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Individual Expression of *Candida tropicalis* Peroxisomal and Mitochondrial Carnitine Acetyltransferase-Encoding Genes and Subcellular Localization of the Products in *Saccharomyces cerevisiae*

Hiroyuki Kawachi,* Haruyuki Atomi,* Mitsuyoshi Ueda,* Noriko Hashimoto,† Keiko Kobayashi,† Tomoko Yoshida,† Naomi Kamasawa,† Masako Osumi,† and Atsuo Tanaka*,[1]

*Laboratory of Applied Biological Chemistry, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-01; and †Department of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, Mejiro-dai, Bunkyo-ku, Tokyo 112

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In an *n*-alkane-assimilating yeast, *Candida tropicalis*, carnitine acetyltransferase (CAT; EC 2.3.1.7) was localized in both peroxisomes and mitochondria. Both CATs were encoded by one gene, *CT-CAT*, although the initiation sites of translation were suggested to be different. In the present study, the genes corresponding to the supposed *C. tropicalis* peroxisomal and mitochondrial CATs, which were truncated from the *CT-CAT* gene, were individually expressed in *Saccharomyces cerevisiae*, using the *C. tropicalis* isocitrate lyase promoter (*UPR-ICL*), which is inducible by oleic acid in concert with proliferation of peroxisomes in *S. cerevisiae* [Umemura, K., Atomi, H., Kanai, T., Teranishi, Y., Ueda, M., and Tanaka, A. (1995) *Appl. Microbiol. Biotechnol.* 43, 489-492]. The 71 kDa precursor of mitochondrial CAT, initiating at the first Met, was found to be processed to the mature size (66 kDa) in *S. cerevisiae* and immunoelectronmicroscopical observation revealed that this enzyme was localized in mitochondria. On the other hand, 68 kDa CAT, initiating at the second Met (residue No. 19), had no cleavable signal and was translocated into peroxisomes and cytosol, but not into mitochondria. The amino-terminal amino acid sequences of individually expressed CATs were identical to those of CATs isolated from alkane-grown *C. tropicalis* cells, respectively. These results demonstrated that only the 71 kDa protein yielded the 66 kDa protein and that peroxisomal and mitochondrial CATs arose from the difference in the initiation sites of translation.

Key words: *Candida tropicalis*, carnitine acetyltransferase, initiation of translation, mitochondria, peroxisomes.

Carnitine acetyltransferase (CAT), which catalyzes the reversible transfer of an acetyl group between coenzyme A and carnitine, is localized in two subcellular organelles, peroxisomes and mitochondria, in an *n*-alkane-assimilating yeast, *Candida tropicalis* (*1*). When *C. tropicalis* is grown on *n*-alkanes as the sole carbon source, acetyl-CoA is formed as the final degradation product of the β-oxidation system in peroxisomes. CATs localized in peroxisomes and mitochondria form an "acetylcarnitine shuttle," allowing the transfer of acetyl unit from peroxisomes to mitochondria (*2, 3*).

In our previous paper (*4*), we found that the subunits of mitochondrial CAT of *C. tropicalis* had the molecular mass of 66 kDa, while that of the peroxisomal CAT subunits was 68 kDa. Screening from a λ EMBL genomic DNA library of the yeast led to the isolation of DNA fragments all containing the same open reading frame corresponding to a protein of 71 kDa and showing homology with previously reported CAT genes from other sources. When we introduced the gene under the control of its own promoter into the yeast *Saccharomyces cerevisiae*, an increase in CAT activity was found in the cells, and proteins with sizes of 66 and 68 kDa could be detected by Western blot analysis using the antibody against *C. tropicalis* CAT. This suggested that the two proteins localized in different organelles were the products of a single gene. Furthermore, we separated and purified CATs from peroxisomes and mitochondria of *C. tropicalis* and analyzed the amino-terminal amino acid sequences of each CAT. The amino-terminus of peroxisomal CAT was "Pro-Ile-Leu-Lys" which directly followed the second Met in the open reading frame. That of mitochondrial CAT was "Asp-Leu-Phe-Lys," further downstream in the open reading frame and not adjacent to Met. Based on these results, we proposed that the translation of peroxisomal CAT was initiated at the second Met (residue No. 19) of the open reading frame, and that, in the case of mitochondrial CAT, the initiation of translation was at the first Met, because there was a putative mitochondrial targeting sequence between the first and second Met, an amphiphilic helix, where basic residues form one side, while hydrophobic residues form the other side (*4*).

