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**REDESIGN OF CARNITINE ACETYLTRANSFERASE
SPECIFICITY BY PROTEIN ENGINEERING**

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INTRODUCTION

1. MODULATION OF COENZYME A POOLS IN THE CELL

Cells contain limited pools of sequestered coenzyme A (CoA) that are essential for the activation of carboxylate metabolites. Esterification of carboxylic acids to CoA through the formation of a thioester bond is a common strategy used in metabolic processes to ‘activate’ the relevant metabolite. The process requires an input of energy in the form of the hydrolysis of nucleotide triphosphate. In general, it represents the first step through which the metabolite enters a particular pathway (e.g., Krebs cycle or synthesis of fatty acids and cholesterol). This activation has two universal consequences: 1) it renders the metabolite (in the form of the CoA ester) impermeant to cell membranes and 2) it sequesters CoA from the limited pools that exist in individual subcellular compartments. As a result, the pools of acyl-CoA esters remain separate in the different cellular compartments and may have specific properties and exert different effects in their respective locations. In the case of acyl-CoA esters, it is imperative that the concentration of individual esters is controlled, since many exhibit high biological activity, including the regulation of gene expression, membrane trafficking and modulation of ion-channel activities. Consequently, the cell has two requirements: 1) a mechanism for the control of CoA-ester concentrations that is rapid and does not involve the energetically expensive hydrolysis and resynthesis of the esters from the free acid, and 2) a system that, after the initial synthesis of the CoA ester, enables the acyl moiety to permeate membranes without the need to re-expend energy (Zammit, 1999; Ramsay, 2004).

The cell achieves these requirements through the carnitine system, which consists of carrier proteins that transport carnitine across the membranes and enzymes, carnitine acyltransferases, that catalyse the reversible transesterification reaction between acyl-CoA esters and L-carnitine to form the corresponding carnitine ester and regenerate unesterified CoA (Fig. 1). As a whole, the carnitine system both connects the various acyl-CoA pools and buffers fluctuations in their acylation state that would be detrimental to cell homeostasis (Ramsay, 2001).

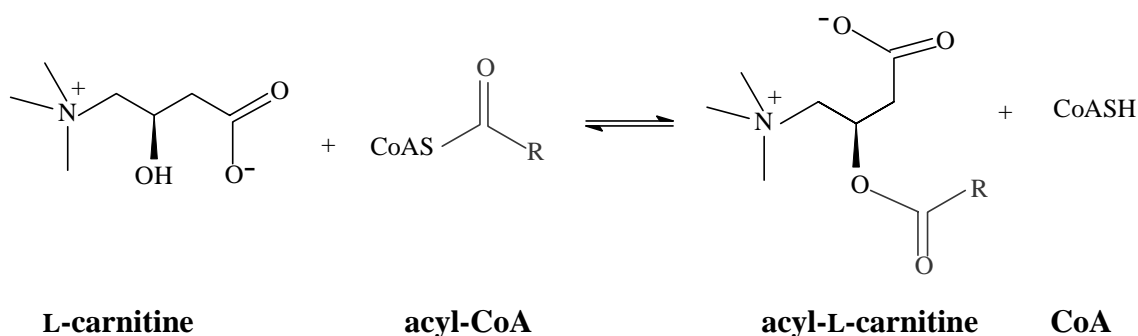


Fig. 1. The reaction catalyzed by carnitine acyltransferases.

2. THE CARNITINE SYSTEM

2.1. CARNITINE

L-Carnitine (L-3-hydroxy-4-*N*-trimethylaminobutyric acid) is a highly polar zwitterionic compound present in some prokaryotes and all eukaryotes. It is synthesized by most eukaryotic organisms from the precursor trimethyllysine (Vaz, 2002). In humans, endogenous synthesis occurs mainly in the liver but is complemented by dietary uptake. Dietary carnitine is especially required in early infancy when the capacity for carnitine biosynthesis is limited (Borum, 1983) and for patients on hemodialysis. Carnitine is found at the highest concentrations in heart and skeletal muscle of vertebrates, and unusually high concentrations have also been measured in the flight muscle of the fly (Fraenkel, 1954).

The most important function of this compound is to facilitate the transport of fatty acids into the mitochondria. The realisation that carnitine plays a central role in cellular fatty acid metabolism dates back to the early 1950s when two key observations were published. Friedman (1955) discovered that carnitine can be reversibly acylated with acetyl-CoA, while Fritz (1955) showed that carnitine markedly stimulates fatty acid oxidation in liver homogenates. These studies led to the discovery that carnitine is somehow involved in the transport of long-chain fatty acids from the cytosol to the mitochondrial matrix where β -oxidation occurs.

2.2. CARNITINE UPTAKE AND TRANSPORT

Since carnitine and acylcarnitines cross membranes, there must be a non-saturable component in cell uptake (Shennan, 1998). However, proteins mediate both the cellular accumulation of carnitine across the plasma membrane and the rapid flux across the mitochondrial inner membrane (MIM) required for β -oxidation.

The sodium-dependent organic cation transporter (OCTN2) is the high-affinity carnitine plasma membrane transporter for uptake of carnitine into the cell (Fig. 2). This transport has an apparent K_m value of 4.3 μ M (Tamai, 1998). OCTN2 is presumably responsible for the accumulation of carnitine across a concentration gradient from about 50 μ M in plasma to millimolar levels in the cells (Bremer, 1983).

The MIM carnitine/acylcarnitine translocase (CACT) transfers carnitine and its esters across the membranes (Fig. 2). CACT is a member of the mitochondrial carrier family. Mammalian CACT proteins contain 301 amino acid residues and occupy the MIM. Similar transport proteins are suggested for the other organelles, like peroxisomes or endoplasmic reticulum (ER), although there is no direct experimental proof for the existence of such carriers (Fraser, 1999).

2.3. CLASSIFICATION OF CARNITINE ACYLTRANSFERASES

Carnitine acyltransferases belong to the carnitine/choline acyltransferase family, which also includes choline acetyltransferase (ChAT). The three known classes of carnitine acyltransferase differ in their acyl-chain length selectivity (Fig. 3), sub-cellular compartmentalization, kinetics and physiological function. Fig. 2 shows the locations of the acyltransferases in the cell.

Carnitine acetyltransferase (CrAT; E.C. 2.3.1.7) has a substrate preference for short-chain acyl-CoAs and is found within the mitochondrial matrix, in the lumen of the endoplasmic reticulum (ER), in the peroxisomes (Colucci, 1988), and has also been reported in the nucleus.

