Biosynthesis of soluble carnitine acetyltransferases from the yeast *Candida tropicalis*

Branko KOZULIĆ,* Klaus MOSBACH and Franz MEUSSDOERFFER

Institute of Biotechnology, ETH Hönggerberg HPT, CH-8093 Zürich, Switzerland

Soluble carnitine acetyltransferase from *Candida tropicalis* is synthesized as a 76 kDa precursor, which is monomeric and possesses no or very little carnitine acetyltransferase activity. Maturation of the enzyme begins with proteolytic processing of the 76 kDa precursor to 64 and 57 kDa subunits. The processed subunits subsequently associate into two kinds of active oligomers; the 57 kDa subunits are assembled into a tetramer and the 64 kDa subunits into an octamer. Formation of these oligomers depends apparently on growth conditions, since both oligomers were present in cells grown in continuous culture, but cells grown batchwise contained only the tetrameric form of carnitine acetyltransferase.

INTRODUCTION

Carnitine and carnitine acyltransferases play a central role in the metabolism of fatty acids [1,2], and it has been reported that L-carnitine shows protective effects on ischaemic heart and hyperammonaemia [3,4]. Since optically pure L-carnitine is not readily available, the possibility of its enzymic production has attracted considerable attention [5,6]. Carnitine acetyltransferase (EC 2.3.1.7) (CAT) accepts only L-carnitine as substrate [7], and therefore it can be used for preparation of L-carnitine from the racemic mixture. As a source of the enzyme, we have investigated the alkane-assimilating yeast *Candida tropicalis*.

CAT activity can be detected in soluble and particulate fractions of *C. tropicalis* [8]. The peroxisomal and mitochondrial CATs have been characterized [9–11]. They are both oligomeric proteins of 420 kDa, containing two types of subunits. In addition to 64 kDa subunits that are present in both oligomers, 57 and 52 kDa subunits have been found in the peroxisomal and mitochondrial CATs respectively [10,11]. We have recently described some properties of a soluble octameric CAT purified from the cells grown on alkanes in continuous culture [12]. During that study, analysis of a cell-free extract indicated that CAT is synthesized as a precursor and that the enzyme may exist also as an oligomer smaller than an octamer. In the present paper we report evidence for existence of the CAT precursor and describe various aspects of maturation of the soluble CATs from *C. tropicalis*.

EXPERIMENTAL

Purification of CAT

Details of growth conditions for *C. tropicalis* and CAT purification have been described [12].

Electrophoretic techniques

SDS/polyacrylamide-gel electrophoresis was performed in 10–25% polyacrylamide gradient gels in accordance with ref. [13]. Non-denaturing electrophoresis was done in 3–30% (2.6% cross-linker) polyacrylamide

gels in a continuous buffer system [0.1 M-Tris/borate (pH 8.3)/2.5 mM-EDTA]. The gradient gels were prepared, with minor modifications, in accord with instructions in the Pharmacia Polyacrylamide-Gel Electrophoresis Manual.

Immunoblotting

Proteins were transferred to nitrocellulose membranes under conditions described in ref. [14]. To facilitate the transfer of proteins from the gradient gels run under non-denaturing conditions, before transfer the gel was incubated in SDS [15]. The membrane was then placed in 1% gelatin (Bio-Rad) solution in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.5 M-NaCl. Antibodies against CAT were raised in rabbits and partially purified by chromatography on Affi-Gel Blue (Bio-Rad), as described by the supplier. The nitrocellulose membrane was first incubated with purified antibodies against CAT and then with ¹²⁵I-labelled goat anti-rabbit IgG antibodies (Amersham).