[1] To whom correspondence should be addressed. Tel: +81-75-753-5524, Fax: +81-75-753-5534

Abbreviations: CAT, carnitine acetyltransferase; ICL, isocitrate lyase; SPT, serine:pyruvate aminotransferase; CT-CAT, *Candida tropicalis* carnitine acetyltransferase.

In the case of *S. cerevisiae* CAT, a single gene encodes both peroxisomal and mitochondrial CATs and alternative initiation sites for transcription have been suggested as responsible for the sorting of CATs to peroxisomes and mitochondria. Polypeptides encoded by the longer transcripts, which contain the amino-terminal extension peptide, are targeted to mitochondria, whereas those encoded by the shorter mRNA, which lack the amino-terminal extension peptide, are targeted to peroxisomes (*5*). But there is no specific information about the amino-terminal amino acid sequences of both CATs.

In this study, we individually expressed *C. tropicalis* peroxisomal and mitochondrial carnitine acetyltransferase-encoding genes in *S. cerevisiae*, followed by Western blot analysis and immunocytochemical study of the subcellular distribution. Based on the results, we discuss the sorting mechanism of peroxisomal and mitochondrial CATs.

MATERIALS AND METHODS

Strains—*S. cerevisiae* MT8-1 (*MATa ade his3 leu2 trp1 ura3*) (*6*) was used to investigate the proliferation of peroxisomes in a medium containing oleic acid (*7*).

Construction of Expression Plasmids pWICM1 and pWICM2—In the previous study (*4*), we isolated the gene *CT-CAT* encoding CATs from the yeast λEMBL library using a CAT cDNA probe (*8*). This *CT-CAT* was used as the template for polymerase chain reaction (PCR). A *Sal*I site at the 5′ end and an *Nco*I site at the 3′ end were introduced for the construction of expression plasmids. M1 fragment, including the first ATG, corresponding to mitochondrial CAT, was amplified with the No. 1 primer (5′-TGTAAGAGAGATCTTCAACCATGTTTAACT-3′) and the No. 2 primer (5′-TTCTCCCATGGACTTACAACTTAGGCTTAG-3′) oligonucleotides. M2 fragment, starting from the second ATG, corresponding to peroxisomal CAT, was made using the No. 3 primer (5′-AATTAGATCTAATCAACCATGCCAATTTTG-3′) and the No. 2 primer. Oligonucleotide primers were synthesized by a Model 381A DNA synthesizer (Applied Biosystems, Foster City, CA, USA). These *Sal*I-*Nco*I fragments, M1 and M2 were subcloned into the *Sal*I-*Nco*I site of pWI3, harboring the ICL promoter gene (*9*) (Fig. 1, A and B). These plasmids, pWICM1 for the expression of mitochondrial CAT and pWICM2 for the expression of peroxisomal CAT, were introduced into *S. cerevisiae* using the electroporation method (*10*).

Cultivation—*S. cerevisiae* was cultivated aerobically at 30°C. After precultivation in YPD medium (1% yeast extract, 1% peptone, and 2% glucose by weight), cells were washed thoroughly with distilled water and transferred to YPO medium (1% yeast extract, 1% peptone, and 0.5% oleic acid by weight).

Preparation of Cell-Free Extracts—Cell-free extracts were prepared by disintegrating the cells (0.1–0.25 g dry cells ml^{-1}) by sonication at 20 kHz for 2.5 min at 0°C in 50 mM potassium phosphate buffer (pH 7.2), followed by centrifugation at 10,000×*g* for 10 min at 4°C.

Subcellular Fractionation—The subcellular fractionation of *S. cerevisiae* cells was carried out by the method described previously with some modifications (*7*): solution A containing 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 50 μg/ml pepstatin A, 50 μg/ml leupeptin, 50 μg/ml antipain, and 50 μg/ml chymostatin (Peptide Institute, Osaka) was used when protoplasts were homogenized. P_2 fraction, 20,000×*g* pellet containing organelles; S_2 fraction, 20,000×*g* supernatant containing cytosol.

Western Blot Analysis—Western blot analysis using anti-*C. tropicalis* CAT antibody was carried out as reported previously (*11, 12*).