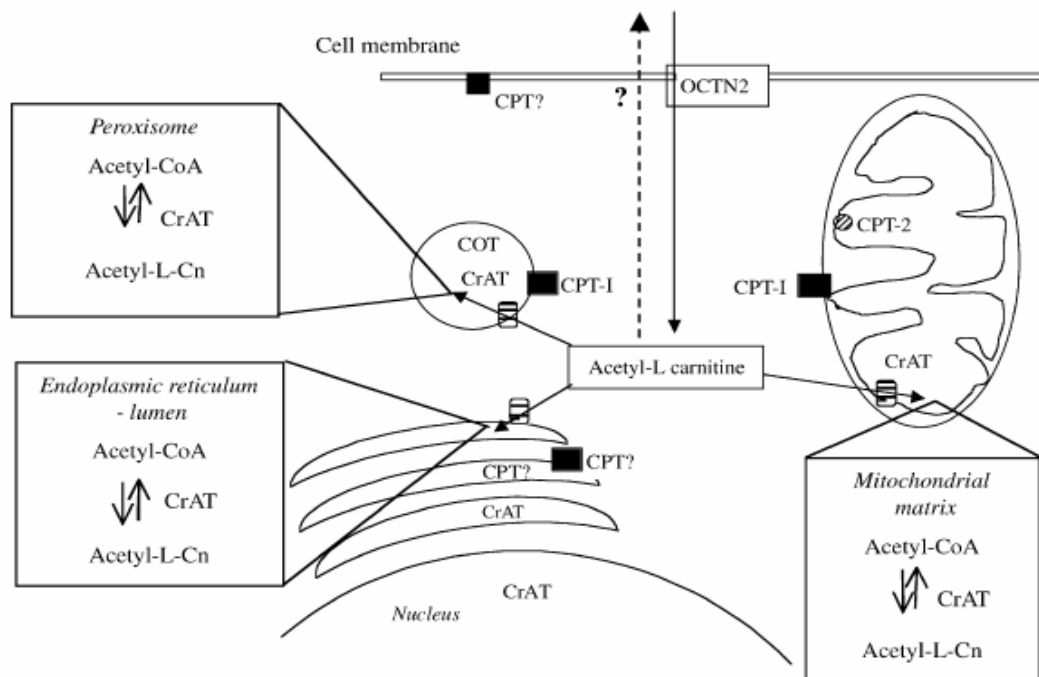


Fig. 2. **Location of carnitine acyltransferases in mammalian cells.** Carnitine/acylcarnitine translocase (CACT) is shown as striped squares. OCTN2 indicates sodium-dependent organic cation transporter; CrAT, carnitine acetyltransferase; COT, carnitine octanoyltransferase; CPT, carnitine palmitoyltransferase; acetyl-L-Cn, acetyl-carnitine. Reproduced from Ramsay (2004).

Carnitine octanoyltransferase (COT; E.C. 2.3.1.137) is localised in the peroxisomal matrix and facilitates the transport of medium-chain fatty acids from peroxisomes to mitochondria through the conversion of acyl-CoA, shortened by peroxisomal β -oxidation, into fatty acylcarnitine (Ferdinandusse, 1999).

Finally, carnitine palmitoyltransferases (CPTs; E.C. 2.3.1.21), CPT I and CPT II, are located in the mitochondrial outer membrane (MOM) and the MIM, respectively. CPT I facilitates the transfer of long-chain acyl groups from the cytoplasm to the mitochondrial matrix, a process that represents the rate-limiting step in β -oxidation (McGarry, 1997). CPT I is the only acyltransferase that has direct access to the cytosolic pool of acyl-CoA.

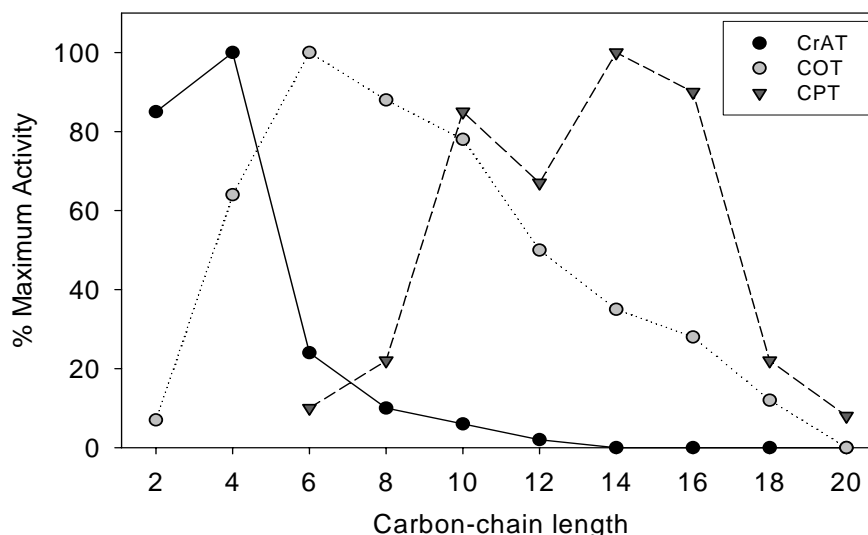


Fig. 3. **Specificity of purified rat liver carnitine acyltransferases for acyl-CoAs of varying chain length.** The forward carnitine acyltransferase reaction is shown (Miyazawa 1983a and 1983b).

Another characteristic that distinguishes the various carnitine acyltransferases activities is their sensitivity to inhibition by malonyl-CoA, the first intermediate in fatty acid synthesis. CPT I and COT are sensitive to malonyl-CoA, while CrAT and CPT II are not. Malonyl-CoA is synthesised from acetyl-CoA through the catalytic activity of acetyl-CoA carboxylases (ACC). A classification of carnitine acyltransferases based on their malonyl-CoA sensitivity is summarised in Table 1.

Abbreviation	Molecular size	Intracellular location	Compartment accessed	Acyl-CoA specificity
<i>Malonyl-CoA sensitive</i>				
CPT I	88 kDa	MOM	Cytosol	LCFA-CoA
COT	69 kDa	Peroxisomes	Peroxisomal lumen	MCFA-CoA
<i>Malonyl-CoA insensitive</i>				
CPT II	69 kDa	MIM	Mitochondrial matrix	LCFA-CoA
CrAT mit	67.5 kDa	Mitochondrial	Mitochondrial matrix	SCFA-CoA
CrAT per	69 kDa	Peroxisomal	Peroxisomal lumen	SCFA-CoA

Table 1. **Carnitine acyltransferases characterised in rat liver.** SCFA-CoA indicates short-chain acyl-CoA; MCFA-CoA, medium-chain acyl-CoA; LCFA-CoA, long-chain acyl-CoA; CrATmit, mitochondrial CrAT; CrAT per, peroxisomal CrAT.

3. CARNITINE PALMITOYLTRANSFERASE

3.1. THE CARNITINE PALMITOYLTRANSFERASE SYSTEM

The activation of long-chain fatty acids (LCFA) to long-chain fatty acyl-CoAs (LCFA-CoA) takes place in the MOM and is catalysed by long-chain acyl-CoA synthetase (LCAS). However, the MIM is not permeable to these acyl-CoAs. The carnitine-dependent shuttle system facilitates entry of activated LCFA into the mitochondrial matrix, where β -oxidation takes place. This transport system consists of three proteins, CPT I, CACT and CPT II, each with a different submitochondrial localization (Kerner, 2000). As a first step, LCFA-CoAs formed by the catalytic action of LCAS in the MOM are converted to acylcarnitines. This transesterification is catalysed by transmembrane CPT I protein, also localized in the MOM. The long-chain acylcarnitine reaction products are then translocated into the mitochondrial matrix in an exchange reaction catalysed by CACT, an integral inner membrane protein. The acylcarnitines are then reconverted in the matrix to the respective acyl-CoAs by CPT II, an enzyme associated with the inner leaflet of the MIM (Fig. 4).

