydrolyzates were analyzed with a Hitachi 835 amino acid analyzer, according to the method of Spackman *et al.*[10] The amount of each amino acid was corrected based on the recovery of the internal standard. Half-cystine was determined by titration with 5,5'-dithiobis(2-nitrobenzoic acid),[11] and tryptophan was assayed by the method of Edelhoch.[12] Amino acid composition was computed from the average values from the duplicate experiments.

Amino-terminal analysis by the dansylation method was performed according to the procedure of Gray[13] with the use of the solvent systems described by Hartley.[14]

4. Determination of isoelectric point

Alkane-grown cells (1 g dry cells) harvested at the middle exponential growth phase were washed twice, suspended into 25 mM imidazole-HCl buffer (pH 7.4) and homogenized with a Braun homogenizer for 150 s per 1 g dry cells. The homogenized cell suspension was centrifuged at 10,000 x *g* for 15 min. The resulting supernatant was further centrifuged at 139,000 x *g* for 1 h to obtain the cell-free extract. An aliquot of the cell-free extract (45 mg protein) was applied to a PBE (poly buffer exchange) 94 column (1 x 40 cm) equilibrated with 25 mM imidazole-HCl buffer (pH 7.4), and proteins were eluted with

the eighth diluted solution of poly buffer 74-HCl buffer (pH 4.0). Fractions (each 4 ml) were collected and the activity of carnitine acetyltransferase and the content of protein were assayed.

5. Preparation of antibody

Rabbit antiserum against each carnitine acetyltransferase isoenzyme was prepared in the following manner. A solution of the purified enzyme (1.75 mg protein) was mixed with an equal volume of complete Freund's adjuvant (Difco, Detroit, USA). This mixture was injected into the footpads of each rabbit. Four weeks later, 1.5 mg of the each purified enzyme was injected subcutaneously as a booster. Blood was obtained from vein of the ears of immunized animals 5-7 days after the booster injection, and antisera were collected by means of centrifugation. Control sera were similarly obtained from a nonimmunized rabbit.[15] Ouchterlony double diffusion analysis was performed by the method of Ouchterlony.[16]

6. Chemicals

Phosphorylase b was obtained from Boehringer Mannheim, West Germany; Thyroglobulin, catalase, aldolase, cytochrome c, DNP-alanine, bovine serum albumin, ovalbumin, and chromato-focusing chromatography kits from Sigma, St. Louis, USA;

lactate dehydrogenase from Wako Pure Chemicals, Osaka, Japan. Other chemicals were purchased from commercial sources.

RESULTS

1. Estimation of molecular weight of peroxisomal and mitochondrial carnitine acetyltransferases

Polyacrylamide slab gel electrophoresis (10 % acrylamide gel) with sodium dodecylsulfate was used for determining the molecular weight of the subunits. The results obtained were 64,000 and 57,000 for peroxisomal carnitine acetyltransferase (Fig. 1, lane 3), and 64,000 and 52,000 for the mitochondrial enzyme (Fig. 1, lane 1), respectively. When both enzymes were mixed and applied to the electrophoresis (Fig. 1, lane 2), the larger subunits of the enzymes were indistinguishable, while the smaller ones were obviously different.

In the analytical ultracentrifugation, both enzymes gave the sedimentation patterns with a single symmetrical boundary (data not shown) and showed almost the same $\underline{s}_{20,w}$ value of 16s. By the meniscus depletion technique, the molecular weights were estimated to be 415,000 for peroxisomal carnitine acetyltransferase and 426,000 for the mitochondrial enzyme.

In the gel filtration chromatography, both enzymes were eluted in the void volume with the elution buffer of 10 mM

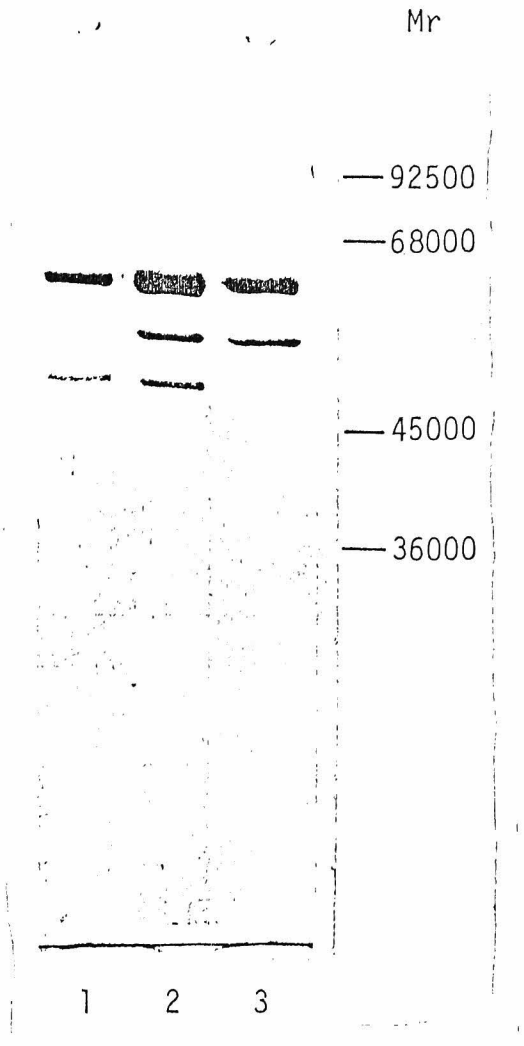


Fig. 1. Sodium dodecylsulfate/polyacrylamide slab gel electrophoresis of purified peroxisomal and mitochondrial carnitine acetyltransferases.

Electrophoresis was performed under the conditions described in 'MATERIALS AND METHODS'. Lane 1, mitochondrial carnitine acetyltransferase; lane 2, a mixture of mitochondrial and peroxisomal carnitine acetyltransferase; lane 3, peroxisomal carnitine acetyltransferase. The marker proteis used were

phosphorylase b (Mr 92,500), bovine serum albumin (Mr 68,000), ovalbumin (Mr 45,000) and lactate dehydrogenase (Mr 36,000). Bromophenol blue served as the tracking dye.

Tris-HCl buffer (pH 8.0) containing 1 mM dithiothreitol and potassium chloride below the concentration of 0.3 M. When the concentration of potassium chloride was raised to 1 M, both enzymes were eluted between thyroglobulin (Mr 669,000) and catalase (Mr 232,000), showing the molecular weight of 42,000 (Fig. 2). These results suggest that the enzymes would be in aggregated forms at a lower ionic strength region. The chromatographic patterns also indicate that both enzymes have hetero-oligomeric structures composed of subunits of different molecular weight, respectively.

2. Amino acid composition and amino terminus

The results of amino acid analysis of both enzymes are summarized in Table 1. The amino acid composition of peroxisomal and mitochondrial carnitine acetyltransferases were similar within the limits of experimental error. However, the contents of several amino acids, such as glutamic acid, aspartic acid, valine and glycine were not identical.

As shown in Fig. 3, amino-terminal analysis by dansylation

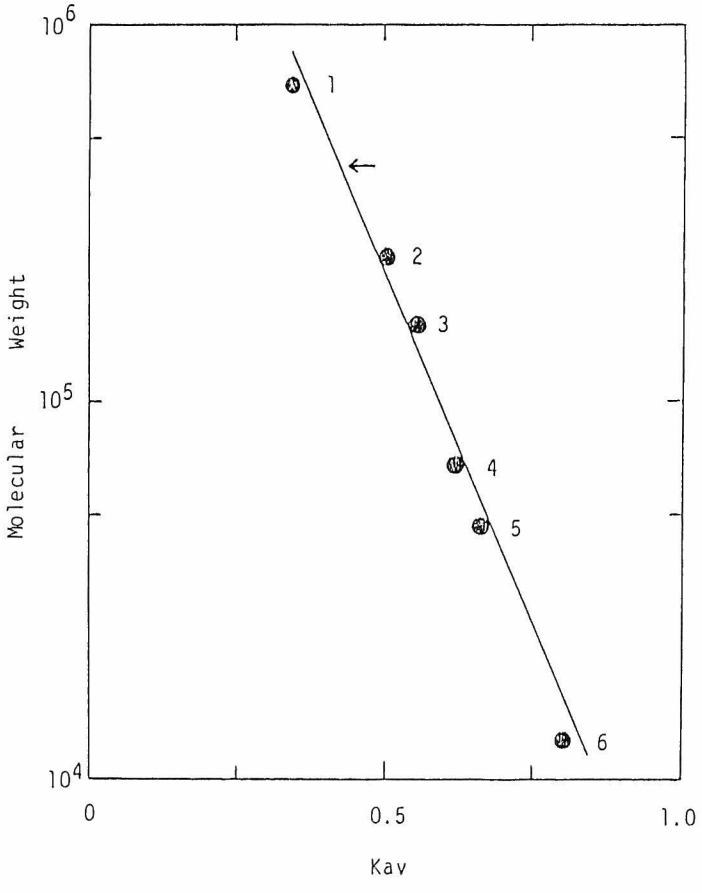


Fig. 2. Estimation of the molecular weight of peroxisomal and mitochondrial carnitine acetyltransferases by gel chromatography.

Elution was performed under the conditions described in 'MATERIALS AND METHODS'. The arrow indicates the elution point of both enzymes. Following proteins were used as markers: (1) thyroglobulin (Mr 669,000); (2) catalase (Mr 232,000); (3) aldolase (Mr 158,000); (4) bovine serum albumin (Mr 68,000); (5) ovalbumin (Mr 45,000); and (6) cytochrome c (Mr 12,500).