Electronmicroscopy and Immunoelectronmicroscopy—For electronmicroscopy, the cells were prefixed with 2.5% glutaraldehyde, and post-fixed with $KMnO_4$ (*13*). For immunocytochemical labeling, yeast cells were fixed with a mixture of 0.5% glutaraldehyde (Nisshin EM, Tokyo) and 3% paraformaldehyde (TA AB Laboratories Equipment, Berkshire, England) in PBS buffer [0.1 M potassium phosphate buffer (pH 7.6) containing 0.8% NaCl]. A gold-particle-labeled goat anti-rabbit IgG was used as the second antibody (*14*). As pWICM1 transformant cells, the cells cultivated for 2 h were used, because poor morphology of mitochondria was observed in the cells cultivated for the same period as the pWICM2 transformant cells (20 h).

Measurement of CAT Activity and Protein—CAT activity and protein were assayed by the methods described previously (*2*).

RESULTS AND DISCUSSION

Expression of CAT Using the Upstream Region of C. tropicalis Isocitrate Lyase Gene (UPR-ICL) in S. cerevisiae—Peroxisomal and mitochondrial CT-CATs have been suggested to be translated from different initiation sites in one chromosomal gene as described in the previous paper (*4*) based on the results of analysis of *CT-CAT* gene and the amino-terminal amino acids of the purified enzymes. Therefore, we constructed plasmids with which we could individually initiate expression from the first and second ATG codons of the *CAT* gene, and then observe the size and the localization of the protein products. We used the isocitrate lyase promoter from *C. tropicalis* (*UPR-ICL*), which induces gene expression in *S. cerevisiae* when the cells are grown on oleic acid (*15, 16*). *S. cerevisiae* MT8-1 bearing pWICM1 starting from the first Met codon or pWICM2 starting from the second Met codon (Fig. 1, A and B) was cultivated in the oleic acid medium to induce CAT synthesis and to proliferate peroxisomes. As shown in Table I, the activity of the cells bearing pWICM1 was 10.8 times higher than that of the cells containing pMW1. Much higher-level induction of CAT was observed in the cells bearing pWICM2, initiating the translation at the second Met (residue No. 19), the amount of recombinant CAT being estimated to be as much as 31% of the total extractable proteins in *S. cerevisiae* from densitometric measurement using a Shimadzu CS-9000 and the enzyme activity in this pWICM2 transformant being 67.2 times higher than that in the pMW1-transformed cells.

Detection of 71 kDa Precursor of Mitochondrial-Type CAT—We suggested previously (*4*) that the translation of mitochondrial CAT was initiated at the first Met, because the protein seemed to be processed after translocation into mitochondria and to have a putative mitochondrial targeting signal located between the first and second Met. If this is so, a 71 kDa precursor would be produced in cytosol and would be processed to the 66 kDa protein found in mito-

