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Expression of Acetoacetyl-CoA Thiolase Isbzyme Genes of n-Alkane-Assimilating Yeast, *Candida tropicalis:* Isozymes in Two Intracellular Compartments Are Derived from the Same Genes[1]

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In the n-alkane-assimilating yeast *Candida tropicalis,* there are two isozymes of acetoacetyl-CoA thiolase, peroxisomal acetoacetyl-CoA thiolase (peroxisomal Thiolase I), and cytosolic acetoacetyl-CoA thiolase (cytosolic Thiolase I). We have previously isolated two genes *(CT-T1A* and *CT-T1B)* which encode Thiolase I. In order to compare the expressed products of Thiolase I isozyme-encoding genes in *C. tropicalis,* cytosolic Thiolase I was first purified from glucose-grown C. *tropicalis* in which the proliferation of peroxisomes and the expression of peroxisomal Thiolase I were repressed. Cytosolic Thiolase I was virtually identical to peroxisomal Thiolase I in molecular mass, kinetic and immunochemical properties, and primary structure at the N-terminus. Amino acid sequence analysis revealed that cytosolic Thiolase I was the mixture of products of two genes *(CT-T1A* and *CT-T1B),* as in the case of the peroxisomal enzyme. *CT-T1A* and *CT-T1B* were expressed independently in the yeast *Saccharomyces cerevisiae* and the recombinant proteins were purified. Recombinant Thiolase IA and IB exhibited practically identical enzymatic properties to cytosolic and peroxisomal Thiolase Is from *C. tropicalis.* These results revealed that cytosolic Thiolase I and peroxisomal Thiolase I were encoded not by different genes, but by the same genes *(CT-T1A* and *CT-T1B)* and are present as a mixture of products expressed by both genes, although their subcellular localizations are different.

Key words: acetoacetyl-CoA thiolase, *Candida tropicalis,* cytosol, isozyme, peroxisome.

There have been many reports of isozymes localized in distinct compartments of eukaryotic cells. In many cases, two counterparts are encoded by different genes with the information needed for their respective localization. In some cases, two isozymes are encoded by the same gene (reviewed in Ref. *1),* for example, carnitine acetyltransferase of yeast *Candida tropicalis (2).*

Thiolase is a ubiquitous enzyme which catalyzes the thiolytic cleavage of 3-ketoacyl-CoA to acetyl-CoA and acyl-CoA in the final step of the fatty acid y3-oxidation system. It is classified into two types according to substrate specificity. One type is acetoacetyl-CoA thiolase [EC 2.3.1.9], which catalyzes the cleavage of acetoacetyl-CoA and the reverse condensation of acetyl-CoA. The other is 3-ketoacyl-CoA thiolase [EC 2.3.1.16], which has broad substrate specificity for 3-ketoacyl-CoAs in carbon chain length ($^C_4$). It has been reported that thiolases participate in a variety of metabolic pathways as well as y9-oxidation *(3-7),* and that in eukaryotic cells, especially in mammalian cells, thiolases also exhibit diversity in their intracellular localization (6-9) and are encoded by respective genes *(10-14).*

In the n-alkane-assimilating yeast *C. tropicalis* pK233, we have found two thiolase isozymes in peroxisomes (peroxisomal Thiolase I and Thiolase III), which differ in their substrate specificities *(15,16).* Thiolase I corresponds to acetoacetyl-CoA thiolase, and Thiolase **III** to 3-ketoacyl-CoA thiolase. It has been presumed that peroxisomal Thiolase I and Thiolase **III** participate in peroxisomal /J-oxidation because of the exclusive localization of the /3-oxidation system in peroxisomes *(17, 18),* as well as the inductive expression of these peroxisomal isozymes in *n*-alkane-grown cells. This yeast also has cytosolic acetoacetyl-CoA thiolase (cytosolic Thiolase I), which is indistinguishable from peroxisomal Thiolase I in immunochemistry and shows a similar subunit molecular mass to peroxisomal Thiolase I *(19-21).* However, there is a difference in the manner of expression between peroxisomal and cytosolic Thiolase Is. Cytosolic Thiolase I is expressed constitutively during growth on several carbon sources tested, while peroxisomal Thiolase I is expressed inductively *(19).* Therefore, it remains unclear whether they are encoded by the same gene or not.

Peroxisomal Thiolase I is encoded by two genes [Thiolase

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Abbreviations: *CT-T1A, Candida tropicalis* Thiolase IA gene; *CT-TIB, Candida tropicalis* Thiolase IB gene; rThiolase IA, recombinant Thiolase IA; rThiolase IB, recombinant Thiolase IB.