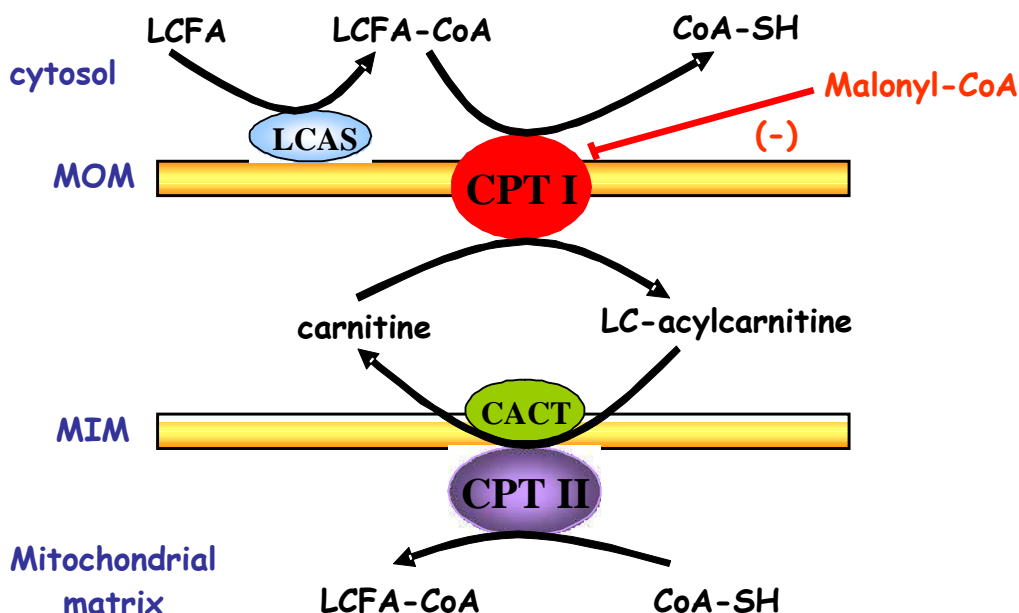


Fig. 4. Long-chain fatty acyl-CoA (LCFA-CoA) translocation into the mitochondria by the CPT system. LCAS indicates long-chain acyl-CoA synthetase; CACT, carnitine/acylcarnitine translocase; MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane.

3.2. CPT I ISOFORMS AND DISTRIBUTION

Mammals express two isoforms of CPT I, a liver isoform L-CPT I (Esser, 1993) and a heart/skeletal muscle isoform M-CPT I (Yamazaki, 1995), which are the products of two different genes (*CPT1A* and *CPT1B*, respectively). The human genes are located at 11q13 (L-CPT I) and 22q13.3 (M-CPT I) (Britton, 1997). L-CPT I protein contains 773 amino acids (88 kDa) and M-CPT I contains 772 amino acids (88 kDa). The amino acids identity is high (62%) but they are differentially regulated by malonyl-CoA. The L-CPT I isoform is inhibited by malonyl-CoA to a much lesser extent than the M-CPT I isoform. The IC_{50} value for M-CPT I is about 2 orders of magnitude lower than for L-CPT I (Esser, 1996).

A novel CPT I family member, CPT Ic, which is mainly expressed in brain and testis, has recently been described (Price, 2002). The protein sequence contains all the residues known to be important for both carnitine acyltransferase activity and malonyl-CoA binding in other family members. However, when the protein was expressed in yeast it had no detectable catalytic activity with several different acyl-CoA esters that are good substrates for other carnitine acyltransferases, but displayed high-affinity malonyl-CoA binding. It is hypothesized that this novel CPT I related protein may be specialized for the metabolism of a distinct class of fatty acids involved in brain function and/or appetite control. The human *CPT1C* gene is located at 19q13.33.

3.3. PHARMACOLOGICAL REGULATION OF CPT I

CPT I is tightly regulated by its physiological inhibitor malonyl-CoA, and thus, CPT I is the most physiologically important regulatory step in mitochondrial fatty acid oxidation (McGarry, 1980). This process allows the cell to signal the relative availability of lipid and carbohydrate fuels in liver, heart, skeletal muscle, and pancreatic β -cells, and converts CPT I into a potential pharmacological target for the treatment of metabolic disorders such as coronary diseases, obesity and diabetes (Ruderman, 1999).

Inhibition of fatty acid regulation is clearly an effective strategy for lowering blood glucose levels in diabetic animal models and in non-insulin dependent diabetes (NIDDM) itself. However, approaches to the direct inhibition of fatty acid oxidation at the level of β -oxidation have so far been unsuccessful due to toxicity and uncontrolled hypoglycaemia. Attention has been directed towards an intermediate step, the CPT system. Inhibition of CPT I seems to be a viable target for the treatment of type 2 diabetes (Anderson, 1998).

Etomoxir is one of the most extensively studied CPT I inhibitors (Fig. 5). It acts as an irreversible, active site-directed inhibitor of liver and skeletal muscle CPT I, and is functionally active only after metabolic conversion to its CoA-ester etomoxiryl-CoA (Weis, 1994). Etomoxir is an orally effective inhibitor of fatty acid oxidation in liver and muscle tissues, and gives rise to antiketogenic and hypoglycaemic activity in animal models of NIDDM. It has been shown to improve insulin sensitivity in NIDDM patients (Hubinger, 1992). However, the drug has not been developed as an antidiabetic agent, probably because mechanism-based myocardial hypertrophy was associated with its use.

A novel compound, the fatty acid synthase (FAS) inhibitor C75, has been proposed to pharmacologically regulate CPT I activity (Price, 2001). Structurally, C75 is a cell-permeable α -methylene- γ -butyrolactone designed to be less reactive and potentially safer than cerulenin, a natural product obtained from the fungus *Cephalosporium caerulens*. C75 lacks the reactive epoxide present in cerulenin, which enhances chemical stability (Fig. 5). C75 has been proposed for two therapeutic applications. Firstly, as an anti-tumour agent, since it has cytostatic and cytotoxic effects in cultured tumour cells, where the increase in malonyl-CoA levels caused by FAS inhibition leads to cancer cell-specific apoptosis (Li, 2001). Secondly, it has been proposed as an anti-obesity agent, since it can alter the metabolism of neurons in the hypothalamus, where a rise in malonyl-CoA serves as a second messenger for nutrient status, thereby mediating appetite suppression (Loftus, 2000). In peripheral tissues, C75 has been postulated not only to increase malonyl-CoA, but also to act as a malonyl-CoA analogue that antagonizes the inhibitory effect on CPT I (Thupari, 2002). Both its central and peripheral actions could reduce weight in lean and fat mice.

It has recently been demonstrated that, unlike the activation effect of C75 on CPT I activity, the CoA derivative (C75-CoA) is a potent competitive inhibitor that binds tightly but reversibly to CPT I (Bentebibel, 2006). IC_{50} values for L- or M-CPT I isoforms overexpressed in yeast, as well as for purified mitochondria from rat liver and muscle, were within the same range as those observed for etomoxiryl-CoA. CPT I activity was also inhibited in mitochondria from pancreas, muscle and kidney-derived cell lines incubated directly with C75, as observed with etomoxir. This finding suggests that the CoA-derivatives of both compounds may be produced within the cell. These inhibitory effects were followed by a decrease in fatty acid oxidation. It was concluded that the inhibitory effect of C75-CoA was caused by strong, reversible binding to CPT I inside the palmitoyl-CoA pocket.