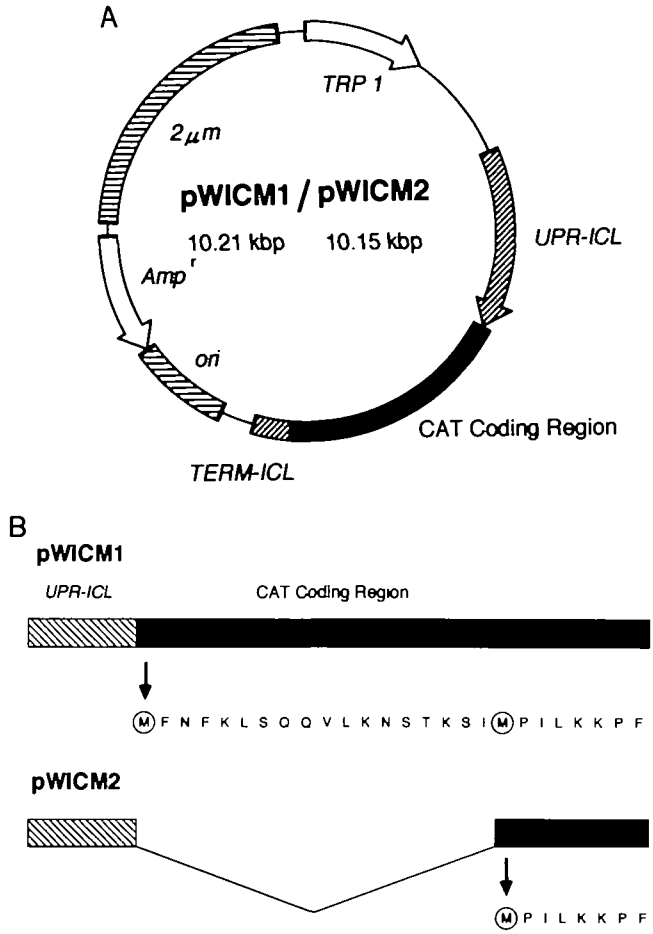

Fig. 1. **Construction of plasmids pWICM1 and pWICM2 (A). The joint sites between *UPR-ICL* and *CAT* gene of pWICM1 and pWICM2 are shown by amino acid residues (B).**

TABLE I. **Activity of CAT in cell-free extracts.**

Plasmid	Specific activity (nmol·min^{-1}·mg^{-1})
pWICM1	56.3
pWICM2	351.0
pMW1	5.22

chondria. By overexpression of the *CAT* gene in pWICM1 followed by Western blot analysis (Fig. 2, lane 2), we were able to detect a 71 kDa precursor protein of CAT, along with the 66 kDa mature CAT protein. This is strong evidence that synthesized 71 kDa precursor of mitochondrial-type CAT, initiated at the first Met, is converted to the mature size (66 kDa) through processing and that the 66 kDa protein is formed by processing of the 71 kDa polypeptide, but is not a processed or degraded product of the 68 kDa polypeptide (peroxisomal CAT) (lane 3). It is interesting that both peroxisomal and mitochondrial CAT proteins appeared when this gene was expressed under the control of the CAT promoter in the previous study (*4*), while the 68 kDa protein was not synthesized under the control of *UPR-ICL*. Therefore, the CAT original promoter seemed to be essential for the simultaneous expression of two types of CATs from one gene, *CT-CAT*. Similar results were also

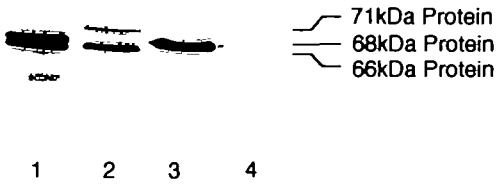

Fig. 2. **Western blot analysis of cell-free extracts (1 μg protein) prepared from *S. cerevisiae* harboring pWICM1 (lane 2), pWICM2 (lane 3), and the control plasmid pMW1 (lane 4), after 20 h cultivation in YPO medium.** *C. tropicalis* CAT was run on lane 1.

reported in the case of *S. cerevisiae* CAT (*5*). In contrast, only the 68 kDa protein was observed in the cells bearing the plasmid pWICM2. There was no cross-reaction of the antibody against CAT of *S. cerevisiae* (lane 4). The amino acid sequences of the amino-terminal regions of these recombinant CATs (66 and 68 kDa proteins) in *S. cerevisiae* analyzed by Edman degradation were identical with those of peroxisomal and mitochondrial CT-CATs in *C. tropicalis*, that is, "Pro-Ile-Leu-Lys-Lys-Pro-Phe-Ser-Thr-Ser" and "Asp-Leu-Phe-Lys-Tyr-Gln-Ser-Gln-Leu-Pro," respectively.

Immunocytochemical Staining of CT-CATs in Transformed S. cerevisiae Cells—In order to investigate the localization of recombinant CT-CATs, electronmicroscopical studies were carried out (Fig. 3). In the pWICM2 transformant cells, the produced CAT protein was detected only in cytosol and peroxisomes, but not in mitochondria (Fig. 3, C and D). Many positive signals observed in cytosol seemed to be due to the overproduction of CAT owing to the use of *UPR-ICL*, as reported (*17*). The results indicated that the peroxisomal targeting signal of *C. tropicalis* CAT seemed to be functional in *S. cerevisiae*. In the case of pWICM1 transformant cells, CAT was localized mostly in mitochondria (Fig. 3, A and B). These results strongly suggested that the 66 kDa CAT protein was localized in mitochondria, after cleavage of a mitochondrial targeting signal of the 71 kDa precursor (Fig. 2, lane 2). We confirmed this by subcellular fractionation of pWICM1 transformant cells (Fig. 4, lane 2). The 71 kDa band seen in Fig. 4, lane 2, seemed to be a precursor CAT before transport into mitochondrial matrix; this view is supported by the signals observed in mitochondrial membranes in Fig. 3B. The 66 kDa CAT protein was actually detected only in the P_2 fraction containing organelles (mitochondria) and not in the S_2 fraction (cytosol).