Immunoaffinity purification of CAT

Glycerol/glass beads (5 g; Fluka; pore size 100 nm) were activated with 400 µl of 2,2,2-trifluoroethanesulphonyl (tresyl) chloride (Fluka) as described in ref. [16]. CAT (200 µg, in 0.1 M-sodium phosphate buffer, pH 7.5, containing 0.5% SDS) was coupled to the activated beads (2 ml) at room temperature overnight. The remaining active groups were blocked with 0.1 M-ethanolamine/HCl, pH 8.5, for 4 h at room temperature. Partially purified antiserum (2 ml) was applied (in 0.1 M-phosphate, pH 7.5) to the beads with immobilized CAT and, after washing with the same buffer (3 column vol.), antibodies were eluted with 0.2 M-KCl/HCl, pH 2.5. The pH of this solution was adjusted immediately to 7.5 with Na₂HPO₄ and the antibody solution was continuously (by means of a pump) passed over tresyl-activated glass beads in a small column (2 ml) overnight. The remaining tresyl groups were made to react with ethanolamine, and the resulting affinity gel was used for purification of CAT from a crude cell extract. Bound CAT was eluted with 0.2 M-KCl/HCl, pH 2.5, mixed with 2 vol. of 0.375 M-Tris/HCl, pH 8.8, containing 2% SDS, boiled

Abbreviations used: CAT, carnitine acetyltransferase; tresyl, 2,2,2-trifluoroethanesulphonyl.

* To whom correspondence and reprint requests should be addressed.

Fig. 1. Appearance of CAT activity after transfer of stationary cells to fresh medium

The medium contained glucose without (●) or with (○) 20 μg of cycloheximide/ml. In the latter case, stationary-phase cells were incubated with cycloheximide 30 min before transfer. At the intervals indicated, the cells were disrupted and, after centrifugation (25000 g , 15 min), the activity of CAT was measured.

for 3 min, concentrated with dry Sephadex G-15 and, before electrophoresis, dialysed against 0.065 M-Tris/HCl (pH 6.8)/0.1 % SDS.

RESULTS

We have recently described an octameric CAT purified from the soluble fraction of *C. tropicalis* grown on alkanes in continuous culture [12]. The enzyme is composed of 64 kDa subunits. Non-denaturing gradient-gel electrophoresis and Western blotting of the starting material used for CAT purification revealed also the presence of a polypeptide, somewhat larger than the subunit, interacting with CAT antibodies [12]. This was an indication that CAT was synthesized as a precursor. We have tried to prove the existence of this precursor and to elucidate the way it is transformed into the mature enzyme.

In accordance with results of other authors [9], we have found that CAT activity decreases several-fold when cells enter the stationary phase. At this stage, the remaining CAT activity was found in the particulate fraction, with only traces of activity in the supernatant. When stationary-phase cells were transferred to fresh medium containing glucose as carbon source, CAT activity rapidly increased and reached a maximum after about 30 min (Fig. 1). Addition of cycloheximide, a well-known inhibitor of protein biosynthesis, did not prevent this increase in CAT activity. Samples were taken from stationary cells and, at three intervals, from the cells transferred to the new medium. After SDS/polyacrylamide-gel electrophoresis followed by immunoblotting, the results shown in Fig. 2(a) were obtained. In the stationary-phase cells there is one very intense band with molecular mass higher than that of the CAT subunit, as well as two rather weak bands with sizes corresponding to 64 and 57 kDa polypeptides. Immediately (10 min) after transfer to the new medium the

Fig. 2. SDS/polyacrylamide-gel electrophoresis followed by immunoblotting

(a) Samples were taken from stationary cells (lane 2) and from the cells transferred to the new medium after 10 min (lane 3), 30 min (lane 4) and 60 min (lane 5). The cells were disrupted in 65 mM-Tris/HCl, pH 6.8, containing a few grains of phenylmethanesulphonyl fluoride. After centrifugation (25000 g , 15 min) the sample was heated in the same buffer containing 2 % SDS and 5 % mercaptoethanol. Purified CAT (lane 1) and standard proteins were run in the same gel. The positions (after electrophoresis and blotting) of the standard proteins (phosphorylase *b*, bovine serum albumin, ovalbumin and carbonic anhydrase) are shown on the left of the Figure. (b) Samples were taken from stationary cells (lane 2) and the cells transferred to the new medium containing cycloheximide (20 $\mu\text{g}/\text{ml}$) after 30 min (lane 3). Cycloheximide was added to stationary cells 30 min before transfer. Purified CAT was also applied to this gel (lane 1).

relative intensity of these two bands increases dramatically (lane 3), and remains strong after 1 h (lane 5). A similar, although much slower, response was observed when the cells were transferred to fresh medium containing *n*-alkanes (results not shown). The same transition was also noticed when the cells were transferred to the fresh medium which contained cycloheximide (Fig. 2b).