In the present study we have attempted to examine the C75-CoA/CrAT interaction *in vitro*.

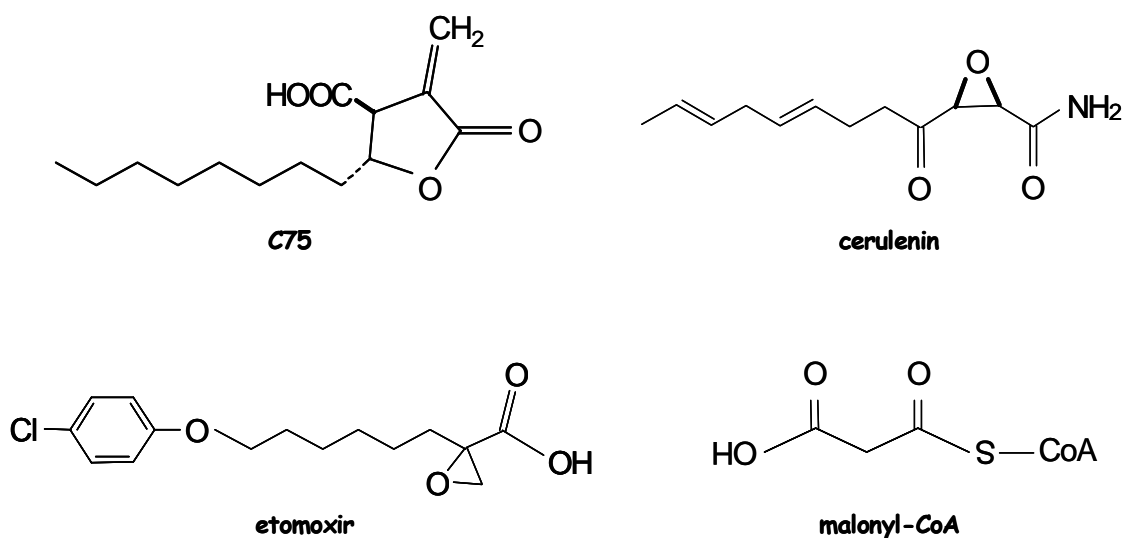


Fig. 5. Structures of C75, cerulenin, etomoxir and malonyl-CoA.

4. CARNITINE OCTANOYLTRANSFERASE

Carnitine octanoyltransferase (COT) is a 69 kDa protein that contains 612 amino acid residues and is localised in the peroxisomes. The human, rat and bovine proteins contain different C-terminal sequences (THL, AHL, and PHL, respectively), thought to serve as peroxisomal targeting signals (PTS). *CROT* is the only gene for carnitine octanoyltransferase and has been mapped in humans to chromosome 7q21.1 (van der Leij, 2000).

Expression of the rat gene for COT is subject to *trans*-splicing, i.e., splicing of different primary transcripts to form one mRNA (Caudevilla, 1998). In the case of rat COT, the different mRNAs that are generated by *trans*-splicing contain either a direct repeat of exon 2 or a direct repeat of exons 2 and 3. Therefore, including the normal transcript, the three mRNA sequences lead to the expression of two COT proteins: one of normal size (69 kDa) and one of 79 kDa. A frameshift caused by the number of nucleotides in exon 2 not being a multiple of three means that the transcript containing the repeat of exon 2 does not produce a COT protein.

COT catalyses the interconversion of acyl-CoA and acylcarnitine esters over a wide range of acyl chain lengths: the enzyme is most active with medium-chain length substrates (C₆ to C₁₀) and less active with long-chain length substrates (C₁₂ to C₁₆) (Farrell, 1984) (Fig. 3). In peroxisomes, COT activity can be inhibited by malonyl-CoA in a way similar to the mitochondrial CPT I (a Bhaird, 1992).

4.1. ROLE OF CARNITINE OCTANOYLTRANSFERASE IN THE PEROXISOMAL β -OXIDATION

The β -oxidation of fatty acids in mammalian cells takes place in both mitochondria and peroxisomes. Long-chain fatty acids are oxidized primarily in mitochondria, whereas very long-chain and branched-chain fatty acids are handled primarily by peroxisomes. Since mammalian peroxisomes do not fully β -oxidize medium-chain fatty acids intermediates (C₈-C₁₀), the medium-chain acyl-CoA molecules (MCFA-CoA) generated by chain shortening are further metabolised

elsewhere in the mitochondria, where they can undergo the final steps of β -oxidation. MCFA-CoA are converted to carnitine esters by COT, allowing their transport out of the peroxisomes to the cytosol and ultimately to the mitochondria (Fig. 6). This transport is most probably mediated by the same carrier as the mitochondrial CACT (Fraser, 1999). The short-chain acyl-CoAs acetyl- and propionyl-CoA formed during incomplete peroxisomal β -oxidation are exported out of the peroxisomes by CrAT for further oxidation (Fig. 6).

5. CARNITINE ACETYLTRANSFERASE

CrAT catalyses the interconversion between acetyl-CoA and acetylcarnitine with carnitine and CoA, respectively, as the co-substrates. However, the enzyme is also able to use other short-chain CoA esters (e.g., propionyl- and butyryl-CoA) as substrates (Miyazawa, 1983a). The kinetic and chemical mechanism has been thoroughly characterized (Colucci, 1988) and sequences have been reported from several organisms including yeast, mouse, rat, fruit fly and human (van der Leij, 2000).

5.1. TISSUE AND SUBCELLULAR LOCATION

CrAT is widely distributed in a variety of rat tissues, with the highest concentrations in heart and brown adipose tissue. In addition, unusually high CrAT activity is observed in testicular tissue (Marquis, 1965). In rat liver, the enzyme is found in the mitochondrial matrix (50%), peroxisomes (30%), and also associated with microsomes (20%) (Kahonen, 1976; Markwell, 1973). Mammalian studies performed in rat hearts reveal no convincing evidence that any form of carnitine acetyltransferase is expressed with its active site available for interaction with cytosolic acetyl-CoA (i.e., either in the cytosol or overtly on the cytosolic face of any membrane system) (Abbas, 1998). The absence of CrAT activity in mammalian cytoplasm means that activated acetyl groups are transferred only between the intra-organelle pools (Ramsay, 2003).

5.2. MOLECULAR GENETICS OF CARNITINE ACETYLTRANSFERASE

CRAT appears to be the only gene for carnitine acetyltransferase and has been mapped in humans to 9q34.1 (Corti, 1994a). Differential targeting of the protein to the peroxisomal lumen and mitochondria arises through differential mRNA splicing during transcription, such that two transcripts are produced, only one of which contains a 28- or 29 residue N-terminal mitochondrial matrix-targeting signal (MTS), which is clipped off during translocation through the MIM (Corti, 1994b). In human CrAT, cleavage of the mitochondrial precursor (626 amino acids), which probably occurs between residues 29 and 30, generates a mature mitochondrial protein (CrAT mit.) of 597 amino acids (67.5 kDa). Translation of the peroxisomal form of CrAT (CrAT per.) starts at a second start codon, and for human CrAT, produces a protein of 605 amino acids (69 kDa). Both proteins are predicted to contain an “AKL” peroxisomal targeting signal (PTS), suggesting that the presence of a mitochondrial signal peptide overrides the simultaneous presence of the PTS sequence and that mitochondrial targeting prevails when the leader sequence is present in the nascent protein. It is also suggested that microsomal targeting of one of the transcripts could be due to the presence of the KVEL motif in the CrAT sequence, since this motif may function like the ER retention signal KDEL (Corti, 1994b).