IA and Thiolase IB genes *(CT-T1A* and *CT-T1B)],* and the A and B genes show an extremely high degree of identity to each other (>96%) *(22).* Both *CT-T1A* and *CT-T1B* were shown to be translated in *C. tropicalis. CT-T1A* and *CT-T1B* have been shown to be located on one pair of chromosomes in this diploid yeast (23), and they are thought to be allelic.

In the present study, we purified cytosolic Thiolase I from *C. tropicalis* and recombinant thiolases expressed independently from Thiolase I genes in the yeast *Saccharomyces cereuisiae,* and characterized the enzymatic properties of these proteins. Based on a comparison with previously purified peroxisomal Thiolase I, we discuss the mode of expression of cytosolic and peroxisomal Thiolase I isozymes in *C. tropicalis.*

MATERIALS AND METHODS

Strains and Media—Candida tropicalis pK233 (ATCC 20336) was used as a source of Thiolase I. *Escherichia coli* strain *DK5a [F~, endAl, hsdR17* (r_k~, m_k~), *supE44, thi-1, l~, recAl, gyrA96, AlacU169 (<j>80lacZ4M15)]* was used for gene manipulation. *Saccharomyces cerevisiae* strain MT8-1 *(MATa ade his3 Ieu2 trpl ura3) (24)* was used as the host cell for the expression of each thiolase isozyme gene.

C. tropicalis was cultivated aerobically at 30°C in a medium containing glucose (16.5 g/liter) or n-alkane mixture (Ci_O-Ci_3) (10 ml/liter) as described previously *(25).* Media used for S. *cerevisiae* were as follows: YPD (1% yeast extract, 2% peptone, and 2% glucose); a chemically defined medium (SA-W) (0.67% yeast nitrogen base without amino acids; 2% anhydrous sodium acetate; 0.002% adenine sulfate dihydrate, 0.002% uracil, 0.002% L-histidine monochloride monohydrate, and 0.003% L-leucine).

Construction of Expression Plasmids—Coding regions and their flanking regions of Thiolase I genes were subcloned into pUC19 from clones in A.EMBL3 *(22).* pT16DB containing *CT-T1A* was constructed by insertion of a *Hindlll-BamHl* fragment of A.T16 into pUCl9. pT18DX containing *,CT-T1B* was constructed by insertion of a *Hindlll-Xbal* fragment of AT18 into pUC19. *Hindlll* sites of pT16DB and pT18DX were filled with T4 DNA polymerase and ligated with *BamHI* linker (named pT16B and pT18B). A multicopy shuttle vector pMWl *(26)* carrying *TRP1* as a selection marker was used to introduce and express thiolase isozyme genes in *S. cerevisiae. BamHI* fragments containing *CT-T1A* and *CT-T1B* with their promoter and terminator regions derived from pT16B and pT18B were inserted into the *BamHI* site of pMWl (named pWTIA and pWTIB, respectively, Fig. 2). These recombinant plasmids were introduced into *S. cerevisiae* by electroporation using an EasyJect electroporater (Cosmo Bio, Tokyo) *(27).*

Preparation of Cell-Free Extracts—S. cerevisiae carrying plasmids for expression of thiolase genes were cultivated aerobically at 30°C. After precultivation in 10 ml of YPD medium for 24 h, cells were harvested, and then inoculated in 100 ml of SA-W medium for expression of Thiolase I genes at initial A_{660} of 0.1 and cultivation was carried out at 30°C for 25 h. Cells were harvested and disintegrated by sonication (20 kHz, 2.5 min, 0°C) in PGD buffer [50 mM potassium phosphate buffer (pH 7.2), 10% glycerol, and 1 mM dithiothreitol] containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.05 g/liter of pepstatin A, 0.05 g/liter of leupeptin, 0.05 g/liter of antipain, and 0.05 g/liter of chymostatin). Cell-free extracts were obtained by centrifugation at 20,000 X *g* for 20 min at 0°C.

Purification of Cytosolic Thiolase I and Recombinant Thiolases— C. tropicalis grown on glucose was harvested at mid-exponential growth phase. Protoplasts prepared from glucose-grown cells were homogenized with a Teflon homogenizer and then fractionated by differential centrifugation *(18, 28).* The fractions obtained were as follows: P_2 fraction (20,000 *Xg* pellet), peroxisomes and mitochondria; S_2 fraction (20,000*Xg* supernatant), cytosol and microsomes; S_3 fraction (139,000 Xg supernatant), cytosol; P_3 fraction (139,000Xg pellet), microsomes. S_3 fraction was used as a crude enzyme preparation of cytosolic Thiolase I for further purification.

