Cloning and Sequencing of a cDNA Encoding \textit{Saccharomyces cerevisiae} Carnitine Acetyltransferase

USE OF THE cDNA IN GENE DISRUPTION STUDIES*

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cDNA encoding for carnitine acetyltransferase (CAT) of yeast \textit{S. cerevisiae} was isolated by screening a yeast cDNA Agt11 library with antibody. The whole coding sequence was obtained from the cDNA and from a YEP 15 DNA clone identified using the cDNA as probe. The coding sequence consists of 670 residues, which amount to a molecular mass of 77,300 kDa. This cDNA was used successfully to disrupt the gene for the mitochondrial isoenzyme of CAT, which was shown by measuring the enzyme activity and by immunoblot. The acetylcarnitine content of these cells decreased significantly. A search in the PIR protein data base revealed that besides the known carnitine acyltransferases, choline acyltransferases are highly homologous to yeast CAT. The mitochondrial CAT-deficient (CAT\(^-\)) cells were able to grow on different fermentable and nonfermentable carbon sources, even on acetate at the same rate as the parental strain. In contrast to these, \(\text{\textsuperscript{13}}\text{C}\) NMR studies revealed significant differences between parental and CAT\(^-\) cells. In CAT\(^-\) cells \(\text{[3-\text{\textsuperscript{13}}\text{C}]pyruvate\)} was converted mainly to lactate and acetate, whereas in the parental cells alanine and tricarboxylic acid cycle intermediates were found as the main products of pyruvate metabolism beside acetate. These results suggest diminished flux through the pyruvate dehydrogenase complex in the absence of mitochondrial CAT in yeast cells.

Carnitine acetyltransferase belongs to that group of enzymes which catalyzes the reversible acylation of \(L\)-carnitine (1). Members of this group differ from each other in chain length specificity. Carnitine palmitoyltransfersases are specific for long chain fatty acids and participate in the transport of long chain acyl-CoA derivatives across the mitochondrial inner membrane to the site of \(\beta\)-oxidation (2). Medium chain fatty acids are transferred from CoA to carnitine by carnitine octanoyltransferase. This enzyme facilitates the transport of medium chain fatty acids produced by the peroxisomal chain shortening system to the mitochondrial matrix (3). Carnitine acetyltransferase (CAT\(^1\)) is specific for short chain fatty acids and has the highest activity with acetyl moiety (4). CAT activity has been detected in most of the examined eukaryotic cells including different yeast strains, plants, and mammals. Brown adipose tissue and heart muscle contain the highest CAT activity in mammalian organisms, but even the brain has significant amount of this enzyme (5).

The function of CAT has not been described in as great detail as that of carnitine palmitoyltransferase or carnitine octanoyltransferase. In mammalian cells CAT is supposed to buffer against rapid changes in acetyl-CoA, thus preventing the depletion of free CoA; on the other hand the product of this enzyme, the acetylcarnitine, can serve as an easily accessible acetyl pool (6). CAT may participate in the elimination of xenobiotic fatty acids as well, since CAT can transfer short, branched chain fatty acid to carnitine, thus promoting their urinary excretion (7).

The metabolic significance of this enzyme was best demonstrated by the discovery of a patient with ataxic encephalopathy, in whom the activity of CAT was markedly decreased (8).

In lower eukaryotes, the transport of the acetyl moiety in or out of the mitochondria has been considered as a possible function for CAT. In the alkane growing yeast, \textit{Candida tropicalis} acetyl-CoA forms in the cytosol, so acetyl group can be transported to the mitochondria using the carnitine shuttle system (9).

In the carnitine-deficient yeast, \textit{Torulopsis bouina}, carnitine requirement could be demonstrated even under anaerobic conditions. Since in this organism CAT is the only known carnitine-specific enzyme a biosynthetic role of CAT can be suggested in addition to its function in the aerobic acetate metabolism (10).

We decided to study the function of CAT in the yeast \textit{Saccharomyces cerevisiae} because in this organism mutations can be introduced to specific sites, thus the role of an enzyme can be examined under \textit{in vivo} conditions by comparing the mutant and parental cells (11). The physiological significance of the supposed CAT functions in buffering the acetyl-CoA level or in acetate transport can be determined this way. Moreover, this type of study frequently reveals new, unexpected functions of an enzyme.