5.3. CARNITINE ACETYLTRANSFERASE FUNCTION

Due to the impermeability of organelle membranes to CoA, CrATs function in a compartmental buffering system by maintaining the appropriate levels of acetyl-CoA and CoA in cellular compartments.

In peroxisomes they remove excess activated acetyl groups releasing free CoA, which can then accept more acetyl groups produced by peroxisomal β -oxidation, thereby allowing the oxidation to proceed (Fig. 6). This indirectly facilitates the transport of acetyl moieties to the mitochondria for β -oxidation (Bieber, 1988).

Mitochondrial CrAT plays a major role in modulating matrix acetyl-CoA concentration. The production and utilisation of acetyl-CoA in the mitochondrial matrix lie at a major metabolic crossroads. Acetyl-CoA can be produced as a result of either glucose metabolism (via pyruvate) or fatty acid β -oxidation, and it can be used for a

number of pathways including oxidation in the Krebs cycle or the synthesis of ketone bodies. Regulation of the fate of acetyl-CoA is mediated, to a large extent, by the effects of the molecule itself on pyruvate dehydrogenase (PDH) kinase, which is inhibited by a high acetyl-CoA/CoA ratio (Zammit, 1994). This effect is thought to be an important mechanism through which the inhibition of the oxidation of glucose by fatty acids is achieved in tissues like heart and skeletal muscle. In the liver, mitochondrial acetyl-CoA also activates the key gluconeogenic enzyme pyruvate carboxylase. Therefore, high rates of β -oxidation of fatty acids result in the activation of gluconeogenesis from pyruvate and its precursors.

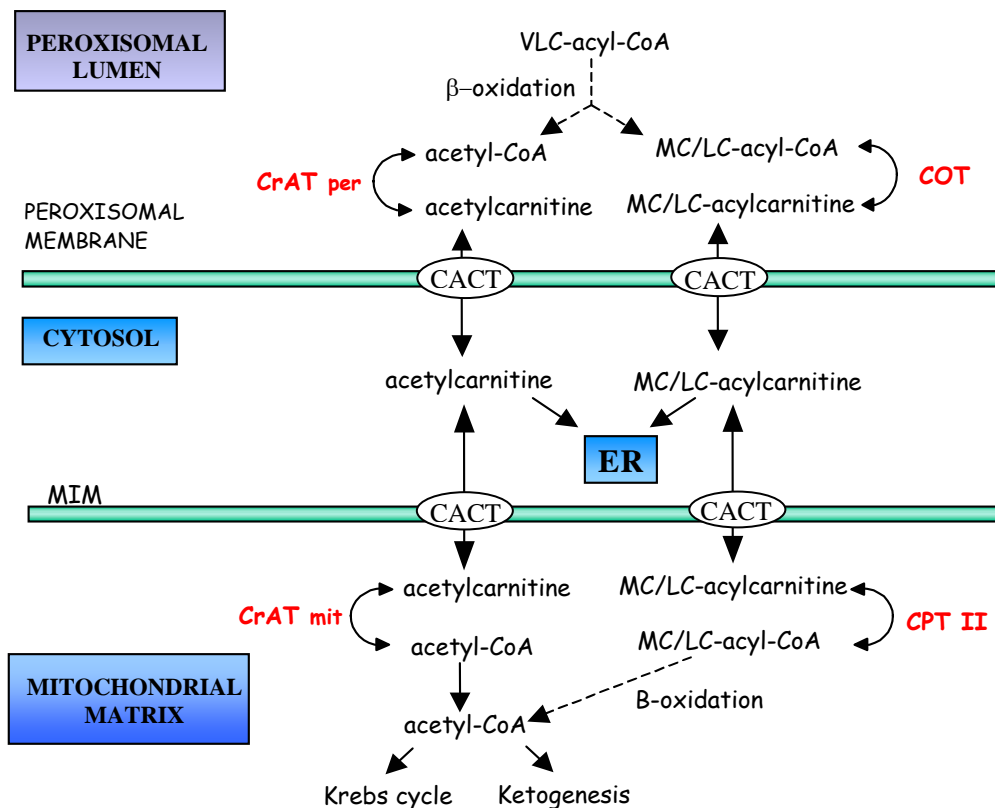


Fig. 6. Role of peroxisomal carnitine acyltransferases in the supply of chain-shortened acyl moieties for either complex lipid formation in the endoplasmic reticulum (ER) or β -oxidation in mitochondria. VLC indicates very-long-chain; MC, medium-chain; LC, long-chain. CrATmit, mitochondrial CrAT; CrATper, peroxisomal CrAT.

In mammalian tissues, CrATs can also contribute to the excretion of excess or harmful acyl molecules as acylcarnitines (Bieber, 1988). The elimination of toxic propionyl residues and other acyls groups in patients who are deficient or seriously

compromised in the enzymes needed for metabolism of the activated acyl groups, represents a situation in which carnitine and its associated acyltransferases both eliminate selective acyl residues and increase the amount of CoA, thereby modulating the CoA/acyl-CoA ratio. For example, diabetic patients excrete more long-chain carnitine esters (C₁₂-C₁₆) than controls, leading to the suggestion that the urinary acyl-carnitine pattern might be a useful tool for diagnosis and monitoring therapy in diabetes mellitus (Moder, 2003). CrAT activity has also been implicated in the cell cycle from G1 to S phase. The progression of the cell towards S phase is dependent on the proper induction of CrAT transcripts and on normal translation into functional protein. It has been demonstrated that antisense-induced reduction of CrAT expression produces a significant loss of growth potential in S3T3 mouse fibroblasts (Brunner, 1997).

5.4. ROLE OF CARNITINE AND CARNITINE ACETYLTRANSFERASE IN HUMAN HEALTH

5.4.1. Carnitine acetyltransferase deficiencies

CrAT appears to play an important role in human health. Isolated cases of carnitine acetyltransferase deficiency have been reported, although no disease-causing mutations are known at the molecular level for CrAT.

DiDonato (1979) observed carnitine acetyltransferase deficiency in brain, liver, kidney, and cultured fibroblasts in a girl suffering from intermittent ataxia, hypotonia, mental confusion, and disturbances of consciousness. Przyrembel (1987) described a baby with CrAT deficiency in brain, heart, and kidney in association with poor respiration and failure to thrive. Moreover, decreased CrAT activity has been reported in Alzheimer's disease patients (Kalaria, 1992; Makar, 1995), and in several vascular diseases (Brevetti, 1991; Melegh, 1999).

5.4.2. Therapeutic use of carnitine and short-chain acylcarnitine esters

The therapeutic use of acetyl-L-carnitine has opened a fascinating window on the role of acetyl-CoA in brain function. Acetyl-L-carnitine, which can be a precursor for acetylcholine synthesis catalyzed by ChAT, is thought to slow the rate of mental deterioration in Alzheimer patients (Ando, 2001; Montgomery, 2003).