Table 1. Amino acid composition of peroxisomal and mitochondrial carnitine acetyltransferases.

The mean values from duplicate analyses. Cysteine and tryptophan were also determined as described in 'MATERIALS AND METHODS'. Ps, peroxisomal; Mt, mitochondrial.

	Ps type	Mt type
	(mol/100 mol)	
Aspartic acid	11.6	12.1
Threonine	5.1	5.2
Serine	7.3	7.5
Glutamic acid	10.4	11.0
Proline	4.9	4.7
Glycine	7.4	6.8
Alanine	8.5	8.1
Cystine	0.3	0.3
Valine	5.2	4.5
Methionine	1.1	1.2
Isoleucine	4.8	4.6
Leucine	7.9	8.1
Tyrosine	4.3	4.5
Phenylalanine	5.6	6.0
Lysine	7.8	7.6
Histidine	2.0	2.0
Arginine	3.1	3.0
Tryptophan	2.3	2.5
Cysteine	0.6	0.5

showed that glutamic acid (or glutamine) occupied the amino-terminal position of each enzyme. The dansylated derivative of glutamic acid was detected with essentially no accompanying traces of other amino acid derivatives even the enzymes were hetero-oligomers.

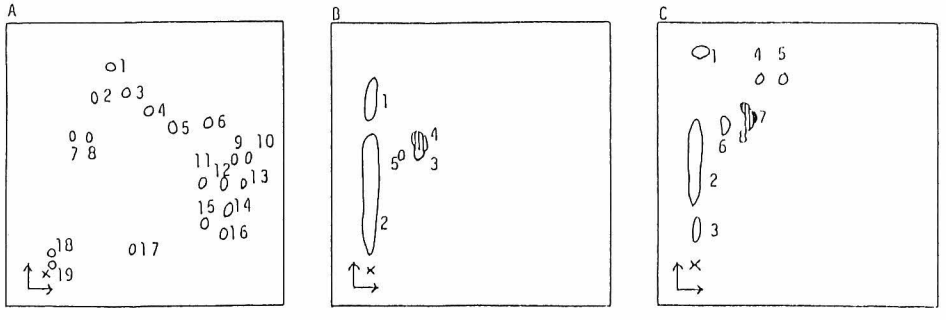


Fig. 3. Analysis of amino-terminus of peroxisomal and mitochondrial carnitine acetyltransferases.

Samples dansylated as described in 'MATERIALS AND METHODS' were spotted on the origin. Each polyamide sheet was developed to both directions shown with arrows. A (standard samples): 1, asparagine; 2, serine; 3, threonine; 4, hydroxyproline; 5, glycine; 6, alanine; 7, aspartic acid; 8, glutamic acid; 9, valine; 10, proline; 11, methionine; 12, norvaline; 13, isoleucine; 14, leucine; 15, phenylalanine; 16, norleucine; 17, tryptophan; 18, cysteine; 19, cystine. B (a sample from peroxisomal carnitine acetyltransferase and several standard samples): 1 and 2, DNS-OH; 3, glutamic acid; 4, a sample

from the peroxisomal enzyme; 5, aspartic acid. C (a sample from mitochondrial carnitine acetyltransferase and several standard samples): 1,2 and 3, DNS-OH; 4, serine; 5, threonine; 6, aspartic acid; 7, glutamic acid; 8, a sample from the mitochondrial enzyme.

3. Measurement of isoelectric point

By the chromatofocusing chromatography of the cell-free extracts from alkane-grown cells of C. tropicalis, two peaks with that of the peroxisomal and mitochondrial enzymes in the cell-free extract, it was confirmed that the enzyme eluted faster was mitochondrial and the enzyme eluted slower was peroxisomal. The isoelectric point of the peroxisomal enzyme was 5.11 and that of the mitochondrial one 5.22 (Fig. 4).

4. Comparison of heat stability

Each enzyme solution containing an equal amount of protein was treated at indicated temperatures for 10 min. As shown in Fig. 5, there was no significant difference between the heat stability of both enzymes.

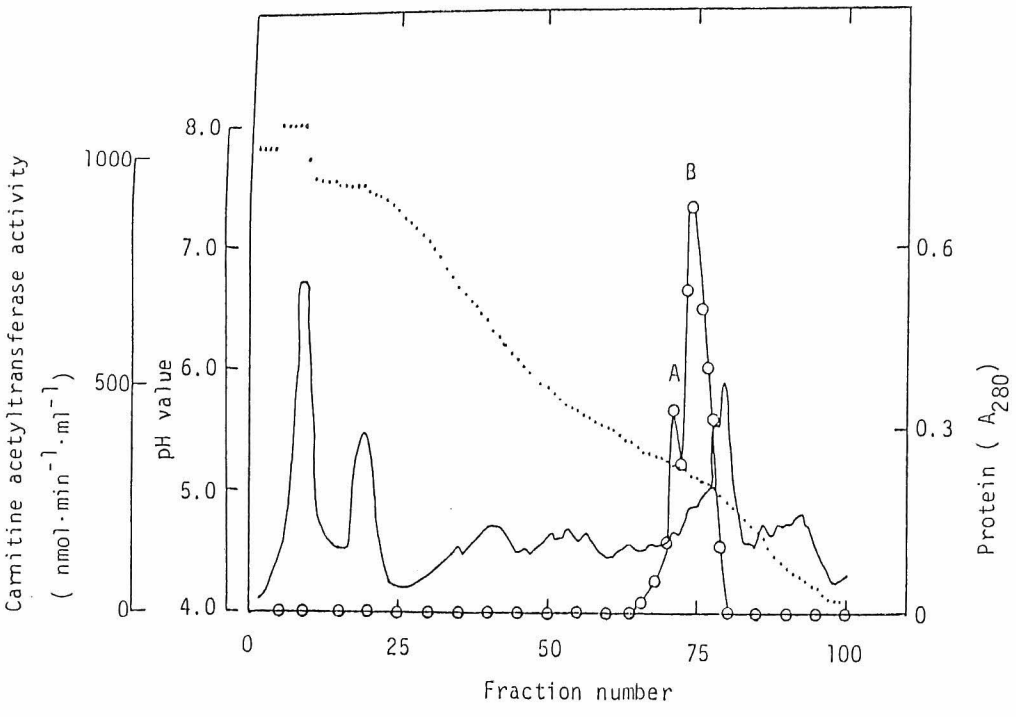


Fig. 4. Determination of isoelectric point of peroxisomal and mitochondrial carnitine acetyltransferases by chromatofocusing chromatography.

The volume of each fraction was 4 ml. The marks A and B show mitochondrial and peroxisomal enzymes, respectively. (o), Enzyme activity; (—), protein; (•), pH value.

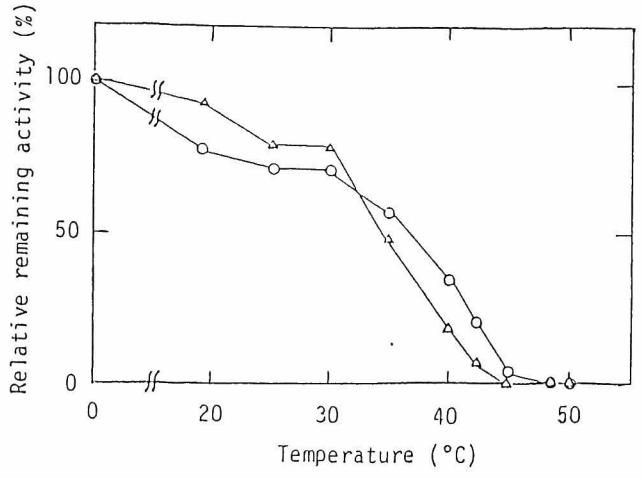


Fig. 5. Heat stability of peroxisomal and mitochondrial carnitine acetyltransferases.

Each enzyme solution (1 ml) containing 15 μg of protein was treated at indicated temperatures for 10 min and cooled immediately to 0 °C before assay of the enzyme activity. (○), Peroxisomal carnitine acetyltransferase; (△), mitochondrial carnitine acetyltransferase.

5. Immunochemical properties of peroxisomal and mitochondrial carnitine acetyltransferases

Respective antisera were prepared against peroxisomal and mitochondrial carnitine acetyltransferases. In the Ouchterlony double diffusion tests, each antiserum gave a single precipitin band toward both peroxisomal and mitochondrial carnitine acetyltransferases. Figs. 6 and 7 illustrate the immunochemical

titration curves of peroxisomal and mitochondrial carnitine acetyltransferases with the respective antisera. In these studies, a different amount of anti-mitochondrial carnitine acetyltransferase antiserum, anti-peroxisomal carnitine acetyltransferase antiserum or control serum was reacted with a fixed amount of the antigens. Each mixture of antigen antiserum was kept at 0 °C for 3 h, then centrifuged at 20,000 x g for 20 min, and the supernatant obtained was assayed for the enzyme activity. The results showed the cross reaction between the respective antisera and antigens.

Moreover, the immunochemical analysis was performed with a fixed amount of each antiserum by changing the amount of each antigen. The equivalent point for both enzymes against the respective antisera were shown to be almost equal (Figs. 8 and 9).

Judging from these results, peroxisomal and mitochondrial carnitine acetyltransferase were immunologically indistiguishable.