In the case of rat serine:pyruvate aminotransferase (SPT), when a cDNA encoding the precursor of mitochondrial SPT was expressed in COS cells, SPT was present predominantly in mitochondria, but small numbers of peroxisomes were also positively stained (*18, 19*), owing to leaky scanning by the 40 s ribosomal subunit (*20*). However, we could not detect leaky scanning in the cells bearing the plasmid pWICM1. This is also the case in *S. cerevisiae* CAT that was found to be localized only in mitochondria by subcellular fractionation, when the gene starting from the first ATG codon was expressed under the control of the *S. cerevisiae* catalase A promoter (*5*).

Based on the results obtained, we can conclude that

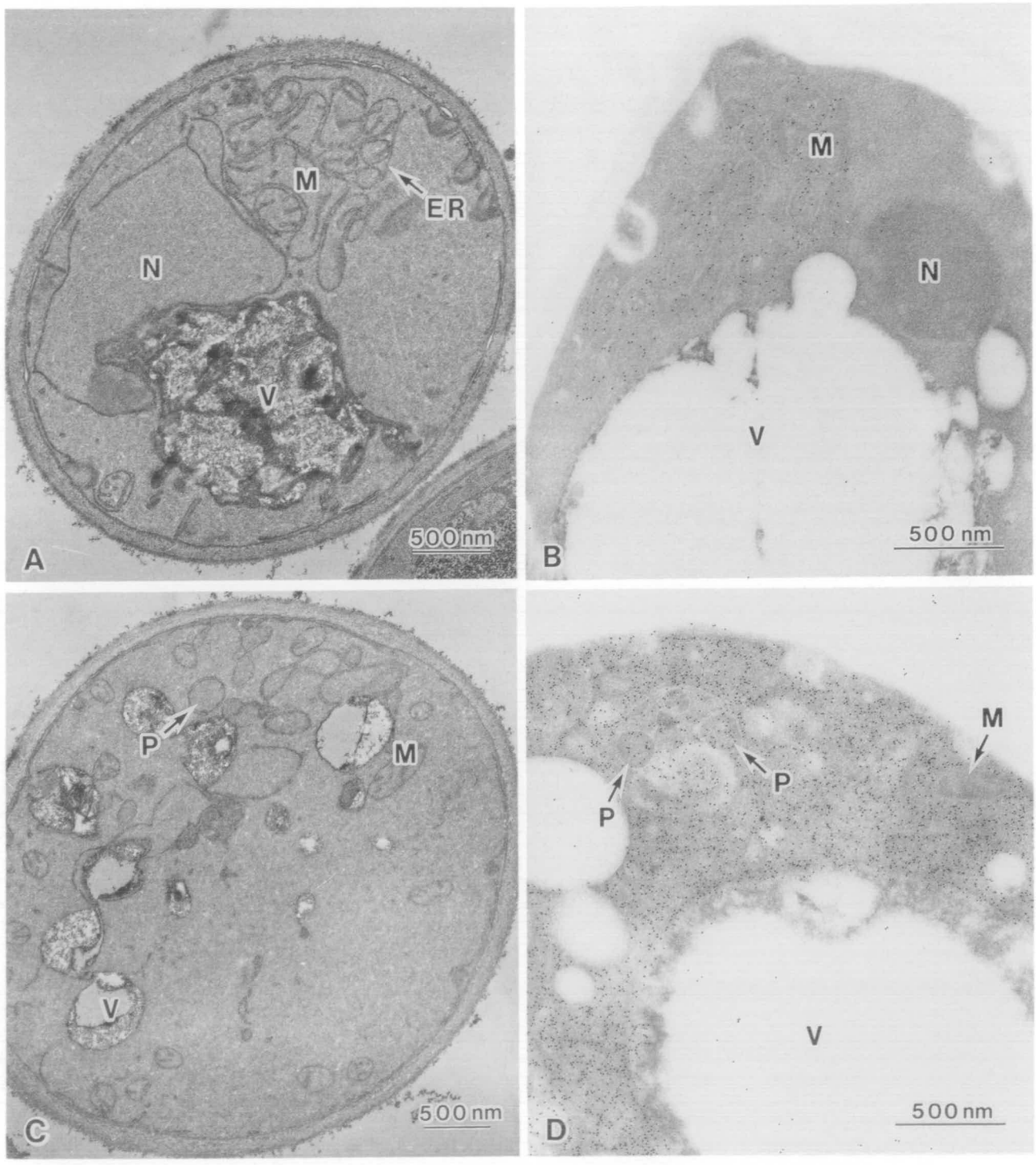

Fig. 3. **Electronmicrographs of whole cells fixed with KMnO$_4$ (A, C) and immunoelectronmicrographs (B, D) with anti-*C. tropicalis* CAT antibody in *S. cerevisiae* MT8-1 harboring pWICM1 (A, B) and pWICM2 (C, D), respectively.** M, Mitochondrion; N, nucleus; P, peroxisome; V, vacuole; ER, endoplasmic reticulum.

peroxisomal and mitochondrial CATs arise from the different initiation of translation on a single gene, *CT-CAT*. When 71 kDa CAT, initiated at the first Met, was expressed, CAT immunoreactivity was observed in mitochondria. Moreover, the amino-terminal mitochondrial signal must be removed from the precursor after the translocation into mitochondria (Fig. 5). Only