In the heart, carnitine and propionyl-L-carnitine are beneficial for cardiac performance in *in vitro* and *in vivo* models of ischemia and hypertrophy (Calvani, 2000). The effects of ischemia and post-ischemia are exacerbated by the continued high utilisation of fatty acids by the myocardium (Lopaschuck, 1999). Carnitine has been shown to inhibit fatty acid oxidation by the myocardium, and may thus have a beneficial effect under these conditions. The increased availability of carnitine inside mitochondria will reduce acetyl-CoA levels at the site through the action of CrAT, thus de-inhibiting PDH and increasing the oxidation of pyruvate, while lowering the rate of formation of lactate and normalising pH (Fig. 7) (Zammit, 1998). Propionyl-L-carnitine would also be predicted to have the same effect, but with the added benefit of generating ATP through metabolism of the propionyl moiety. This has been shown experimentally in hypertrophied hearts (Schonekess, 1995) and following ischaemia (Felix, 2001).

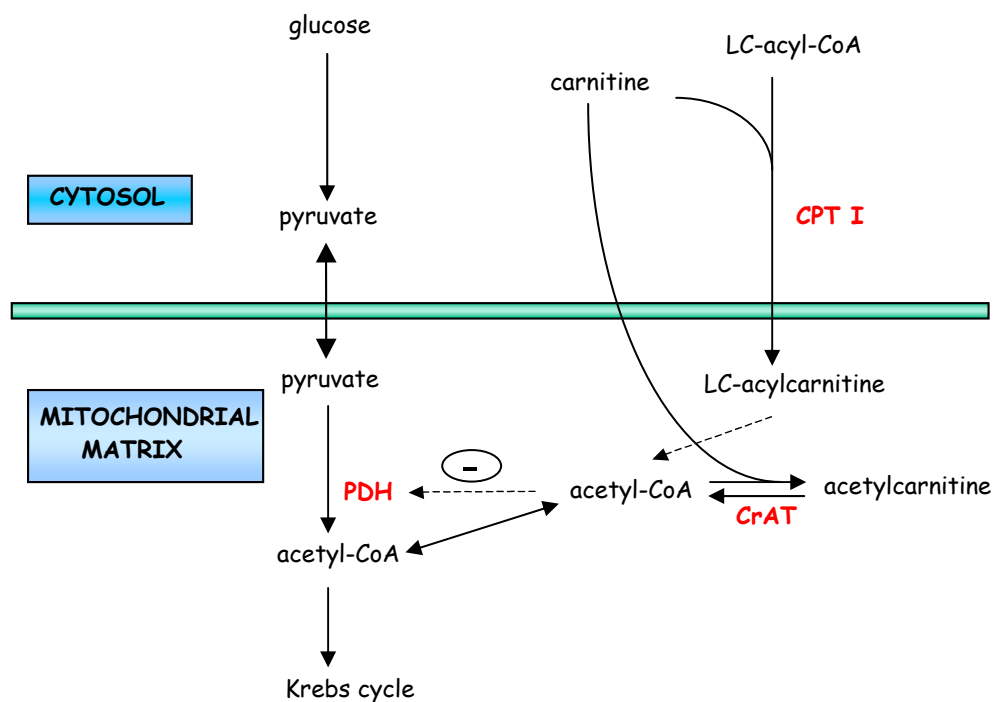


Fig. 7. **Anticipated effects of increased L-carnitine availability on cardiac glucose-fatty acid interactions.** The mass-action effect of carnitine on CPT I activity is counteracted by that on CrAT, which lowers intra-mitochondrial acetyl-CoA, and thus releases PDH inhibition, to allow higher rates of pyruvate oxidation.

6. ENZYMOLOGY OF CARNITINE ACETYLTRANSFERASE

The reaction catalyzed by CrAT is fully reversible and has an equilibrium constant of 1.7 (Fritz, 1963; Chase, 1966). For pigeon CrAT, the reaction follows a random-order equilibrium (random Bi-Bi) mechanism (Chase, 1966). In this mechanism, the Michaelis constants (K_m), approximate true dissociation constants (K_s), and binding of one substrate has little or no effect on binding of the second (Fig. 8).

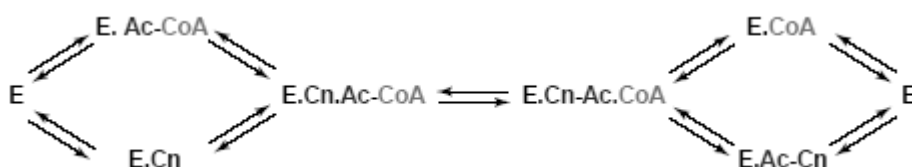


Fig. 8. **Enzyme kinetics of carnitine acyltransferases.** This diagram emphasizes the random order of substrate addition and that the reaction is freely reversible. Cn, carnitine; Ac, acetyl group.

Table 2 shows the K_m for purified CrATs obtained from several sources. All CrATs bind CoA (or acyl-CoA) more tightly than they bind carnitine (or acetylcarnitine), probably because of the greater molecular surface area of CoA.

Organism	Tissue-organelle	K_m (μM)			
		Acetyl-L-carn	CoA	L-carnitine	Acetyl-CoA
Pigeon ¹	Breast muscle homogenate	350	37	120	34
Mouse ²	Liver peroxisomes	700	180	86	15.3
Rat ³	Liver mitochondria	280		720	30
Yeast ⁴	Mitochondria	639	257	622	36

Table 2. **Comparison of the K_m for substrates of purified carnitine acetyltransferases.** References: (1) Chase, 1967; (2) Farrell, 1984; (3) Mittal, 1980; (4) Ueda, 1982.

6.1. ACYL-GROUP SELECTIVITY

CrAT has a substrate preference for short-chain acyl-CoAs. Yeast CrAT has exceptional selectivity for acetyl (C₂), whereas for most of the isolated CrATs, propionyl (C₃) or butyryl (C₄) rather than acetyl is the optimal substrate (Table 3). Chain lengths greater than C₄ show diminished activity. Neither palmitoylcarnitine (C₁₆) nor palmitoyl-CoA is a substrate, but the latter is a potent inhibitor. Palmitoyl-CoA binds quite tightly (its inhibition constant or $K_i = 0.43 \mu\text{M}$), about two orders of magnitude stronger than acetyl-CoA ($K_m = 34 \mu\text{M}$), with which it competes reversibly. Palmitoyl-CoA also competes with L-carnitine and inhibition is observed with other medium- and long-chain acyl-CoAs (see section 6.2). Unsaturated esters such as acryloyl-, 4-pentenoyl-, and sorboyl-CoA bind as tightly to CrAT as their saturated analogs propanoyl-, pentanoyl-, and hexanoyl-CoA, although they exhibit diminished activities compared to their saturated counterparts (Chase, 1967; Holland, 1973).

Organism	Tissue-organelle	Acyl-chain length								
		C ₂	C ₃	C ₄	C ₆	C ₈	C ₁₀	C ₁₂	C ₁₄	C ₁₆
Pigeon ¹	Breast muscle homogenate	100	77	41	13	8	4	< 0.1	0	0
Pig ²	Heart	100	123	100	23	13	3	-	-	0
Mouse ³	Liver peroxisomes	100	121	132	26	24	16	1	0	0
Rat ⁴	Liver mitochondria	100	-	124	15	7	2	0	0	0
Yeast ⁵	Mitochondria	100	15	0.26	0	-	0	-	-	0

Table 3. Acyl-group selectivity of different purified CrATs measured as a percentage of the velocity of the acetyl reaction. References: (1) Chase, 1967; (2) Fritz, 1963; (3) Farrell, 1984; (4) Miyazawa, 1983a; (5) Ueda, 1982.