S. *cerevisiae* carrying each thiolase gene was cultivated aerobically in SA-W medium for 25 h (early stationary growth phase, an optimum phase for expression as tested in this study), harvested, suspended in approximately 30 ml of the PGD buffer containing protease inhibitors (described above), and disintegrated in a Braun cell homogenizer using glass beads *(25).* Cell-free extracts were prepared by centrifugation at 20,000 X *g* for 20 min, followed by ultracentrifugation at 139,000Xg for 1 h. These supernatants were used as crude enzyme preparations.

Purification of cytosolic Thiolase I and recombinant Thiolase IA and Thiolase IB (rThiolase IA and rThiolase IB) was carried out at 0-5°C as described by Kurihara *et al. (16),* using a DEAE-Sepharose CL-6B column (2.5 X 17.5 cm, Pharmacia Fine Chemicals, Uppsala, Sweden), a Butyl-TOYOPEARL 650M column (2.0x15.4 cm, Tosoh, Tokyo), and a Cellulofine GCL-2000m column (2.0x85.5 cm, Seikagaku Kogyo, Tokyo), sequentially. Peroxisomal Thiolase I was purified as described previously *(16).*

Assay of Enzymes and Protein—Thiolase activities were measured using acetoacetyl-CoA (for acetoacetyl-CoA thiolase activity) or 3-ketooctanoyl-CoA (for 3-ketoacyl-CoA thiolase activity) as a substrate *(15, 16).* The activity for the condensation reaction of two molecules of acetyl-CoA was assayed by measuring the oxidation of NADH, coupled with the reduction of the formed acetoacetyl-CoA by 3-hydroxyacyl-CoA dehydrogenase *(29, 30).* The reaction mixture (300 //I) was composed of 100 mM Tris-HCl (pH8.1), 2.4 mM acetyl-CoA, 300 mM NADH, 2.5 U of 3-hydroxyacyl-CoA dehydrogenase from porcine heart and enzyme solution. Protein concentrations were measured with the method by Lowry *et al. (31).*

Electrophoresis and Western Blot Analysis—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a slab-gel (12.5% acrylamide) as described previously *(28).* Phosphorylase *b* (94,000 Da), albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da), and <z-lactalbumin (14,400 Da) were used as molecular size markers. After electrophoresis, gels were stained with Coomassie Brilliant Blue or subjected to Western blot analysis using antiserum against Thiolase I *(15, 16, 28).*

Amino Acid Sequence Analysis and Determination of Native Molecular Mass—After electrophoresis, proteins

were transferred to a sheet of ProBlott (Applied Biosystems, Foster City, CA, USA). The filter after transfer was set in a protein sequencer 610A (Applied Biosystems) and analyzed as recommended by the vendor. Gel filtration to estimate the molecular masses of the native forms of the enzymes was carried out on a Cellulofine GCL-2000m column, using Calibration protein II for chromatography (Boehringer Mannheim, Mannheim, Germany) as size markers.

Enzymes and Chemicals—Restriction enzymes and modifying enzymes for gene manipulation were purchased from Toyobo (Osaka) and Takara Shuzo (Shiga). Acetoacetyl-CoA, acetyl-CoA, NADH, and 3-hydroxyacyl-CoA dehydrogenase were purchased from Sigma (St. Louis, MO, USA). CoA was donated by Kojin (Tokyo). 3-Ketooctanoyl-CoA was prepared enzymatically from octenoyl-CoA synthesized by the mixed anhydride method *(32, 33).* All other chemicals were obtained from commercial sources.