Taken together, these results (Figs. 1 and 2) demonstrate that CAT is synthesized as a larger precursor which possesses no or very little catalytic activity. Despite addition of cycloheximide, processing of the precursor took place, strongly indicating that it was performed by an already existing proteinase (not synthesized *de novo*). Several metabolites (glucose, glucose 6-phosphate, ATP, NADH and NADPH) were tested, but none of them was able to bring about the appearance of CAT activity in a cell-free extract of the stationary cells.

As determined by SDS/polyacrylamide-gel electrophoresis, the molecular mass of the CAT precursor was about 12 kDa larger than that of the mature subunit (64 kDa). The CAT precursor showed a molecular mass in that range also on gradient-gel electrophoresis under non-denaturing conditions (Fig. 7 in ref. [12]). Therefore, it is obvious that the precursor of CAT exists as a monomer in the native form. On the other hand, active CAT is oligomeric [12]. We have tried to monitor its formation in the stationary cells which were transferred to fresh medium. Non-denaturing gradient-gel electro-

Fig. 3. Non-denaturing gradient (3–30%) gel electrophoresis and immunoblotting

The samples were from stationary cells (lane *b*) and from cells transferred to the fresh medium after 10 min (lane *c*), 30 min (lane *d*), and 60 min (lane *e*). Cells were disrupted in 50 mM-Tris/borate, pH 8.3, containing a few grains of phenylmethanesulphonyl fluoride. Electrophoresis was performed at 130 V for 1800 V·h. Purified CAT (lane *a*) was also run in the same gel. The strong band above the octameric CAT in lane (*a*) represents aggregates which tend to form in the preparations of pure CAT upon storage [12]. The letters indicate the octameric CAT (O) and the precursor (P).

phoresis followed by immunoblotting was used and, as shown in Fig. 3, a strong band representing oligomeric CAT was detected shortly after the transfer. From a densitometric scan of the autoradiogram (not shown) it was calculated, assuming the same efficiency of blotting and equal affinity of CAT antibodies for both forms, that in stationary cells around 90% of total soluble CAT is present as inactive precursor. This decreases to 61% within only 10 min after transfer to the new medium. At the same time the amount of active CAT tripled (from 12 to 39%). These data clearly show that CAT maturation consists of two stages, namely a proteolytic cleavage of the inactive precursor and association of the subunits into the active oligomer.

The molecular mass of rapidly formed active CAT (Fig. 3) was smaller than the molecular mass of the enzyme which we have purified from the cells grown on alkanes in continuous culture. However, continuous-culture cells did contain such an oligomeric protein of 240 kDa in size [12]. We decided to find out the number and size of subunits present in this oligomer. Since two polypeptides of different sizes were formed after pro-

Fig. 4. Second-dimension SDS/polyacrylamide-gel electrophoresis and immunoblotting of the proteins resolved by first-dimension non-denaturing gradient gel electrophoresis

The strip of the first-dimension 3–30% gradient gel (0.7 mm thick) was equilibrated in 65 mM-Tris/HCl buffer, pH 6.8, and then in the same buffer containing 1% SDS. The gel strip was subsequently placed on top of the second-dimension gel, which was 1 mm thick. The arrows at the top indicate positions of the octameric CAT (O), the tetrameric CAT (T) and the precursor (P) in the first-dimension gel. The lines on the left side indicate positions, after SDS/polyacrylamide-gel electrophoresis, of the precursor (76 kDa) and 64 and 57 kDa CAT subunits respectively.