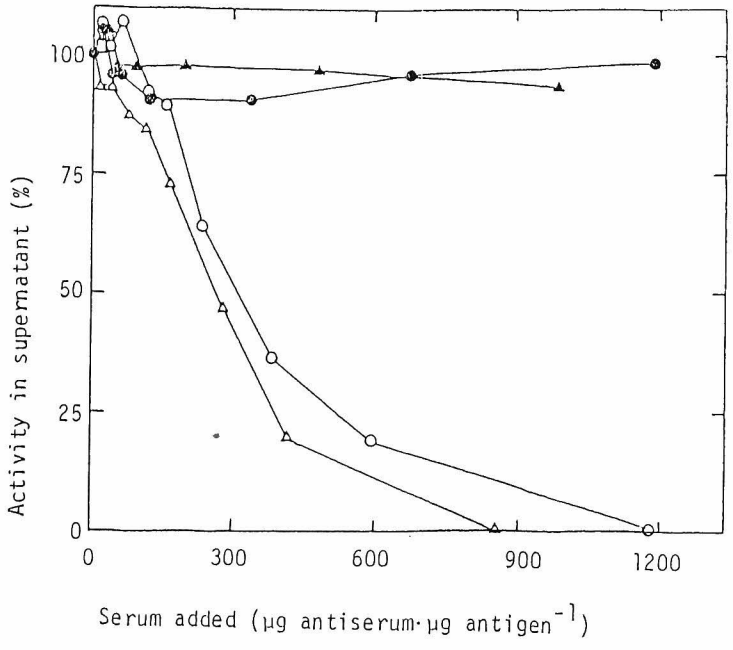

100
75
50
25
0
Activity in supernatant (%)
0
300
600
900
1200
Serum added (μg antiserum·μg antigen^{-1})

Fig. 6

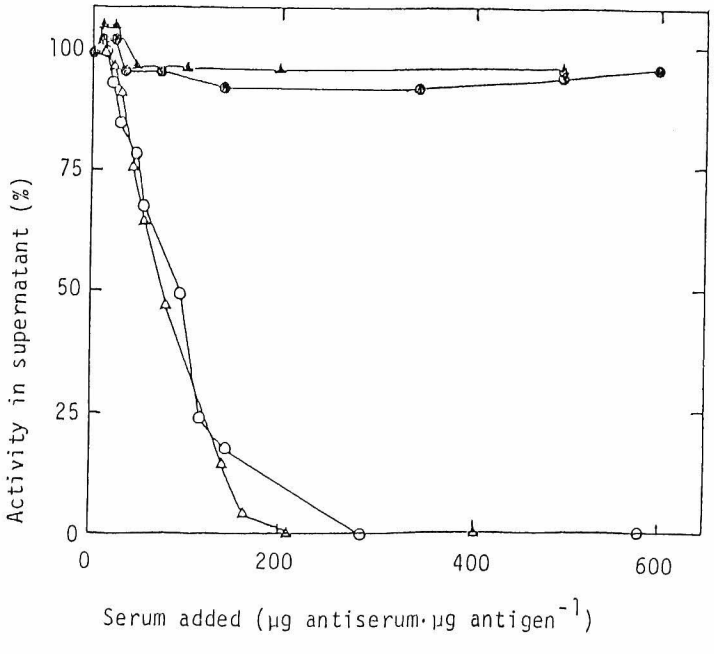

100
75
50
25
0
Activity in supernatant (%)
0
200
400
600
Serum added (μg antiserum·μg antigen^{-1})

Fig. 7

Fig. 6. Immunochemical titration of peroxisomal and mitochondrial carnitine acetyltransferases with anti-peroxisomal carnitine acetyltransferase antiserum.

Peroxisomal (2.75 μg) or mitochondrial carnitine acetyltransferases (1.94 μg) was incubated at 0 °C for 3 h with a various amount of anti-peroxisomal carnitine acetyltransferase antiserum (○, △) or control serum (●, ▲) in 1 ml of 100 mM Tris-HCl buffer (pH 8.0). After removal of the antigen-antibody precipitates by centrifugation, carnitine acetyltransferase activity in the supernatant solutions was determined. (○, ●), Peroxisomal carnitine acetyltransferase; (△, ▲), mitochondrial carnitine acetyltransferase.

Fig. 7. Immunochemical titration of peroxisomal and mitochondrial carnitine acetyltransferases with anti-mitochondrial carnitine acetyltransferase antiserum.

All the experimental conditions except for the antiserum, and the symbols used were the same as those described in Fig. 6.

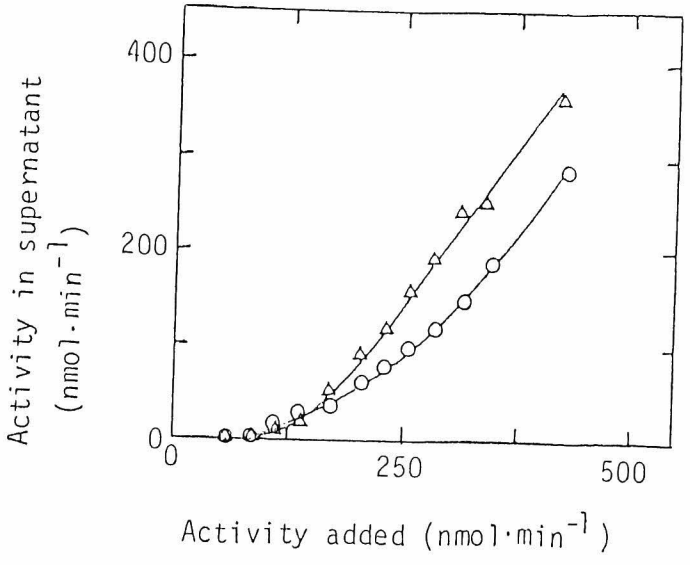

400
200
0
0
250
500
Activity in supernatant
(nmol·min⁻¹)
Activity added (nmol·min⁻¹)

Fig. 8

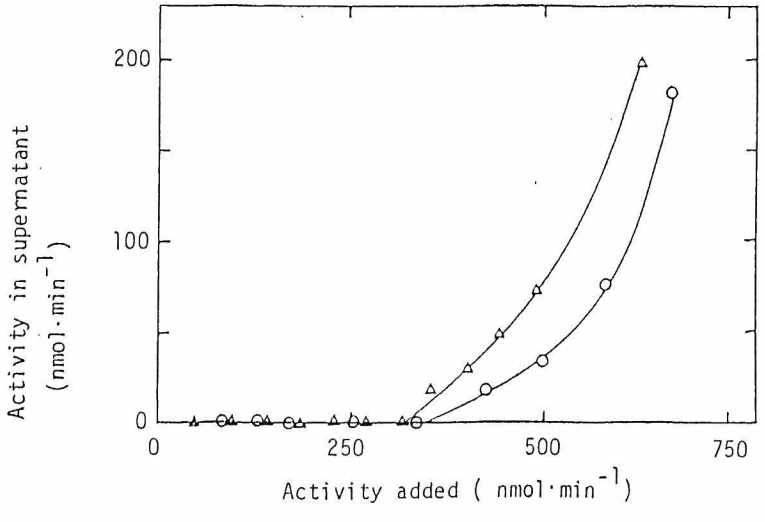

200
100
0
0
250
500
750
Activity in supernatant
(nmol·min⁻¹)
Activity added (nmol·min⁻¹)

Fig. 9

Fig. 8. Immunochemical analysis of peroxisomal and mitochondrial carnitine acetyltransferases with anti-peroxisomal carnitine acetyltransferase antiserum.

The abscissa represents each carnitine acetyltransferase activity before immunochemical reaction. The ordinate represents each enzyme activity remaining in the supernatant solutions after removal of the antigen-antibody precipitates by centrifugation. A constant amount of anti-peroxisomal carnitine acetyltransferase antiserum (235 μg protein) was used. (○), Peroxisomal carnitine acetyltransferase; (△), mitochondrial carnitine acetyltransferase.

Fig. 9. Immunochemical analysis of peroxisomal and mitochondrial carnitine acetyltransferases with anti-mitochondrial carnitine acetyltransferase antiserum.

All the experimental conditions, except that 266 μg of anti-mitochondrial carnitine acetyltransferase antiserum was used, were the same as those described in Fig. 8. (○), Peroxisomal carnitine acetyltransferase; (△), mitochondrial carnitine acetyltransferase.

DISCUSSION

It has been reported that carnitine acetyltransferase is present in peroxisomes and microsomes as well as in mitochondria of mammalian cells,[1-4] and the activity increases in peroxisomes of tumor cells.[17] Although its important role has been suggested in fatty acid metabolism, various problems remain unsolved to elucidate the function of this enzyme. One of those problems of physiological significance is why the enzyme localizes in both peroxisomes and mitochondria. Another question is whether the molecular properties of these distinctly localized enzymes are different or not.