6.2. EFFECT OF CHAIN LENGTH ON CrAT KINETIC CONSTANTS

It has been reported that the binding of medium-chain acyl-CoAs (C₈, C₉ and C₁₀) to CrAT lowers its affinity for L-carnitine, that is, the K_m of the enzyme for L-carnitine is positively correlated with acyl-CoA concentration (Chase, 1967). The interaction

between acyl-CoA and L-carnitine binding for medium-chain acyl-CoAs is in marked contrast to the independent characteristic of the short-chain substrates.

In the same study, Chase demonstrated that K_m 's for acyl-CoA are invariant over the range C₂ to C₇ ($38 \pm 6 \mu\text{M}$), but as their chain length increases from C₈ to C₁₆, acyl-CoAs bind more tightly to the free enzyme (K_m 's drop rapidly) (Table 4). This is due to the fact that the ΔG° of binding ($\Delta G^\circ = -RT \ln K_m$) changes linearly with each additional methylene ($-0.31 \text{ kcal/mol per CH}_2$) beyond C₈. It was concluded that CrAT contains a hydrophobic region that interacts with the side chain of medium- and long-chain acyl-CoAs and thereby hinders the binding of carnitine. An inhibition mechanism for palmitoyl-CoA was proposed involving carnitine and hydrocarbon recognition sites. Competition between the sites is regulated by conformational changes in the protein (Tipton, 1969). Because palmitoylcarnitine is not an inhibitor or substrate, binding at the carnitine site may disrupt the remote hydrocarbon recognition site. Correspondingly, a filled hydrocarbon site may either hinder access to the carnitine site or prevent conformational changes of the enzyme that are required for proper carnitine binding. The finding that the palmitate ion only moderately inhibits pigeon CrAT and is not an inhibitor of rat liver CrAT (Mittal, 1980) suggest that a filled CoA site is required for the overall inhibition mechanism.

Acyl-CoA	K_m for L-carnitine (μM)	K_m for acyl-CoA (μM)	K_i for acyl-CoA (μM)
C ₂ -CoA	120	34	-
C ₄ -CoA	159	44	-
C ₆ -CoA	172	33	-
C ₈ -CoA	131	32	-
C ₁₀ -CoA	100	11	8.8
C ₁₂ -CoA	-	-	3.4
C ₁₄ -CoA	-	-	1.5
C ₁₆ -CoA	-	-	0.43

Table 4. Kinetic constants (K_m and K_i) for the reaction between L-carnitine and a range of acyl-CoAs for pigeon CrAT. Data taken from Chase (1967).

7. STRUCTURE AND FUNCTION OF CARNITINE ACYLTRANSFERASES

Although the first crystals of CrAT were observed in 1965 (Chase, 1965), it was not until 2003 that the first structure of an enzyme of the carnitine/choline acyltransferase family was solved. That year, the three-dimensional (3-D) structure of mouse (Jogl, 2003) and human CrAT (Wu, 2003) were solved almost simultaneously. Mouse CrAT was crystallised alone and in complex with its substrate CoA or carnitine. Although human CrAT was first crystallised alone, more recently the 3-D structure of the complex with L-carnitine has also been reported (Govindasamy, 2004a). The crystal structure of mouse COT has been solved recently, alone and in complex with octanoylcarnitine, showing for the first time the structure of the acyl moiety binding site in the carnitine acyltransferase family (Jogl, 2005). Moreover, the 3-D structure of rat ChAT has also been reported by two independent groups (Cai, 2004; Govindasamy, 2004b). A summary of the crystal structures of the carnitine/choline family is shown in Table 5.

Currently, no 3-D structures of the members of the family that catalyses long-chain acyl-CoAs as substrates (CPT I and CPT II) have been elucidated. However, a structural model for L-CPT I was recently published based on the similarity (~30%) of this enzyme to the crystallized mouse CrAT (Morillas, 2004).

The structure of CrAT reveals a monomeric protein composed of two domains, N and C domains (Jogl, 2003; Wu, 2003). Surprisingly, the N and C domains share the same backbone fold, despite the lack of any recognizable amino acid sequence homology between them (Fig. 9A). These domains are interconnected and create a tunnel that passes through the centre of the enzyme. This tunnel defines a solvent-accessible surface in the centre of the protein, which constitutes the active site.

The overall structure of COT (Jogl, 2005) is remarkably similar to that of CrAT, consistent with the fact that the two enzymes share 36% amino acid sequence identity. However, significant differences are observed in the acyl group binding region of the active site, which defines the differing acyl-CoA substrate specificity of the two enzymes.

	Mouse CrAT	Human CrAT	Mouse COT	Rat ChAT
Resolution (free enzyme)	1.8 Å [1NDB] ¹	1.6 Å [1NM8] ²	2.0 Å [1XL7] ³	2.5 Å [1Q6X] ⁴ 1.55 Å [1T1U] ⁵
Residues in atomic model	30-625 (of 626)	30-620 (of 626)	11-612 (of 612)	18-617 (of 645)
Substrate cocrystallised	Carnitine [1NDF] ¹ CoA [1NDI] ¹	Carnitine [1S5O] ⁷	Octanoylcarnitine [1XL8] ³	
Secondary elements	16 β-strands / 20 α-helices	14 β-strands / 17 α-helices	16 β-strands / 20 α-helices	18 β-strands / 22 α-helices
Mutants crystallised	M564G [1T7N/1T7O] ⁶ F565A [1T7Q] ⁶		C323M [1XMC] ³ M335V [1XMD] ³	

Table 5. **Summary of the crystal structures in the carnitine/choline acyltransferase family.** Protein Data Bank (PDB) accession number is shown in square brackets. References: (1) Jogl, 2003; (2) Wu, 2003; (3) Jogl, 2005; (4) Cai, 2004; (5) Govindasamy, 2004b; (6) Hsiao, 2004; (7) Govindasamy, 2004a.

Despite the lack of any sequence conservation, the backbone fold of the N and C domains of CrAT is similar to that of chloramphenicol acetyltransferase (CAT) (Leslie, 1988) as well as the catalytic domain of dihydrolipoyl transacetylase (E2pCD) (Mattevi, 1993). Both CAT and E2pCD function as trimers and catalyse the transfer of an acetyl group from acetyl-CoA to an organic substrate. The active site of these enzymes is located at the interface between neighbouring monomers of the trimer, and the catalytic histidine residue is located at the same position in all these enzymes. The carnitine acyltransferases may have evolved by gene duplication of a single domain enzyme such as E2pCD or CAT.

7.1. THE ACTIVE SITE TUNNEL AND THE CATALYTIC HISTIDINE

The active site of CrAT and COT is located at the interface between the N and C domains (Fig. 9A). The catalytic base of carnitine acyltransferases has been identified by biochemical and mutagenesis studies as a histidine residue, equivalent to His³⁴³ in CrAT. Removal of the corresponding homologous residues in rat CPT I, CPT II, and COT has been shown to inactivate these enzymes completely (Morillas, 2001; Brown, 1994; Morillas, 2000).