cessing of the precursor (Fig. 2a), this oligomer could contain various numbers of both or of only one subunit. A sample from alkane-grown continuous-culture cells was taken for analysis because it contained both oligomers. Proteins were first resolved by non-denaturing gradient-gel electrophoresis, which was followed by second-dimension SDS/polyacrylamide-gel electrophoresis (Fig. 4). Both oligomeric CATs as well as the precursor gave only one spot. This clearly demonstrates that the active CATs were composed only of the processed subunits of equal size. The 540 kDa oligomer contains subunits of 64 kDa, as already shown after purification and analysis of this protein [12]. In the smaller oligomer only 57 kDa subunits were present (Fig. 4). It can be easily calculated that there are four such subunits in this 240 kDa CAT. By the same two-dimensional electrophoretic technique we have also analysed the oligomer (shown in Fig. 3) formed in batch culture on glucose. This oligomer also contained only 57 kDa subunits (results not shown). Tailing of the precursor spot is caused by its slow elution from the first-dimension gradient gel, where it was stacked in a high concentration (approx. 25%) of polyacrylamide (Fig. 4). In contrast with the precursor, oligomeric CATs were dissociated by SDS and their subunits were eluted more easily from the first-dimension gel. Since we found that 1% SDS without 2-mercaptoethanol dissociated the enzyme into subunits, disulphide bridges cannot be involved in maintenance of the quaternary structure of CAT.

Fig. 5. Second-dimension SDS/polyacrylamide-gel electrophoresis and immunoblotting of the fragments produced by CNBr cleavage

The sample shown in Fig. 2(a), lane 3, was used. The polyacrylamide strip (0.7 mm thick), containing resolved proteins from the cell-free extract of *C. tropicalis*, was incubated with CNBr [19], and placed on top of the second-dimension gel (1 mm thick). The top of the first-dimension (1-D) gel was in the upper left corner.

Several smaller polypeptides also interacted strongly with CAT antibodies (Figs. 2a and 2b). It is unlikely that they are, together with larger polypeptides, constitutive parts of active CATs since polypeptides of such sizes could not be detected in oligomeric CATs (Fig. 4). These shorter peptides could be either some cross-reacting proteins or degradation products of CAT. In the latter case, it is reasonable to expect that CAT fragments produced by chemical cleavage of peptide bonds will be also recognized by CAT antibodies. To verify this hypothesis, a strip of polyacrylamide gel containing proteins separated by electrophoresis in the presence of SDS (as shown in Fig. 2a, lane 3) was treated with CNBr and applied to the top of a second gel. SDS/polyacrylamide-gel electrophoresis was then performed under identical conditions (Fig. 5). The spots on the diagonal represent uncleaved bands separated by the first-dimensional electrophoresis. Two of these spots, which could be easily identified as a CAT subunit and CAT precursor, gave fragments reacting with CAT antibodies. CNBr degradation of CAT resulted in 11–12 fragments [12], but apparently only the larger ones retained sufficient tertiary structure of intact subunits to be recognized by CAT antibodies (Fig. 5).

However, on the basis of these results we could not rule out with certainty the existence of yet another polypeptide in the newly formed CAT, since it is likely that it would not be recognized by antibodies raised against the 64 kDa polypeptide present in octameric CAT. If present, the second type of subunit could be detected after isolation of the native oligomeric CAT by

Fig. 6. SDS/polyacrylamide-gel electrophoresis of CAT purified by immunoaffinity chromatography

Low-molecular-mass standard proteins (Pharmacia) are shown in the left-hand lane and purified CAT in the right-hand lane. Proteins were detected by silver staining [20]. Standard proteins were: 1, phosphorylase *a* (94 kDa); 2, bovine serum albumin (67 kDa); 3, ovalbumin (43 kDa); 4, carbonic anhydrase (30 kDa); 5, trypsin inhibitor (20 kDa); 6, lactalbumin (14.4 kDa).

immunoaffinity chromatography. For this purpose, antibodies against CAT were purified on a column with immobilized 64 kDa CAT subunits (as described in the Experimental section) and then immobilized on tressyl-activated glass beads. This affinity matrix was consequently used for one-step purification of CATs from a crude cell extract. SDS/polyacrylamide-gel electrophoresis of the material eluted from the immuno-affinity column showed only two bands after silver staining (Fig. 6). These two polypeptides had the same molecular masses as subunits present in octameric (64 kDa) and tetrameric (57 kDa) CAT respectively. Therefore we conclude that 240 kDa active CAT contains no other but 57 kDa subunits.