The author has previously reported the existence of carnitine acetyltransferases in both peroxisomal and mitochondrial fractions from alkane-grown yeast cells, and proposed that an "acetylcarnitine shuttle" system between these organelles would play an important role in the complete operation of the glyoxylate cycle localized between peroxisomes and mitochondria in an alkane-grown yeast, Candida tropicalis.[18,19] These two enzymes have been separated on a DEAE-Sephacel column chromatography and purified individually.[8] Detailed studies on the properties of peroxisomal and mitochondrial carnitine acetyltransferases may offer one of the clues to solve the mechanism of biogenesis and development of cellular organelles, especially that of peroxisor

Although carnitine acetyltransferase has been isolated from pigeon breast muscle and rat liver mitochondria, there is a complicating feature in the molecular structure of this enzyme, for example, a single peptide form,[5] a hetero-dimeric form[6] or multi-forms.[20] No information had been available about the isolation and characterization of the enzyme from distinct organelles in the same eukaryotic cells before the author's work[8] (Part II), reporting the isolation of peroxisomal and mitochondrial carnitine acetyltransferases from alkane-grown C. tropicalis cells. For the separation of these two isoenzymes, DEAE-Sephacel column chromatography was found to be very useful.

The enzymes isolated from alkane-grown yeast cells were hetero-oligomers even when purified in the presence of protease inhibitors. Molecular weight of the larger subunits was 64,000 in both cases. Molecular weight of the smaller subunit of the peroxisomal enzyme was 57,000, while that of the mitochondrial enzyme was 52,000. Such difference in the molecular weight is very interesting in relation to the distribution of the respective enzymes into the corresponding subcellular compartments. There might be a possibility that the difference results from an oligosaccharide side chain of the enzymes. However, the absence of an oligosaccharide moiety in the enzyme was confirmed by the reactivity toward the Schiff reagent as well

as the phenol-sulfuric acid reagent, and the absence of amino-sugars was demonstrated by amino acid analysis. These results indicate that peroxisomal and mitochondrial carnitine acetyl-transferases comprise the respective polypeptides of different molecular weight. The molecular weight of each native enzyme was estimated to be about 420,000 by the ultracentrifugal analysis nd the gel filtration chromatography at a high ionic strength. At present, we can not conclude whether this oligomeric form is a real form of the native enzyme in the cells or a result of aggregation. The number of subunits in both enzymes is not clear in vivo.

In spite of the many similarities between two enzymes, such as substrate specificity, optimum pH,[8] heat stability, amino terminus and antigenesity, there exist several differences in the isoelectric point and amino acid composition in addition to the molecular weight of the smaller subunits. The separation of peroxisomal and mitochondrial carnitine acetyltransferases upon DEAE-Sephacel column chromatography might be attributed to the difference in the electrostatic properties of these isoenzymes.

The results of the immunochemical study suggest that carnitine acetyltransferases located in peroxisomes and mitochondria might be originated from a common product of the same nuclear gene. If so, the yeast systems seem to be an excellent tool for investigating where peroxisomal and mitochondrial

carnitine acetyltransferases are biosynthesized, and how the enzymes are sorted and distributed into each organelles. Especially, the difference in the molecular weight of the smaller subunits of each enzyme might correlate to the sorting mechanism of these two isoenzymes. Investigation on the molecular property of nascent polypeptides of carnitine acetyltransferase will be one of the most promising approaches to elucidate a probable post-transcriptional or post-translational modification process required for localization of the enzyme into different subcellular compartments. In the following part, translation of carnitine acetyltransferase mRNA from C. tropicalis in the reticulocyte lysate cell-free translation system will be reported.

SUMMARY

Properties of peroxisomal and mitochondrial carnitine acetyltransferases purified from an alkane-grown yeast, Candida tropicalis, were compared each other. The molecular weight of both enzymes was estimated to be about 420,000 by the analytical ultracentrifugation and the gel filtration chromatography with Sepharose 6B. However, each enzyme gave two subunits on the polyacrylamide slab gel electrophoresis in the presence of sodium dodecylsulfate: the peroxisomal enzyme, 64,000 and

57,000; and the mitochondrial enzyme, 64,000 and 52,000. The subcellularly distinct enzymes gave a similar amino acid composition except for the contents of several amino acids: glycine, valine, glutamic acid and aspartic acid. Their isoelectric point was somewhat different: Both enzymes had the same amino-terminal residue (glutamic acid or glutamine) and the heat stability, and was indistinguishable immunochemically. These results suggest that peroxisomal and mitochondrial carnitine acetyltransferases of C. tropicalis cells may be products of the same nuclear gene. Difference in the molecular weight of the subunits of peroxisomal and mitochondrial carnitine acetyltransferases would result from modification or processing of the common protein in the step of distribution to the respective organelles, — that is, so-called post-translational modification.

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Part V. Synthesis *in vitro* of precursor-type carnitine acetyltransferase with messenger RNA from *Candida tropicalis*

INTRODUCTION

Recently, many reports have been published about the protein secretions across membranes and the protein insertion into membranes. In this connection, studies called "intracellular protein topogenesis"[1] concerning the transport of specific proteins into cellular organelles, such as nuclei, lysosomes, chloroplasts, mitochondria, glyoxysomes or peroxisomes, have been actively carried out.[2] On the study of the biogenesis of cellular organelles, it would be necessary to resolve the mechanism of sorting, distribution and transport of specific proteins into their own subcellular compartments. Since the biogenesis and development of cellular organelles require a highly specific distribution of proteins synthesized outside the organelles, a unique localization of isoenzymes in different organelles could be regarded as the most useful tool for studying the mechanism of protein sorting and transport.

The author has proved that peroxisomal and mitochondrial carnitine acetyltransferases in an alkane-grown yeast, *Candida tropicalis*, constitute an "acetylcarnitine shuttle" system between these organelles[3] and play an important role in the

complete operation of the glyoxylate cycle localized between peroxisomes and mitochondria.[4,5] Peroxisomal and mitochondrial carnitine acetyltransferases isolated from the alkane-grown yeast cells were immunochemically indistinguishable but showed a characteristic difference in the molecular property, especially the molecular weight of subunits.[6] The difference in the molecular property of these isoenzymes was expected to be derived from the modification during sorting and transport. Therefore, it would be very important to study whether or not a precursor-type protein of mature carnitine acetyltransferase is synthesized in the cells.

This part deals with the isolation of mRNA from alkane-grown cells or propionate-grown cells of C. tropicalis, in which carnitine acetyltransferase was induced markedly, and the in vitro synthesis of the nascent carnitine acetyltransferase in the mRNA-dependent reticulocyte lysate translation system. Comparison of the molecular weight between the in vitro translation products and purified peroxisomal and mitochondrial carnitine acetyltransferases was also carried out, the results strongly suggesting the existence of a common precursor of the peroxisomal and mitochondrial enzymes.

MATERIALS AND METHODS

1. Purification and assay of carnitine acetyltransferases and preparation of antibodies

Peroxisomal and mitochondrial carnitine acetyltransferases from *Candida tropicalis* were purified as described in Part II,[7] and their homogeneity was confirmed by polyacrylamide gel electrophoresis, analytical ultracentrifugation, and Ouchterlony double-diffusion analysis. Measurement of enzyme activity and preparation of rabbit antisera against each carnitine acetyltransferase were carried out as described in Part IV.[6] The antisera as well as serum from a nonimmunized rabbit were subjected to ammonium sulfate fractionation[8] followed by DEAE-Sephacel column chromatography[9] to obtain the respective antibodies or control immunoglobulin G. Protein was determined by the method of Lowry *et al.*[10] with bovine serum albumin as standard.

2. Cultivation of yeast and isolation of RNA

C. tropicalis Berkhout strain pK 233 (ATCC 20336) was cultivated aerobically for the indicated period at 30 °C in a medium containing 1.0 % (v/v) alkane mixture (C_{10}- C_{13}) or 1.0 % (w/v) sodium propionate as sole carbon source, as described in Part III.[6,7]

All procedures to isolate RNA from C. tropicalis cells were performed at 0 - 4 °C, unless stated otherwise, under the conditions following the method of Horikawa et al.[11] Yeast cells were harvested by centrifugation at 2200 x g for 5 min, and washed once with an appropriate volume of buffer A (10 mM Tris-HCl buffer, pH 7.5, containing 10 mM Nacl, 10 mM $MgCl_2$, 0.5 mg/ml heparin and 0.1 mg/ml cycloheximide) in the presence of 0.1 % diethylpyrocarbonate. Cells, resuspended in the same solution (twofold of the cell volume), were disrupted with glass beads (d = 0.45 - 0.50 mm, 1 g beads/g wet cells) in a Braun cell homogenizer (Braun, Melsungen, West Germany) for 60 s under cooling with liquid CO_2. The cell homogenate was centrifuged at 12,000 x g for 10 min. The supernatant obtained was diluted with 5 volume of buffer A containing 0.1 % diethylpyrocarbonate, and sodium dodecylsulfate was added to give a final concentration of 1 %. An equal volume of phenol/chloroform/isoamyl alcohol (50/49/1, v/v) was added, and the mixture was shaken at room temperature for 10 min and then centrifuged at 7500 x g for 10 min. The aqueous phase was carefully collected and reextracted four or five times in the same manner until the turbidity of the interface phase disappeared, and 20 % sodium acetate (pH 5.5) was added to the aqueous phase to give a final concentration of 2 %. RNA was allowed to precipitate at -20 °C overnight by adding 2 vol.

ethanol chilled previously at -20 °C. The resulting precipitates were collected by centrifugation at 16,300 x g for 20 min, washed twice with 70 % ethanol and dried under N_2 gas. The pellet was dissolved in distilled water, 4 M sodium acetate buffer (pH 6.0) was added to give a final concentration of 3 M, and the solution was allowed to stand at -20 °C overnight. RNA precipitated was collected by centrifugation at 16,300 x g for 1 h at -20 - -10 °C, washed twice with 70 % ethanol, dried and dissolved again in distilled water. RNA so obtained was used as a source of mRNA. Total RNA preparations with A_{260}/A_{280} value above 1.85 were used for the *in vitro* translation without further purification. RNA concentration was determined by assuming an $A^{1\ cm}_{1\ \%}$ at 260 nm to be 250.[12]

3. Poly(U)-Sepharose chromatography

Poly(A)-containing RNA was prepared by subjecting the RNA obtained to poly(U)-Sepharose chromatography according to the procedures described by Taylor and Tse[13] except that 20 mM Tris-HCl buffer (pH 7.5) was used as a buffer solution.