the 71 kDa protein gave rise to 66 kDa protein. On the other hand, CAT was found to be localized in peroxisomes and cytosol, but not in mitochondria and vacuoles, when 68 kDa CAT, initiated at the second Met (residue No. 19), was expressed.

In *C. tropicalis* CAT, *S. cerevisiae* CAT, and rat SPT, the mitochondrial proteins have a putative peroxisomal targeting signal in addition to the mitochondrial targeting signal.

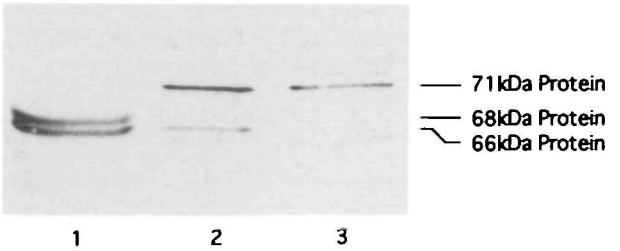

Fig. 4. **Western blot analysis of P_2 fraction (3 μl) (lane 2) and S_2 fraction (13.6 μl) (lane 3) prepared from *S. cerevisiae* harboring pWICM1, after 2 h cultivation in YPO medium.** *C. tropicalis* CAT was run on lane 1.

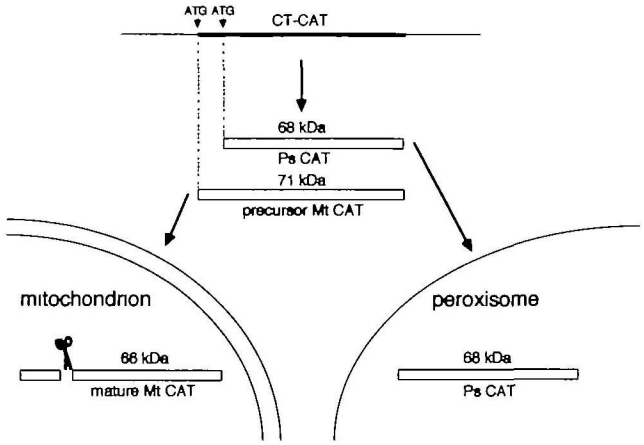

Fig. 5. **Model for the generation and sorting of peroxisomal and mitochondrial CATs.** Mt, Mitochondrial; Ps, peroxisomal.

Since the 68 kDa CAT protein starting from the second Met localizes to peroxisomes, the targeting signal must be present in this protein. The mitochondrial targeting signal located at the N-terminus of the protein seems not only to direct protein transportation to mitochondria, but also to abolish the effect of the peroxisomal targeting signal which still should be present in the protein molecule. It would be interesting to clarify how the mitochondrial targeting signal completely overrides the peroxisomal targeting signal.

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