We have already carried out the first steps leading to the isolation of the gene encoding for \textit{S. cerevisiae} CAT (YCAT), necessary to generate disruption of mutant yeast cells (12).

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\(\text{\textsuperscript{1}}\) The abbreviations used are: CAT, carnitine acetyltransferase; YCAT, yeast carnitine acetyltransferase; kb, kilobase(s); bp, base pair(s).
CAT was isolated from baker's yeast with a subunit molecular weight of approximately 65,000 as determined by SDS-polyacrylamide gel electrophoresis. Antibodies were raised against this protein in rabbit, which were specific and sensitive enough to be used in screening a λgt11 yeast cDNA library.

The identification and sequencing of cDNA and nuclear DNA encoding for YCAT and the phenotype of CAT- cells are described in this report.

MATERIALS AND METHODS

The yeast strains used were: PSY 142; Mat a, ura 3-52, leu 2-2, ade2-12, lys 2-801, MMY 63; Mat a, ade2-1, leu 2-3, ade2-12, his 3-11, his 3-15, trp 1-1, ura 3-5. Cells were grown at 30 °C in complete medium (2% peptone, 1% yeast extract, 0.5% glucose, 0.1% yeast nitrogen base without amino acids, 2% agar).

Transformation of yeast cells was done as described by Ito et al. (1983).

Plasmid Vector.

Plasmids from the positive clones were isolated under the conditions suggested by the suppliers. Digested DNA samples were subcloned in the plasmid vectors pBR322 or pUC18. Restriction enzyme mapping. We found the characteristic restriction sites on all of these clones, which proved the identity of the inserts. The inserts were liberated and their size determined. The longest insert, 2.2 kb, was subcloned in pUC18 plasmid vector and analyzed further. Restriction enzyme mapping allowed us to subclone overlapping fragments of this cDNA to M13 phage vector for sequencing.

Sequencing the 2,125-bp insert revealed a long open reading frame from 1 to 1994 bases and a 118-bp untranslated region followed by the poly(A) tail. To find the translation initiation codon we had to isolate the nuclear DNA encoding for YCAT, since the cDNA was not long enough to ascertain the presence of initiation codon at the 5' end.

We used a YEP 13 nuclear DNA plasmid library, and the 5' EcoRI-BamHI fragment of the cDNA (see Fig. 1A) was used as a probe. Eight 10^6 colonies were examined from which seven positives were isolated and subjected to restriction enzyme mapping. We found the characteristic restriction sites on all of these clones, which proved the identity of the isolated nuclear DNAs (Fig. 1B). One of these nuclear DNAs was sequenced from the unique PvuII site in the upstream direction. No intron in this DNA segment was found. This sequence revealed complete identity with the 5'-300-bp sequence of cDNA in the overlapping region. Moreover, a new translation initiation codon was found 13 bp upstream from the 5' end of the cDNA preceded by a long repeat of TA.

The full-length open reading frame encodes a polypeptide of 670 amino acids with a molecular size of 77.3 kDa. We searched the PIR protein data base (release 28.0) for homologous proteins using the FASTA program (22). The highly homologous proteins were the rat carnitine octanoyltransferase and rat and human carnitine palmityltransferase (23, 24). Surprisingly, choline acetyltransferase (pig and Drosophila) (25, 26) showed high amino acid sequence homology to YCAT, as well as all over the protein sequence, not only in the already known LPXLPXPL (24) motif. Besides this motif we found four others that are conserved in choline and carnitine acetyltransferases, as well, suggesting an important role.
for these amino acid segments (Fig. 3). These conserved regions are found in the second half of the protein sequences.

Fig. 3. Alignment of highly homologous regions present in choline and carnitine acyltransferases. RCOT, rat carnitine octanoyltransferase; HCPT, human carnitine palmitoyltransferase II; RCPT, rat carnitine palmitoyltransferase; PCLAT, pig choline acetyltransferase. Boxes indicate sequence identity; homologous amino acids are shown by a one-letter code, and nonhomologous amino acids are substituted by X. Numbers show the positions of selected peptide fragments in the respective protein.