4. Sucrose density gradient centrifugation

The poly(A)-containing RNA from the poly(U)-Sepharose chromatography steps was fractionated by sucrose density gradient centrifugation. Prior to the centrifugation, the RNA samples

were dissolved in 85 % formamide containing 3 mM Tris-HCl buffer and 3 mM EDTA (pH 7.2) and incubated at 40 °C for 20 min. The mRNA solution (52 μg mRNA/300 μl) was layered on a linear gradient of 5 to 20 % sucrose in 85 % formamide containing the above buffer. Sedimentation was performed at 40,000 rev./min in a Beckman SW 41 rotor at 20 °C for 40 h. Each fraction (0.4 ml) was collected from the bottom. mRNA was collected by precipitation after adjusting gradient fractions to 0.2 M NaCl, addition of 2.5 vol. ethanol, and standing at -70 °C for 10 min. Total Escherichia coli RNA centrifuged in the paralleled tube was used as the size marker of RNA. The size of mRNA was determined by the 2.5 % polyacrylamide slab gel electrophoresis with the marker RNA. Electrophoresis was carried out using the solution containing 100 mM Tris-HCl buffer, 40 mM sodium acetate, 4 mM EDTA and 0.2% sodium dodecylsulfate (pH 7.4) at room temperature for 4 h at 100 V. Xylene cyanol and bromophenol blue served as the tracking dye. The gel was stained with 5 μg/ml ethidium bromide and checked with UV transilluminator.

5. Preparation of the rabbit reticulocyte lysate

The reticulocyte lysate was incubated with micrococcal nuclease at 25 °C for 10 min to reduce endogenous mRNA as described by Pelham and Jackson.[15] The reaction mixture

(total volume, 500 µl) for translation contained 500 µl/ml of nuclease-treated reticulocyte lysate, 20 mM Tris-HCl buffer (pH 7.5), 110 mM KCl, 1 mM magnesium acetate, 2 mM dithiothreitol, 0.02 mM hemin, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 48 µg/ml of creatine kinase, 400 µCi/ml of L-[^{35}S]methionine (760 - 1190 Ci/mmol), 0.1 mM each 19 unlabelled amino acids and specified amounts of RNA. After incubation at 25 °C for 3 h, the reaction was terminated by the addition of an equal volume of a solution containing 10 mM sodium phosphate buffer (pH 7.2), 0.15 M NaCl, 2 % sodium deoxycholate, 2 % Triton X-100, 20 mM unlabelled L-methionine, 50 µg/ml of phenylmethylsulfonyl fluoride, and each 100 µg/ml of leupeptin, antipain, pepstatin A and chymostatin. The diluted sample was centrifuged at 145,000 x g for 1 h, and the resulting supernatant (post-ribosomal supernatant) was collected. An aliquot (5 µl) of the post-ribosomal supernatant was used to determine the radioactivity incorporated into trichloroacetic acid-insoluble material according to the procedure described by Pelham and Jackson.[15]

7. Indirect immunoprecipitation

For immunoprecipitation, 14 µg of antibody against carnitine acetyltransferase (or control immunoglobulin G) was added to 495 µl of the post ribosomal supernatant. After

incubation at 4 °C overnight, 10 mg of Protein A-Sepharose CL-4B, swollen and washed with buffer B (12.8 mM potassium phosphate buffer, pH 7.2, containing 20 mM NaCl, 1 % Triton X-100 and 0.1 % sodium dodecylsulfate) was added, and the mixture was shaken gently ay room temperature for 1 h. After Protein A-Sepharose CL-4B binding the antigen-antibody complex was washed three times with buffer B, the complex was eluted from the adsorbent with buffer C (31.3 mM Tris-HCl buffer, pH 6.8, containing 5 mM EDTA, 10 % glycerol, 5 % 2-mercaptoethanol, 5 % sodium dodecylsulfate and 0.001 % bromophenol blue) by boiling at 100 °C for 3 min.[16] The solution obtained was used for electrophoretic analysis.

8. Gel electrophoretic analysis of the in vitro translation products

Electrophoresis on discontinuous polyacrylamide slab gel in the presence of sodium dodecylsulfate was carried out according to the method of King and Laemmli[17] except that both the stacking and the separating gels contained 0.25 mM EDTA. Acrylamide concentration of 10 % (w/v) was employed. Phosphorylase b, bovine serum albumin, ovalbumin, and lactate dehydrogenase were used as the standard of molecular weight. Fluorography was performed as described by Laskey and Mills.[18]

9. Chemicals

Reagents were obtained as follows: poly(U)-Sepharose CL-4B from Pharmacia (Uppsala, Sweden), sucrose (ribonuclease-free) from Schwarz/Mann (Orangeburg, USA), formamide and lactate dehydrogenase from Wako Pure Chemical (Osaka, Japan), total E. coli RNA from Miles (Elkhalt, USA), phosphorylase b from Boehringer (Mannheim, West Germany), bovine serum albumin and ovalbumin from Sigma (St. Louis, USA), and L-[^{35}S]methionine from Radiochemical Centre (Amersham, England). Other chemicals were also obtained from commercial sources.

RESULTS

1. In vitro translation of carnitine acetyltransferase mRNA

When Candida tropicalis was cultivated on alkanes or propionate as the sole source of carbon and energy, the cellular level of carnitine acetyltransferase was enhanced markedly with growth of the yeast (Fig. 1). mRNA was isolated from the cells harvested at the phase immediately before the maximum enzyme activity, as indicated by arrows in Fig. 1.

mRNA isolated from alkane-grown cells was translated in the mRNA-dependent reticulocyte lysate translation system in the presence of L-[^{35}S]methionine. The total polypeptide products as well as the products isolated by indirect immunoprecipitation

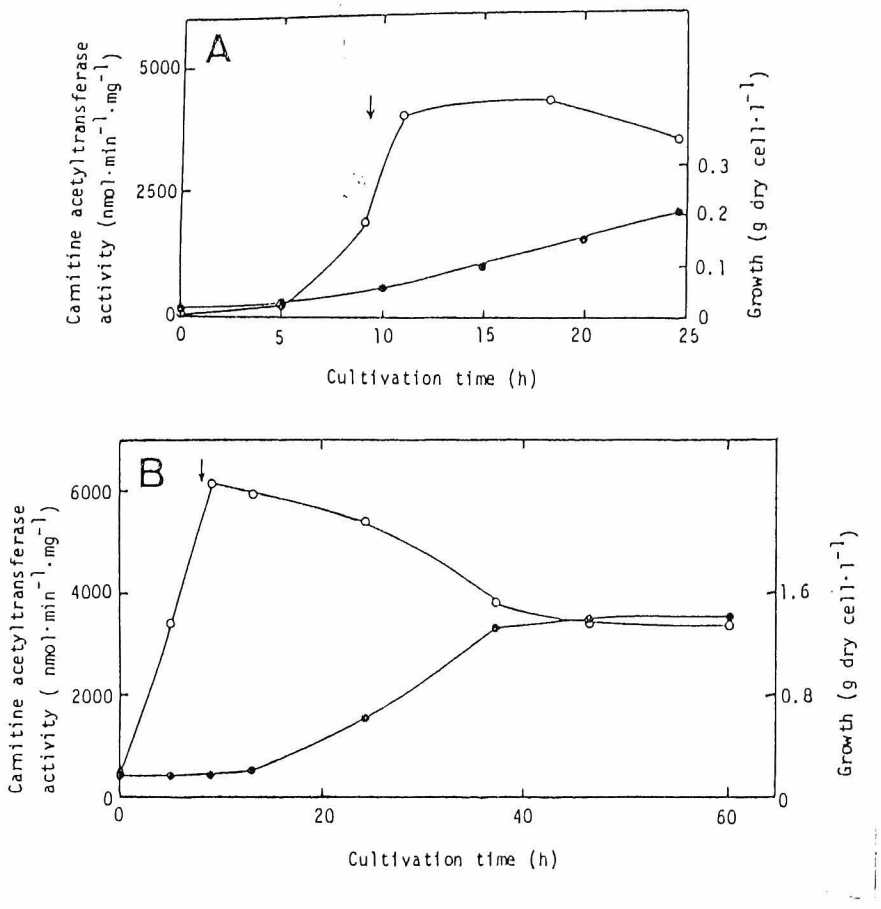


Fig. 1. Time-course change in carnitine acetyltransferase activity of Candida tropicalis.

(A), alkane-utilizing cells; (B), propionate-utilizing cells. (●), Cell growth; (○), carnitine acetyltransferase activity. mRNA was isolated from the cells harvested at the time indicated by arrows.

were analyzed by electrophoresis on a sodium dodecylsulfate/ polyacrylamide slab gel. Anti-(mitochondrial carnitine acetyltransferase) antibody was used in this experiment, because respective antibodies against peroxisomal and mitochondrial carnitine acetyltransferases cross-reacted mutually with each antigen and the antibody against the mitochondrial enzyme had a higher activity as antibody for both enzymes.