RESULTS

Purification of Cytosolic Thiolase I from Candida tropicalis—The enzymatic properties and primary structure of cytosolic Thiolase I should provide a basis for the comparison of cytosolic Thiolase I and peroxisomal Thiolase I. Cytosolic Thiolase I was purified from the cytosolic fraction of glucose-grown *C. tropicalis*, in which most of the acetoacetyl-CoA thiolase activity exists in this fraction *(19).* The protein was purified by the same procedure as used for peroxisomal Thiolase I (Table I and Pig. 1). The native molecular mass of cytosolic Thiolase I was approximately 250,000 Da, essentially identical to that of peroxisomal Thiolase I (240,000 Da). The subunit molecular mass of cytosolic Thiolase I (41,000 Da) was the same as that of the peroxisomal enzyme (Fig. IB) *(20, 21).* This cytosolic protein was cross-reactive to the antiserum against peroxisomal Thiolase I (Fig. IB) and the activity was immunochemically titrated completely (data not shown). The N-terminal amino acid sequence of this protein was NH_2-(Ala/Thr)-Leu-Pro-Pro-Val-Tyr-Ile-Val-Ser-Thr-Ala-(Xaa)-Thr-Pro-Ile-Gly-(Xaa)-Phe-, which coincides with that of peroxisomal enzyme *(22).* It is noteworthy that both Ala for Thiolase IA and Thr for Thiolase IB were detected as the first amino acid after methionine was processed.

Individual Expression of Thiolase Isozyme Genes in Saccharomyces cerevisiae and Purification of Recombinant Thiolases—Examination of the enzyme characteristics of the proteins from *CT-T1A* and *CT-T1B* is also available as a means to compare cytosolic and peroxisomal Thiolase Is. We expressed these genes independently in *S. cerevisiae.* pWTIA and pWTIB were constructed for the expression of Thiolase I genes with their own promoters (Fig. 2). Cell-free extracts from cells carrying the respective plasmids

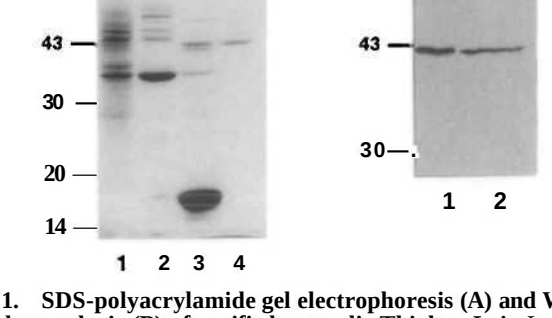

Fig. **1. SDS-polyacrylamide gel electrophoresis (A) and Western blot analysis (B) of purified cytosolic Thiolase I.** A: Lane 1, S_3 fraction (50 *fig);* lane 2, DEAE-Sepharose CL6-B column eluate (25 *us);* lane 3, Butyl-TOYOPEARL 650M column eluate (25,ug); lane 4, Cellulofine GCL-2000m column eluate (5//g). B: Lane 1, cytosolic Thiolase I (0.1 //g); lane 2, peroxisomal Thiolase I (0.1 //g). Antiserum against peroxisomal Thiolase I was used for detection.

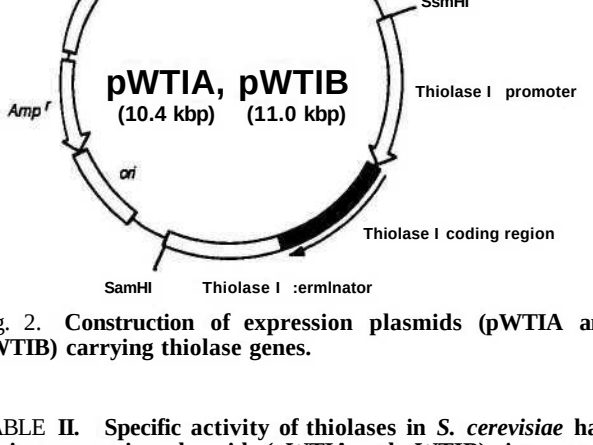

Fig. 2. **Construction of expression plasmids (pWTIA and pWTIB) carrying thiolase genes.**

TABLE **II. Specific activity of thiolases in *S. cerevisiae* harboring expression plasmids (pWTIA and pWTIB).** Acetoacetyl-CoA was used as a substrate. Cells carrying these plasmids were cultivated in SA-W medium. pMWl was a control plasmid.

Plasmid	Specific activity (nmolTnin~'-mg protein"[1])
pMWl	43.5
pWTIA	9,360
pWTIB	10,800

TABLE **I. Purification of cytosolic Thiolase I from glucose-grown *C. tropicalis.*** Thiolase activity was monitored using acetoacetyl-CoA as a substrate.