Fig. 4. Southern blotting analysis of PSY 103 and MY 03 parental and CAT− nuclear DNA. Total nuclear DNA from yeast cells was digested with BamHI, separated on 1% agarose, and blotted to Hybond nylon membrane. Lane 1, PSY 103 parental; lane 2, PSY 142 CAT−; lane 3, MY 03 parental; lane 4, MY 03 CAT+. Probes were the random prime-labeled Ura− DNA from parental and CAT− isolates (Fig. 3). Hybridization and washing conditions were done at very low stringency conditions (2 × SSC, 0.1% SDS at 42°C) the simple YCAT cDNA hybridization pattern suggested that this Cat gene is isolated by us is present only in one copy per haploid yeast genome. CAT enzyme activity was measured in the whole cell lysate, isolated mitochondria, and in the mitochondrial supernatant (cytosol) of the transformed cells the enzyme activity decreased to less than 5% of the parental value. An even more pronounced decrease in the enzyme activity was observed in the mitochondrial supernatant. Since the genome of the CAT− isolate was derived from the wild-type isolate by a single-site transposition into the Cat locus of yeast chromosome, the Cat− isolate is not isogenic to the parental isolate.

Fig. 5. Alignment of highly homologous regions present in choline and carnitine acyltransferases. RCOT, rat carnitine octanoyltransferase; HCPT, human carnitine palmitoyltransferase II; RCPT, rat carnitine palmitoyltransferase; PCLAT, pig choline acetyltransferase. Boxes indicate sequence identity; homologous amino acids are shown by a one-letter code, and nonhomologous amino acids are substituted by X. Numbers show the positions of selected peptide fragments in the respective protein.
CAT activity was observed in the isolated CAT mitochondria; however, in the cytosol we found no change in the enzyme activity (Table I). The low residual CAT activity in the mitochondria of CAT cells may be contamination from the cytosol. Immunoblot analysis of the mitochondrial and cytosol fractions of parental and CAT PSY 142 cells revealed that our anti-CAT antibody recognizes only the mitochondrial CAT; no immunoreactive material was found in the cytosol of parental or CAT cells. Moreover, no CAT protein was found in mitochondria of CAT cells (Fig. 5). Free carnitine and short chain acyl-soluble carnitine esters were measured in the perchloric acid extract of parental and CAT cells (Table II). In CAT cells without any significant change in the free carnitine level, the short chain carnitine ester concentration decreased below the detection limit of our radioactive carnitine assay. This result shows that after the elimination of CAT from the mitochondria no other major carnitine acyltransferase activity remained in the yeast cells.

The phenotype of CAT cells was studied by comparing the growth of mutant cells with the parental strain on different carbon sources. CAT cells were able to grow on complete or minimal medium plates containing fermentable or nonfermentable carbon sources such as glycerol, lactate, pyruvate, and acetate (data not shown). No difference was found in the time required for the colonies to reach the visible size, in the logarithmic growth rate, or in the yield of growth between the parental and CAT cells.

The pyruvate metabolism of mutant cells was investigated using NMR spectroscopy after labeling the metabolite pool of complete medium/galactose grown cells with [3-13C]pyruvate. The NMR spectrum of the perchloric acid extract obtained from the labeled parental and CAT cells is shown in Fig. 6. This experiment revealed a striking difference in the metabolism of pyruvate between parental and CAT cells. In the parental cells a significant amount of 13C label still remained in pyruvate. A high amount of added label was found in acetate, alanine, and as expected in the citric acid cycle intermediates, glutamate and malate, and in the oxaloacetate derivative, aspartate. A signal in the lactate peak could not be detected. In CAT cells a very high signal intensity was found in the lactate peak in addition to acetate. Alanine was labeled much less compared with the acetate peak, and much less pyruvate was converted to the citric acid cycle intermediate in these cells. The decreased 13C incorporation into citric acid cycle intermediates is obvious if the acetate C2 and glutamate C4 or even when alanine C3 and glutamate C4 are found in the lactate peak in addition to acetate. Alanine was labeled much less compared with the acetate peak, and much less pyruvate was converted to the citric acid cycle intermediate in these cells. The decreased 13C incorporation into citric acid cycle intermediates is obvious if the acetate C2 and glutamate C4 or even when alanine C3 and glutamate C4 are compared. Comparison of glutamate C3 and C4 peak intensities can be used as a rough estimation for the turnover rate of the citric acid cycle assuming identical pool size, because as pyruvate enters the cycle through acetyl-CoA, label appears first in glutamate at C4 and then scrambling in the second part of the cycle transfers the label from C4 to the position