As shown in Fig. 2, lane 3, electrophoresis of the immunoprecipitates formed with the antibody revealed several polypeptide bands. To identify the in vitro translation products specific for carnitine acetyltransferase mRNA, competition for the antibody between the in vitro translation products and the authentic enzymes was examined. Addition of an excess amount of the unlabelled authentic enzyme to the translation products prior to immunoprecipitation resulted in the disappearance of the two polypeptide bands indicated by arrows (lanes 3 and 4). The same results were obtained when either purified enzyme was used. The polypeptide bands observed after competition with the authentic enzyme (lane 4) were also detected clearly when the immunoprecipitates obtained with control immunoglobulin G were subjected to electrophoresis (lane 5). These results indicate that the two polypeptide bands shown with arrows (lane 3) represent the in vitro translation products specific for carnitine acetyltransferase mRNA. Similar results were also

obtained when the anti-(peroxisomal carnitine acetyltransferase) antibody was used.

2. Existence of precursor-type of carnitine acetyltransferase

The molecular weights of these two polypeptides were determined using the marker polypeptides shown in Fig. 2, lane 6. The larger polypeptide showed a molecular weight of 71,000 and the smaller one, 57,000. On the other hand, the molecular weight of the purified enzymes was as follows: 64,000 and 57,000 for peroxisomal carnitine acetyltransferase (Fig. 2, lanes 8 and 9); and 64,000 and 52,000 for the mitochondrial enzyme (Fig. 2, lanes 7 and 8). The molecular weight of the larger subunit of the *in vitro* translation product was found to be 7,000 larger than the corresponding subunits of the peroxisomal and mitochondrial enzymes. Difference in the molecular weight between the smaller subunits of the translation product and the mitochondrial enzyme was 5,000, while the molecular weight of the smaller translation product was the same as that of the smaller subunit of the peroxisomal enzyme. These results strongly suggest that the *in vitro* translation product is the common precursor of peroxisomal and mitochondrial carnitine acetyltransferases.

Poly(A)-containig RNA was fractionated by sedimentation in a sucrose gradient, and the RNA fractions were translated

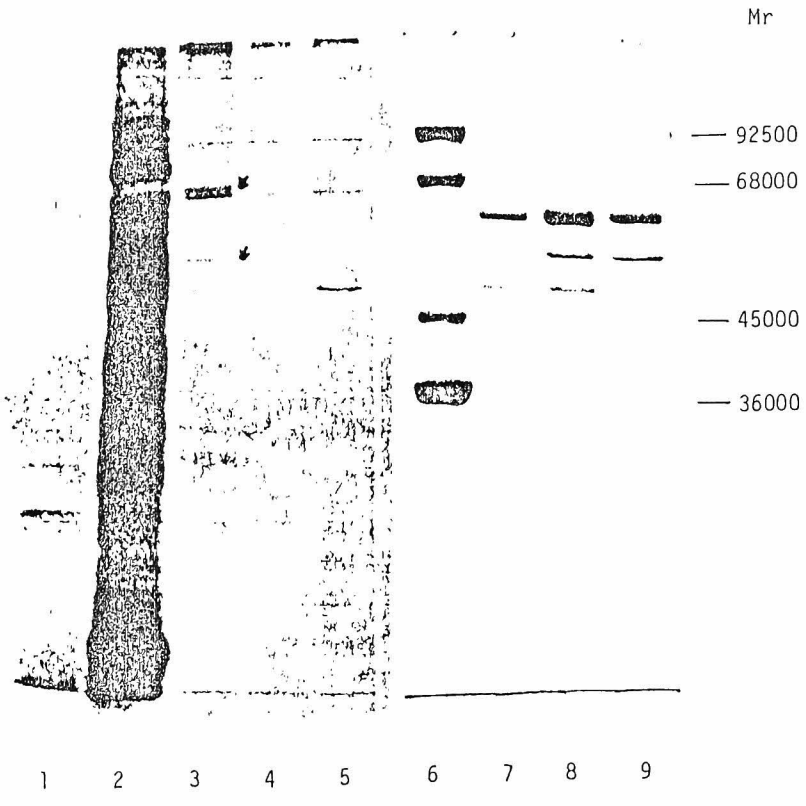


Fig. 2. Sodium dodecylsulfate/polyacrylamide gel electrophoresis of the <u>in vitro</u> translation products of mRNA from alkane-grown cells and purified peroxisomal and mitochondrial carnitine acetyltransferases.

Total RNA (20 µg) from alkane-grown cells was used in 500 µl of the reaction mixture to direct protein synthesis. An aliquot (2.5 µl) of the reaction mixture was applied to electrophoresis without immunoprecipitation (lane 1 and 2), and the rest was subjected to indirect immunoprecipitation (lanes 3 - 5). After electrophoresis, the gel was processed for fluorography (lanes 1 - 5) or stained with 0.1 % Coomassie brilliant blue (lanes 6 - 9).

Lane 1; total translation products formed without yeast RNA.

Lane 2; total translation products.

Lane 3; products immunoprecipitated with antibody against carnitine acetyltransferase (arrows indicate the subunits of carnitine acetyltransferase).

Lane 4; products immunoprecipitated with the antibody in the presence of 300 μg of purified mitochondrial carnitine acetyltransferase.

Lane 5; products immunoprecipitated with control immunoglobulin G.

Lane 6; the marker polypeptides used: Phosphorylase b (Mr 92,500), bovine serum albumin (Mr 68,000), ovalbumin (Mr 45,000) and lactate dehydrogenase (Mr 36,000) from top to bottom of the lane.

Lane 7; purified mitochondrial carnitine acetyltransferase (3.20 μg).

Lane 8; a mixture of purified peroxisomal (3.90 μg) and mitochondrial carnitine acetyltransferases (3.20 μg).

Lane 9; purified peroxisomal carnitine acetyltransferase (3.90 μg).

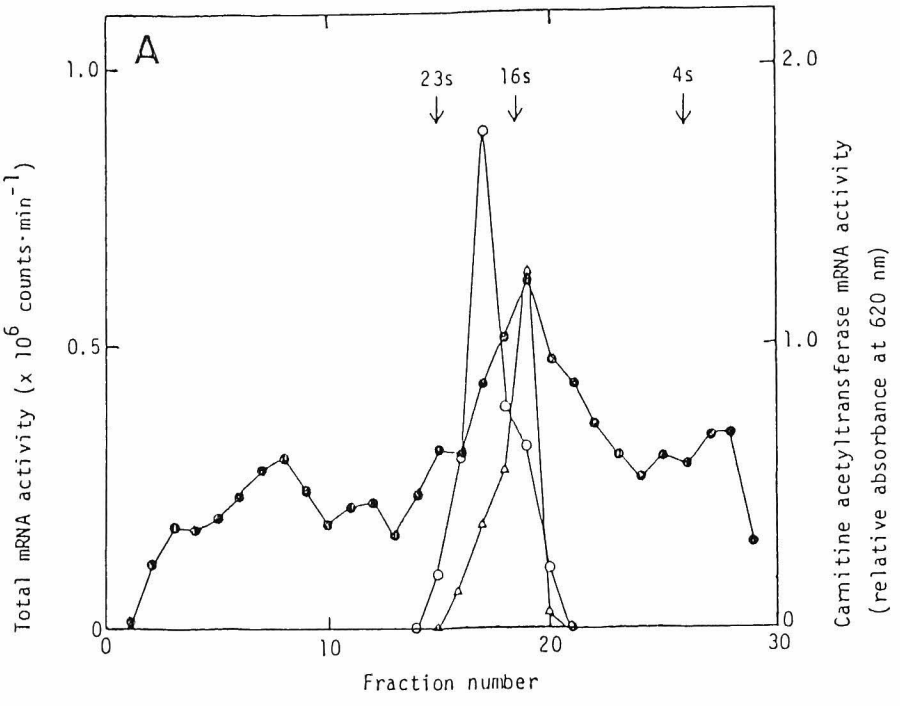
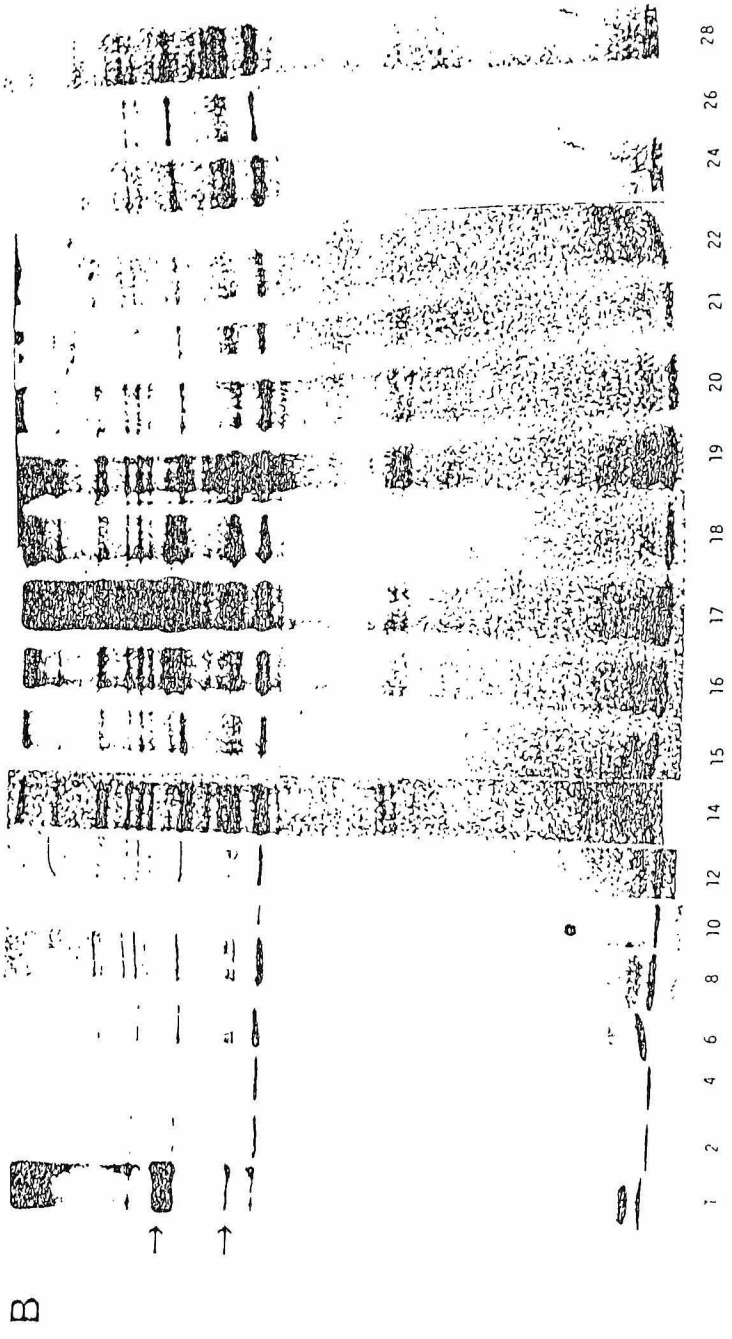