	Total activity (nmol-min"[1])	Total protein (ing)	Specific activity (nmol-mirr'Tng protein"[1])	Recovery (%)	Purification (-fold)
S_3 fraction	18,400	124	148	100	1
DEAE-Sepharose CL-6B	6,710	9.63	697	36.5	4.71
Butyl-TOYOPEARL 650M	5,300	0.732	7,230	28-8	48.9
Cellulofine GCL-2000m	1,970	0.0329	59,800	10.7	404

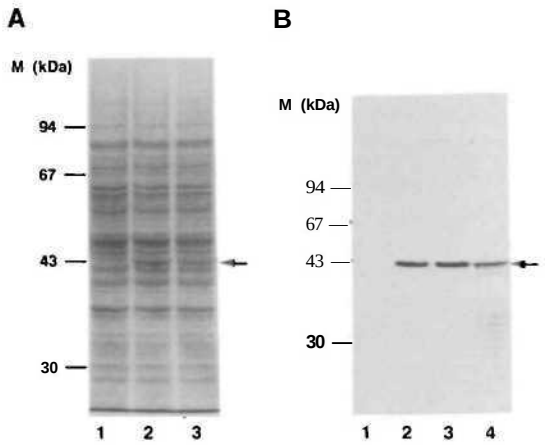

Fig. 3. **SDS-polyacrylamide gel electrophoresis (A) and Western blot analysis (B) of cell-free extracts prepared from S. *cerevisiae* harboring pWTIA and pWTlB.** Cell-free extracts were prepared from acetate-grown S. *cerevisiae* carrying pWTIA (A and B, lane 2), pWTIB (A and B, lane 3), and pMWl as a control (A and B, lane 1). Cell-free extracts (40 //g) were used for protein staining (A), and 80 //g was used for Western blot analysis with antiserum against peroxisomal Thiolase I (B). Cell-free extract of n-alkane-grown C. *tropicalis* was used as a control of Western blot analysis (B, lane 4).

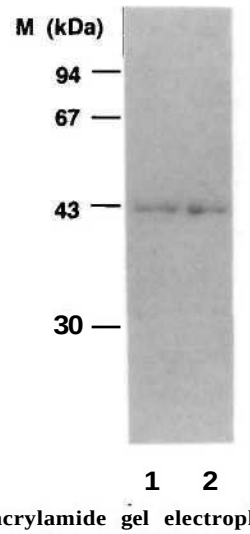

Fig. 4. **SDS-polyacrylamide gel electrophoresis of purified recombinant thiolases.** Lane 1, rThiolase IA; lane 2, rThiolase IB. Purified recombinant thiolase (3 *fig)* was run on each lane.

showed higher activity of the cleavage reaction of acetoacetyl-CoA than those from cells harboring pMWl as a control (Table II). The production of recombinant thiolases under optimum conditions of gene expression was also monitored by protein staining and Western blot analysis (Fig. 3). The subunit molecular mass (Fig. 3) and native molecular mass of rThiolase IA and rThiolase IB were shown to be identical to those of both cytosolic and peroxisomal Thiolase Is from *C. tropicalis.*

Each recombinant thiolase was purified by modified purification procedures as described in "MATERIALS AND METHODS" (Fig. 4). The specific activities of rThiolase IA and rThiolase IB were 23 and 26//mol*min~[l]'mg protein"[1], respectively. The N-terminal amino acid sequence analysis of the purified proteins demonstrated

TABLE **III. Summary of kinetic evaluation of thiolases from *C. tropicalis* (cytosolic Thiolase I and peroxisomal Thiolase I) and recombinant thiolases expressed in 5. *cerevisiae* (rThiolase IA and rThiolase D3).** The concentration of CoA was fixed at 50 ^M in the measurement of *IQ,* values for acetoacetyl-CoA. The concentration of acetoacetyl-CoA was set at 40 ^M in the estimation of K_m values for CoA. 0,-CoA, acetoacetyl-CoA; C_2-CoA, acetyl-CoA. "This value was cited **from** the previous report by Kurihara *et* ***al.*** *(16).*

	Cleavage reaction			Condensation reaction	
	Optimum pH	K_m for		Optimum pH	K_m for C_2 (mM)
		CoA	C4-C0A C«M)		
Cytosolic Thiolase I	8.3	37	42	8.1	0.77
Peroxisomal Thiolase I	8.3	30	25"	8.1	1.05
rThiolase IA	8.3	50	23	8.1	0.69
rThiolase IB	8.3	32	57	8.1	0.74

that the N-termini of rThiolase IA and rThiolase IB were the second amino acids after the first methionine was processed (Ala for rThiolase IA and Thr for rThiolase IB).