### Table I

| Cat activities in total homogenate and subcellular fractions of PSY 142 parental and CAT cells |
|-------------------------------------------------|
| Cells were grown in complete medium containing 2% galactose to midlog phase. Total cell homogenate was prepared by disrupting cells with glass beads. Mitochondria and cytosol were separated after lyticase treatment as described by Daum et al. (19). CAT activity in these samples was measured using the 5,5′-dithiobis(nitrobenzoic acid) assay (4). Milliunits/mg protein means nmol of substrate converted/min/mg of protein at 25 °C. Values represent means ± S.D. for three separate cultures. |
| | Total homogenate | Mitochondria | Cytosol |
| Parental | 250 ± 10 | 550 ± 10 | 30 ± 5 |
| CAT | 12 ± 3 | 3 ± 2 | 30 ± 7 |

### Table II

| Carnitine and acetylcarnitine concentration in PSY 142 parental and CAT cells |
|-------------------------------------------------|
| Perchloric acid extract was prepared from cells growing in complete medium/galactose. Carnitine and acetylcarnitine determinations were carried out as described in (21). Values represent means of two determinations. |
| **Concentration** | **Carnitine** | **Acetylcarnitine** |
|-------------------|---------------|-------------------|
| Parental | 73.5 | 43.5 |
| CAT | 63.8 | 0.0 |

**Fig. 5. Immunoblot analysis of PSY 142 parental and CAT cells.** Complete medium/galactose-grown cells were subjected to cell fractionation as described by Daum et al. (19) and analyzed on SDS-polyacrylamide gel electrophoresis. Lanes 1 and 2 are parental mitochondria and cytosol. Lanes 3 and 4 are CAT mitochondria and cytosol, respectively. Separated proteins were transferred to nitrocellulose membrane and were immunodecorated by antiserum raised against yeast CAT in rabbit. The migration position of pure YCAT is shown by an arrow.

**Fig. 6. Pyruvate metabolism of PSY 142 parental and CAT cells.** Cells were grown in complete medium/galactose to midlog phase and then transferred to [3-13C]pyruvate-containing medium. After a 30-min incubation at 30 °C perchloric acid extract was prepared from the cells and subjected to NMR spectroscopy. Panel A, parental; panel B, CAT cells. The assigned peaks were as follows: 1, Glu C2; 2, Asp C2; 3, malate C3; 4, Asp C3; 5, Glu C4; 6, Glu C5; 7, pyruvate C3; 8, acetate C2; 9, lactate C3; 10, Ala C3.
of C3 and C2. The C3/C4 ratio was 0.50 and 0.45 in parental and CAT− cells, respectively, suggesting that the turnover rate of the citric acid cycle is not affected by the absence of CAT.

DISCUSSION

To begin to investigate the function of CAT we set out to determine the primary structure of this enzyme. We chose this protein because despite the fact that it is present with high activity and amount in eukaryotic cells no clear-cut in vivo function could be assigned to it.

We decided to isolate the cDNA encoding CAT from a yeast Agt11 library because we had already isolated CAT from yeast S. cerevisiae and raised antibody against the enzyme in rabbit, which was specific and sensitive enough to screen an expression library. A 2.2-kb insert of one of the positive clones was sequenced and used as a probe to screen a YEP 13 library containing 6–8-kb yeast nuclear DNA fragments. The coding sequence was localized on the nuclear DNA by restriction mapping and sequenced in that region that corresponds to the 5′ end of cDNA. The overlapping region of this nuclear DNA fragment was completely identical to that found in CAT cDNA. Reading the nuclear DNA upstream we found the supposed initiation codon 16 bases from the 5′ end of cDNA. Upstream from this ATG between 2 and 17 bases the long repeat of TA can be assumed to be the TATA box, the transcription initiation site. The ATG found on the nuclear DNA is suggested as the translation initiation site, since yeast favors the initiation codon closest to the TATA box.

The one long open reading frame obtained this way encodes a polypeptide of 670 amino acids with a molecular weight of 77,300. This molecular weight is approximately 12,000 larger than that of the isolated YCAT enzyme (65,000). The measured size of isolated YCAT, however, cannot be used for the prediction of the amino terminus of mature enzyme because of the uncertainty in the determination of molecular weight by SDS-polyacrylamide gel electrophoresis, but it suggests that the enzyme undergoes post-translational processing.