Fig. 3. Sucrose density gradient centrifugation of carnitine acetyltransferase mRNA.

Poly(A)-containing RNA (52 μg) from alkane-grown cells was centrifuged in 11.7 ml of a 5 - 20 % (w/v) linear sucrose gradient in the presence of 85 % formamide. The centrifugation was performed in a Beckman SW-41 rotor (Palo Alto, USA) at 40,000 rev./min and 20 °C for 40 h. Fractions of 0.4 ml were collected from the bottom. RNA from each fraction was concentrated by ethanol precipitation and translated in the in vitro translation system.

(A), Total translation products of each fraction (●). Arrows indicate relative position of standard RNA. The relative activity of carnitine acetyltransferase mRNA was assayed by scanning the fluorogram of electrophoresis of immunoprecipitated samples and measuring the density of radioactivity band of the products with Mr 71,000 (○) and Mr 57,000 (△).


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(B), an aliquot of the L-[^{35}S]methionine-labelled translation products corresponding to 100 µl of the reaction mixture was subjected to indirect immunoprecipitation with the antibody against carnitine acetyltransferase. The immunoprecipitates formed were subjected to electrophoresis on sodium dodecyl/polyacrylamide slab gels and detected by fluorography. Lane T, the products obtained by translation of the total mRNA and indirect immunoprecipitation with antibody against carnitine acetyltransferase. Arrows indicate the subunits of nascent carnitine acetyltransferase. The numbers beneath the lanes corresponded to those of the fractions shown in Fig. 3A.

in the in vitro translation system (Fig. 3A). The L-[^{35}S]methionine-labelled products, immunoprecipitated with the antibody against mitochondrial carnitine acetyltransferase, were subjected to sodium dodecylsulfate/polyacrylamide gel electrophoresis followed by fluorography. Each band ascribable to the corresponding subunit (indicated by arrows in Fig. 3B, lane T) of the precursor polypeptide was observed on the different lanes, although several bands resulting from coprecipitation of nonspecific peptides were observed on the respective lanes (Fig. 3B). mRNA for the larger subunit of carnitine acetyltransferase was detected around Fraction 17, while mRNA for the smaller subunit around Fraction 19. The fact that the respective mRNAs are separable by sucrose density gradient

centrifugation indicates that the smaller subunit is not a degradation product of the larger subunit but the product encoded by different mRNA.

3. Comparison of the *in vitro* translation products of mRNA from alkane-grown cells and propionate-grown cells

mRNA isolated from the propionate-grown cells at the indicated period in Fig. 1 was translated in the mRNA-dependent reticulocyte lysate translation system in the presence of L-[^{35}S]methionine. As shown in Fig. 4, lane 2, the immunoprecipitates formed with the antibody against carnitine acetyltransferase contained two specifically labelled products. In a competition experiment (Fig. 4, lane 1), these two bands, identified as the nascent carnitine acetyltransferase subunits, disappeared. The molecular weight of these two polypeptides was exactly the same as those of the *in vitro* translation products of mRNA from the alkane-grown cells.

These results indicated that mRNA from the propionate-grown cells also directed the synthesis of the precursor-type of carnitine acetyltransferase as did mRNA from the alkane-grown cells. The molecular weights of the nascent and mature carnitine acetyltransferases are summarized in Table 1.

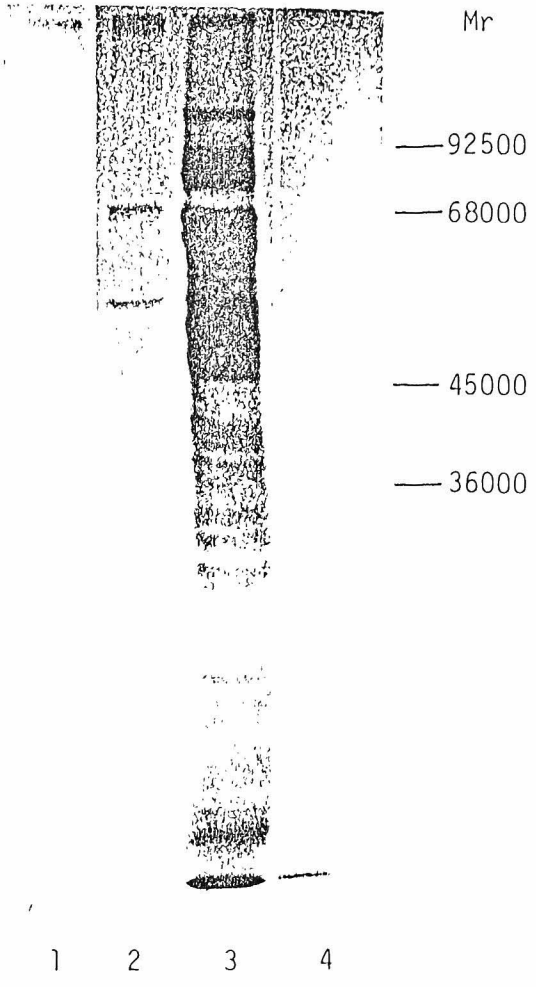


Fig. 4. Fluorogram of sodium dodecylsulfate/polyacrylamide gel electrophoresis of the <u>in vitro</u> translation products of mRNA from propionate-grown cells.

Total RNA (20 µg) from propionate-grown cells was used in 500 µl of the reaction mixture to direct protein synthesis. The procedures were the same as those described in Fig. 2. Lane 1, products immunoprecipitated with antibody against carnitine acetyltransferase in the presence of 300 µg of purified mitochondrial carnitine acetyltransferase; lane 2,

products immunoprecipitated with the antibody; lane 3, total translation products; lane 4, total translation products formed without yeast RNA.

Table 1. Comparison of molecular weights of nascent and mature carnitine acetyltransferase subunits.

	Precursor type	Mature enzyme	
		Peroxisomal	Mitochondri
	(Molecular weight)		
Larger subunit	71,000	64,000	64,000
Smaller subunit	57,000	57,000	52,000

DISCUSSION

Studies on the mechanism of protein secretion across membranes and of protein localization in intracellular organelles have recently been stimulated with the help of *in vitro* translation system with exogenous mRNA.[2] Since the protein localization is directly related to the biogenesis and development of cellular organelles in eukaryotic cells, many studies have been carried out to elucidate the mechanisms of sorting and transport of proteins, such as cytochromes[19] and urea cycle enzymes[20] in mammalian mitochondria, and glyoxylate cycle enzymes in glyoxysomes of *Neurospora crassa* and plant seeds.[21-25] Some mitochondrial proteins that are nuclear gene products and transported post-translationally into the organelles have the respective precursors of higher molecular weight, and these precursors have been suggested to be nascent proteins containing extra peptide chains like signal peptides. The processing from precursor proteins to mature proteins has significant meanings on biogenesis of specific organelles in relation to organization of several enzyme specific for the organelles.

However, it is difficult to study the processing mechanism with mitochodria as a target, because these organelles have a complicated two-membrane system composed of inner-membrane

and outer-membrane. Peroxisomes and glyoxysomes with single bilayer membrane can be regarded as the most suitable organelles for studying the mechanism of localization of specific proteins.

In the case of peroxisomes of mammalian cells, catalase[26] and uricase[27] have been demonstrated to be synthesized by free ribosomes but not by membrane-bound ribosome, and to be transported post-translationally. So far, there have been a few reports suggesting the existence of the precursors of peroxisomal proteins,[28,29] but many problems remain to be solved about the mechanism of the localization of the specific peroxisomal enzyme.