Comparison of Kinetic Properties of Purified Thiolase Isozymes—Optimum pH and K_m values were determined in both cleavage and condensation reactions to compare the recombinant thiolases and the thiolases purified from C. *tropicalis* (Table III). rThiolase IA, rThiolase IB, cytosolic Thiolase I, and peroxisomal Thiolase I could equally catalyze the condensation reaction of acetyl-CoA. rThiolase Is and cytosolic Thiolase I did not degrade a longer-chain substrate, 3-ketooctanoyl-CoA, as in the case of peroxisomal Thiolase I *(16).* rThiolase Is, cytosolic Thiolase I, and peroxisomal Thiolase I had the same optimum pH values of 8.3 and 8.1 in the cleavage reaction of acetoacetyl-CoA and in the condensation reaction, respectively. The K_m values for each substrate were virtually identical among rThiolase IA, rThiolase IB, cytosolic Thiolase I, and peroxisomal Thiolase I.

DISCUSSION

As described above, cytosolic Thiolase I and peroxisomal Thiolase I were substantially identical in molecular mass and kinetic and immunochemical properties. Moreover, the primary structure at the N-terminus of cytosolic Thiolase I was that of a mixture of Thiolase IA and Thiolase IB, as in the case of peroxisomal Thiolase I *(22).* We also examined the functional expression of all two thiolase isozyme genes from *C. tropicalis* independently in *S. cerevisiae.* The rThiolase IA and rThiolase IB assembled correctly in *S. cerevisiae* cytosol. These recombinant thiolases exhibited essentially identical kinetic properties with, and the same molecular masses as, cytosolic Thiolase I and peroxisomal Thiolase I purified from *C. tropicalis.* These results represent strong evidence that cytosolic Thiolase I and peroxisomal Thiolase I are encoded by the same genes, *CT-T1A* and *CT-T1B,* and both are present as a mixture of Thiolase IA and Thiolase IB in *C. tropicalis.*

Previous results suggested that cytosolic acetoacetyl-CoA thiolase was identical in glucose-grown and n-alkane-grown cells and that peroxisomal Thiolase I was the sole peroxisomal acetoacetyl-CoA thiolase in n-alkane-grown cells of *C. tropicalis (15, 16, 19, 21).* In the chromatography of the cytosolic fraction of glucose-grown *C. tropicalis,*

no redundant thiolase activity was detectable other than cytosolic Thiolase I (data not shown). This indicates that the same single thiolase protein, cytosolic Thiolase I, is responsible for the acetoacetyl-CoA thiolase activity in the cytosol of both cells. Peroxisomal acetoacetyl-CoA activity can hardly be detected in glucose-grown cells. Therefore, it is suggested that there is no other acetoacetyl-CoA thiolase than peroxisomal and cytosolic Thiolase Is in *C. tropicalis.* Southern blot analysis with *CT-T1A* gene fragment as a probe also indicated that there were no other genes with high similarity to *CT-T1A* and *CT-T1B (22).*

Peroxisomal Thiolase I and cytosolic Thiolase I exhibited essentially identical kinetic properties in both thiolytic cleavage and condensation reaction. Therefore, their physiological functions should be determined mainly by the difference in their subcellular locations. We have previously suggested that the peroxisomal Thiolase I participates in /?- oxidation in the peroxisome and that cytosolic Thiolase I contributes to sterol synthesis. Our present results along with the previous results on peroxisomal Thiolase I *(19)* indicate that protein products from *CT-T1A* and *CT-T1B* are both localized in dual intracellular compartments, cytoplasm and peroxisomes, and rule out the possibility that one gene corresponds to the peroxisomal enzyme, and the other to the cytosolic enzyme. The compartmentalization mechanism of a single gene product into several cellular locations has been studied extensively (reviewed in Ref. *1), e.g.,* for *C. tropicalis* carnitine acetyltransferase *(2).* In many cases, two distinct protein molecules are produced from a single gene, leading to distinct subcellular localization. In the case of Thiolase I, we could not detect any difference between the peroxisomal and cytosolic enzymes. There are cases where inefficient targeting and translocation may be the mechanism by which dual compartmentalization to cytoplasm and peroxisomes is achieved as in the case of rat epoxide hydratase (34). However, Thiolase I must be sorted to two locations in a quantitatively regulated manner, as the amount of peroxisomal Thiolase I is increased in parallel with peroxisome proliferation, whereas that of cytosolic Thiolase I is constant *(19).* Thiolase I has a putative motif of the peroxisome targeting signal type I *(22)* which has been identified at the C-termini of many peroxisomal proteins *(35, 36).* This sequence of Thiolase I may represent a clue to the mechanism of distribution of Thiolase I to two subcellular locations.

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