DISCUSSION

Active CAT is present in only minor amounts in the soluble fraction of the stationary-phase cells of *C. tropicalis*. However, after transfer to fresh medium, CAT activity increases rapidly (Fig. 1). At the molecular level, this increase in enzymic activity is characterized by proteolytic processing of the 76 kDa precursor and association of the 57 kDa subunits formed into the active tetramer (Figs. 2–4). We have monitored this process by a two-dimensional electrophoretic technique, where in the first dimension proteins were resolved according to

their size by non-denaturing gradient-gel electrophoresis. In the second dimension, subunits present in the oligomers were identified by SDS/polyacrylamide-gel electrophoresis and immunoblotting (Fig. 4). This process can be also monitored by gel filtration in the first step, as done in a study on the assembly of processed ornithine transcarbamoylase subunits into trimers [17]. However, we consider that gradient-gel electrophoresis offers advantages in sensitivity and speed for investigations on the formation of oligomeric enzymes. With 3–30 % polyacrylamide gradient gels, oligomers of up to 1000 kDa can be analysed, but recently introduced poly-*N*-acryloyl-Tris gradient gels expand the useful range to over 3000 kDa [18].

The processing of the CAT precursor yields two subunits, of 64 and 57 kDa. At present it is not clear whether 57 kDa subunit is formed directly from 76 kDa precursor or via the 64 kDa subunit. The 64 kDa subunits are found in the octamer (Fig. 4 and ref. [12]), and therefore absence of the octamer (Fig. 3) under conditions where the 64 kDa subunits were present (Fig. 2) might indicate that the 64 kDa polypeptide is rapidly processed further to the 57 kDa subunit. Alternatively, these data can also mean that additional modifications of the 64 kDa polypeptides are necessary before their assembly into the octamer. An indication in favour of this assumption is our findings that 64 kDa subunits from octameric CAT could be resolved into four homologous charge-modified polypeptides [12].

The association of the subunits into active oligomers certainly depends on growth conditions. Thus in batch culture with glucose as carbon source, active CAT contains four 57 kDa subunits (Fig. 3). Such an enzyme is also present in the cells grown in continuous culture on alkanes [12]. On the other hand, the octameric form of CAT composed of eight 64 kDa subunits, and purified from cells grown in continuous culture on alkanes, was never observed when *C. tropicalis* was grown batchwise on glucose.

It has been reported that mitochondrial and peroxisomal CATs from *C. tropicalis* contain two different subunits, both being synthesized as precursors [11]. Accordingly, the larger precursor (71 kDa) is processed to give 64 kDa subunits which exist in both mitochondrial and peroxisomal enzymes. The smaller precursor (57 kDa) is presumably processed to a 52 kDa subunit present in the mitochondrial enzyme or directly incorporated into the peroxisomal CAT. These post-translational modifications were suggested to be necessary for the correct subcellular localization of CATs [10,11]. There are apparent similarities between these two enzymes and the soluble CAT that we have described. Subunits of the soluble CAT are also synthesized as larger (76 kDa) precursor molecules, and this precursor is then processed to two subunits different in size (64 and 57 kDa) which associate, depending on growth conditions, into octamers (64 kDa subunits) or tetramers (57 kDa subunits; Fig. 4). Relatedness of these three polypeptides was demonstrated by peptide mapping (Fig. 5) and by interaction of antibodies raised against 64 kDa subunit with 76 and 57 kDa polypeptides (Figs. 2, 3 and 4). It is not known whether the two precursors of mitochondrial and peroxisomal CATs show homology, and how many subunits of each type there are in

these two enzymes whose molecular mass was reported to be 420 kDa [9–11]. Therefore, in spite of similarities in size of the subunits in soluble, mitochondrial and peroxisomal CATs (64 and 57 kDa), there are significant differences in size (540 and 240 versus 420 kDa) and structure (the same subunits versus two different subunits) of these oligomeric CATs. It is tempting to speculate that these differences are responsible for different localization of these enzymes. However, the molecular mass of the soluble CAT precursor (76 kDa) is apparently 5 kDa higher than that of the larger of the two precursors of mitochondrial and peroxisomal CATs. Therefore, it is not possible to say whether the 64 kDa subunits in the soluble, mitochondrial and peroxisomal CATs represent the same polypeptide or not, as they are produced from two precursors differing in size. Furthermore, one should be cautious in comparing these enzymes because the strains of *C. tropicalis* used might be genetically different; in line with this is our demonstration of dissimilarity in structures of CATs from two *C. tropicalis* strains [12].

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