Professor Fukui's group has demonstrated that conspicuous number of peroxisomes (microbodies) appear in Candida tropicalis cells grown on alkanes as the sole source of carbon and energy.[30,31] For studying the mechanism of the localization of the specific peroxisomal enzymes in connection with the development of the organelles, yeast cells are one of the excellent tools, because the development and degradation of peroxisomes as well as the induction and repression of peroxisomal enzymes can be easily controlled only by changing the carbon source for growth.[32] As reported previously,[33] we have carried out in vitro translation of the catalase mRNA from alkane-grown cells of C. tropicalis. The results revealed that the nascent and mature proteins were indistinguishable

in molecular weight, suggesting the enzyme transportation into peroxisomes without the processing of the protein. Existence of peroxisomal and mitochondrial carnitine acetyltransferase isoenzymes, which showed characteristic difference in molecular weight but not in immunochemical property,[6,7] seemed to serve as a clue to elucidate the mechanism of sorting, transport and localization of specific proteins into subcellular organelles.

mRNA from C. tropicalis was translated in the mRNA-dependent in vitro translation system derived from reticulocytes. The in vitro translation products obtained by immunoprecipitation with antibody against carnitine acetyltransferase were found to be composed of two polypeptides with molecular weight (Mr 71,000 and 57,000) larger than those of the corresponding mature polypeptide (Mr 64,000 and 57,000 for peroxisomal carnitine acetyltransferase; Mr 64,000 and 52,000 for mitochondrial carnitine acetyltransferase), i. e., the precursor polypeptides. Both precursors seemed to be common to the respective subunits of peroxisomal and mitochondrial carnitine acetyltransferases. The presence of two kind of the nascent subunits was confirmed by the fact that the in vitro synthesis of these two polypeptides was directed by two different mRNAs isolated from the yeast cells. From analysis of molecular weight, the mechanism of processing of the precursor carnitine acetyltransferase to the respective isoenzymes is supposed to

be characteristic of peroxisomes and mitochondria, respectively. The smaller polypeptide of the *in vitro* translation products seems to be directly related to the sorting and transport mechanisms. Based on the results obtained, a proposed model is shown in Fig. 5.

To acquire information about the biogenesis and development of peroxisomes, it will be necessary to study the molecular properties of isoenzymes localized between peroxisomes and cytoplasm or between peroxisomes and mitochondria. These isoenzymes seem to be a clue to investigate the directed distribution of nascent proteins. The same translation products were obtained with mRNA from the propionate-grown cells as well as the alkane-grown cells, both carbon sources being effective for the induction of carnitine acetyltransferase. The propionate-grown cells are characteristic by a small number of large peroxisomes and a high ratio of mitochondrial-type carnitine acetyltransferase, which are in contrast to the alkane-grown cells having a large number of small peroxisomes and a high ratio of the peroxisomal-type enzyme. These differences may be useful for studying the development of the organelles in relation to the distribution of the enzyme.[32)]

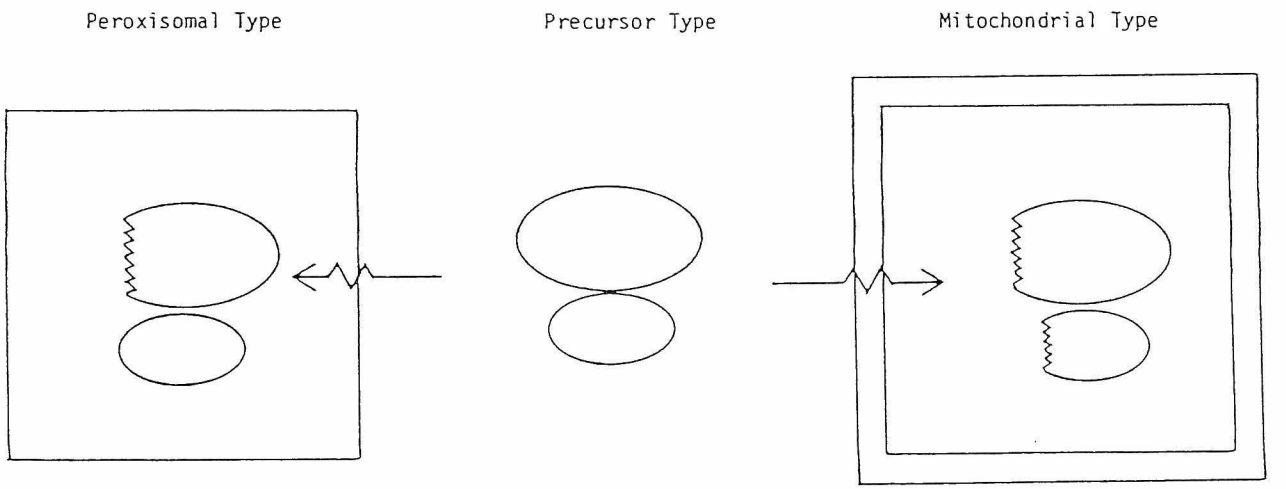


Fig. 5. Proposed model for modification of precursor carnitine acetyltransferase to peroxisomal and mitochondrial enzymes.

SUMMARY

Carnitine acetyltransferase was synthesized *in vitro* in the mRNA-dependent reticulocyte system with mRNA from alkane-grown or propionate-grown cells of *Candida tropicalis*. The protein synthesized *in vitro* was isolated by immunoprecipitation with antibody against peroxisomal or mitochondrial carnitine acetyltransferase and was compared with peroxisomal carnitine acetyltransferase (Mr of subunits, 64,000 and 57,000) and the mitochondrial enzyme (Mr of subunits, 64,000 and 52,000) of *C. tropicalis* by electrophoresis in the presence of sodium dodecylsulfate. Nascent carnitine acetyltransferase prepared *in vitro* showed a hetero-oligomeric property, like the peroxisomal and mitochondrial enzymes isolated from *C. tropicalis*. The molecular weights of the subunits of nascent carnitine acetyltransferase were estimated to be 71,000 and 57,000, indicating the existence of the precursor form of the enzyme. By sucrose density gradient centrifugation of total mRNA, these two subunits proteins were shown to be synthesized with respective mRNAs of different sizes. The same precursor-type of carnitine acetyltransferase was obtained with the mRNAs from the alkane-grown cells and the propionate-grown cells. The results obtained suggest that a common precursor will be post-translationally modified to form the peroxisomal and

mitochondrial enzymes in connection with sorting or distributing into the two distinct subcellular compartments.

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GENERAL CONCLUSION

This study has been attempted to clarify the physiological significance of peroxisomes related to n-alkane assimilation from the aspects of the cooperation among different cellular organelles and to aquire information about the biogenesis and development of peroxisomes in the n-alkane-grown yeast Candida tropicalis.

Carnitine acetyltransferase was induced and localized mainly in peroxisomes and mitochondria in the n-alkane-grown cells, indicating the operation of an "acetylcarnitine shuttle" between peroxisomes and mitochondria.

Peroxisomal and mitochondrial carnitine acetyltransferases could be separated by means of DEAE-Sephacel column chromatography. Such two types of carnitine acetyltransferases were first isolated and purified from a microorganism. Little difference was observed in their kinetic properties and substrate specificity. These two carnitine acetyltransferase preparations were only specific to acetyl and propionyl groups, the final products of peroxisomal β-oxidation of fatty acids with even and odd carbon chains.

The role of carnitine acetyltransferase was investigated under simple conditions where acetate or propionate was the sole source of carbon and energy for growth of the yeast.

The enzyme was induced moderately in acetate-grown cells and extensively in propionate-grown cells. Noticeably, propionate-grown cells contained mainly the mitochondrial-type enzyme which was localized predominantly in cytoplasm. Different from acetate-grown cells which had only a few peroxisomes and n-alkane-grown cells which contained conspicuous number of the organelles, the appearance of the characteristic peroxisomes, considered as immature type of the organelles, was observed electronmicroscopically in propionate-grown cells. These results suggested that carnitine acetyltransferase might be a very useful target enzyme to investigate the biogenesis and development of peroxisomes.

In comparison of molecular properties of peroxisomal and mitochondrial carnitine acetyltransferases purified from the yeast cells, both enzymes had the same amino-terminal residue and the heat stability, and was indistinguishable immunochemically. However, there existed several differences in the isoelectric point (5.11 for the peroxisomal enzyme and 5.22 for the mitochondrial enzyme) and amino acid composition (glycine, valine, glutamic acid and aspartic acid). Moreover, the enzymes composed of two subunits had a significant difference in the molecular weight of subunits: the peroxisomal enzyme, 64,000 and 57,000; and the mitochondrial enzyme, 64,000 and 52,000.

Carnitine acetyltransferase was first synthesized *in vitro* in the mRNA-dependent reticulocyte system with mRNA from *n*-alkane-grown or propionate-grown cells of *Candida tropicalis*. Nascent carnitine acetyltransferase showed a hetero-oligomeric property similar to the peroxisomal and mitochondrial enzymes isolated from *C. tropicalis*. The molecular weight of the subunits was estimated to be 71,000 and 57,000, respectively, indicating the existence of the precursor form of the enzyme. By the sucrose density gradient centrifugation, the respective precursor subunits were clarified to be synthesized by the corresponding mRNAs of different sizes. In addition, the identical precursor-type of carnitine acetyltransferase was obtained with the mRNAs from the *n*-alkane-grown cells and the propionate-grown cells. The presence of a common precursor demonstrated that peroxisomal and mitochondrial carnitine acetyltransferases were post-translationally modified in sorting or distributing into the two distinct subcellular compartments.

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