The identity of this isolated cDNA was proved by the fact that we could use it to disrupt the yeast chromosomal CAT gene, thus giving rise to CAT− cells. The elimination of CAT activity from the mitochondria of the transformed cells was shown by enzyme activity determination and by immunoblot. The result that the acetyl carnitine content of these cells decreased significantly shows that no other major CAT activity remained in the cell. The residual low CAT activity in the cytosol of mutant cells did not change, suggesting that this cDNA codes for the mitochondrial isoenzyme of CAT.

CAT is encoded by the nuclear genome, thus this protein is synthesized in the cytoplasm as precursor and then imported to the matrix space. Import is followed by a proteolytic cleavage by matrix processing proteases that remove the mitochondrial targeting sequence from the amino terminus of the protein; consequently the mature protein that can be isolated is usually smaller than its precursor form (28). We assume that the found nucleotide sequence encodes the precursor of YCAT (pre-YCAT) containing a targeting sequence. The amino terminus of the pre-YCAT contains mostly apolar and positively charged amino acids resembling that of mitochondrial targeting sequences, giving further support to our idea.

A search of the PIR protein data base yielded homologies between the already known carnitine acetyltransferases rat and human carnitine palmitoyltransferase II, rat carnitine octanoyltransferase giving an additional proof for the identity of this isolated cDNA. Two other proteins appeared with the same degree of homology, pig and Drosophila choline acetyltransferases. A highly homologous motif (LPXLPXPXL) (24) in choline and carnitine acetyltransferases has already been described, which is present in YCAT as well. This motif was suggested to be the choline or carnitine binding site, since this fragment is present only in this group of proteins and is missing from other acetyltransferases. Comparing carnitine and choline acetyltransferases with YCAT we found four more highly conserved motifs in the second half of the proteins which was present only in these proteins. Thus, we think that it is very uncertain to assign a specific function to any of these fragments at present.

Based on the high homology between YCAT-choline acetyltransferases and YCAT-carnitine acetyltransferases we propose that CAT of primitive eukaryotic cells might be the common ancestor of both enzymes catalyzing acyltransfer from CoA to a quaternary amine-containing substrate.

The in vivo function of YCAT was examined with the help of mutant cells in which the mitochondrial CAT was deleted by disrupting the CAT gene with Ura 3. Cells with the disrupted gene were able to grow on different fermentable and nonfermentable carbon sources, even on acetate, showing that CAT has no indispensable function in acetate metabolism. The significance of the previously suggested role of CAT in transport of acetyl group in or out of the mitochondria can be questioned based on these results. Participation of CAT in the mitochondrial acetate transport cannot be ruled out in wild type cell; however, yeast cells have enough capacity to bypass a supposed block of acetate metabolism in the absence of mitochondrial CAT using other metabolic pathways.

Surprising differences were in the pyruvate metabolism studied by NMR after labeling cells with [3-13C]pyruvate. In parental cells pyruvate is converted mostly to acetate, alanine, and citric acid cycle intermediates. In the CAT− cells the entry of pyruvate to alanine and citric acid cycle intermediates decreased significantly; instead, pyruvate was converted mostly to lactate and acetate. The turnover rate of the citric acid cycle was not affected in the CAT− cells.

Similar functions of CAT in insect flight muscle (29) and later in rat (30) and human (31) mitochondria have been demonstrated. CAT, in the presence of carnitine, was able to promote the pyruvate dehydrogenase activity in these organisms supposedly by decreasing the pyruvate dehydrogenase inhibitor acetyl-CoA level. Rapid adaptation to a sudden increase of energy demand on the initiation of muscle activity was suggested as the physiological role of this indirect regulatory effect.

In yeast, growing on galactose pyruvate can be converted to citric acid cycle intermediate via pyruvate dehydrogenase or by the cytosolic pyruvate decarboxylase and aldehyde oxidase, in which acetate is formed first and than converted to acetic acid-CoA or directly to a citric acid cycle intermediate by the glyoxylate cycle. Based on these results we cannot decide which pathway is affected most by the absence of CAT, but we clearly show that CAT has an important function in the metabolism which will be studied further using this CAT− cell line.

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