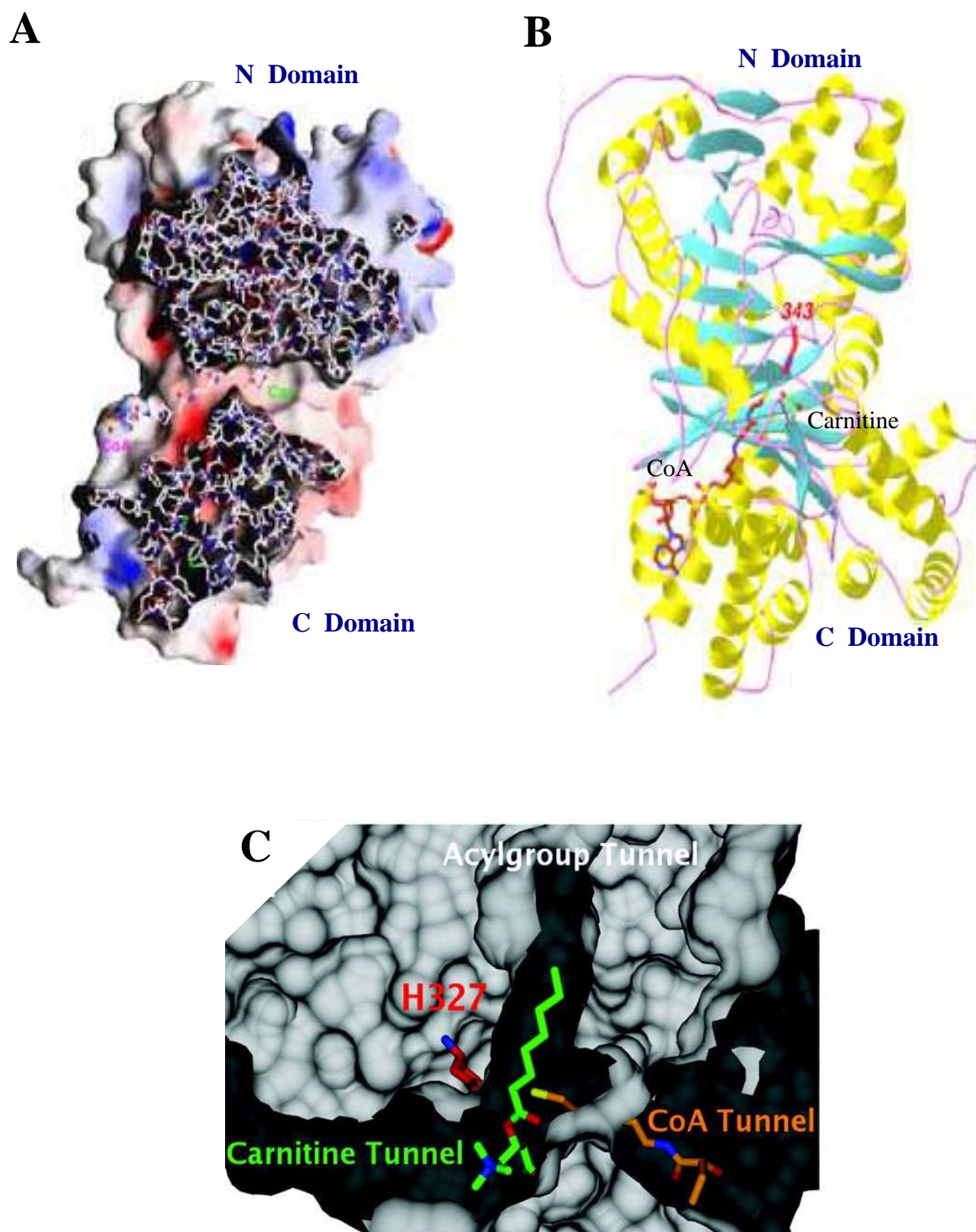


Fig. 9. **The active site tunnel in CrAT and COT.** A) Molecular surface of CrAT showing the tunnel that extends through the center of the molecule. B) Schematic drawing of the structure of CrAT. The side chain of the catalytic His³⁴³ residue is shown in red and carnitine is shown in green. C) The three tunnels for binding carnitine, CoA, and the acyl group in the active site of COT; the catalytic histidine (His³²⁷) is shown in red. Reproduced from Jogl (2004) and Jogl (2005).

The catalytic histidine is positioned at the centre of the active site tunnel and is accessible from opposite surfaces of the enzyme through two channels: one of the channels is for binding the carnitine substrate, whereas the other is for CoA (Fig. 9A and 9B). This is consistent with the rapid equilibrium random order kinetics proposed for CrAT (Fig. 8) (Chase, 1966). In the structure of COT, a third channel extends from the active site towards the core of the N domain. This is the channel for binding the medium-chain acyl group of the substrate (Fig. 9C). The side chain of the catalytic histidine forms a hydrogen bond with the 3-hydroxyl group of carnitine. Hence, the carnitine substrate is positioned perfectly for the withdrawal of its hydroxyl proton by the catalytic base (Jogl, 2003).

7.2. THE CARNITINE BINDING SITE

The crystal structure of mouse CrAT in complex with carnitine was determined at 1.9 Å resolution (Jogl, 2003). Carnitine is bound in a partially folded conformation, which is one of the favoured rotamers of this compound in solution (Colucci, 1986). There are only minor conformational changes in the enzyme upon binding of carnitine.

The carboxylate group of carnitine is involved in a network of hydrogen-bonding interactions, as well as electrostatic interactions with the side chain group of Arg⁵¹⁸. The side chain hydroxyls of Tyr⁴⁵², Ser⁴⁵⁴, and Thr⁴⁶⁵ provide three hydrogen-bonding partners for the carboxylate group, whereas the fourth partner is a water molecule (Fig. 10). Thr⁴⁶⁵ and Arg⁵¹⁸ are conserved among the carnitine acyltransferases, but they are substituted for neutral amino acids (Val⁴⁵⁹ and Asn⁵¹⁴, respectively) in ChAT. Mutation of the homologous Arg to Asn in bovine COT (R505N) led to a 1650-fold increase in the K_m for carnitine but had little effect on the K_m for CoA or the K_{cat} of the enzyme (Cronin, 1997a). This finding indicates that an Arg residue plays a critical role in carnitine binding in the carnitine acyltransferase family.

In comparison, the trimethylammonium group of carnitine does not show many specific interactions with the enzyme, and its positive charge is not recognized by a negatively charged residue from the enzyme. Instead, the group is located in a mostly hydrophobic environment, near the side chains of Ser⁵⁵², Phe⁵⁶⁶, and Val⁵⁶⁹. Ser⁵⁵² is

the first residue of the strictly conserved STS motif among the carnitine acyltransferases. Mutation of this Ser to Ala in bovine COT (S542A) caused a 17-fold increase in the K_m for carnitine (Cronin, 1997b). Structural analysis suggests the positive charge on carnitine may be important for the catalytic activity of these enzymes (see section 7.4).

Recently, Govindasamy (2004a) crystallised human CrAT in complex with L-carnitine and characterised some of the L-carnitine binding residues via site-directed mutagenesis. Replacement of the amino acid Tyr⁴⁵² with Phe (Y452F) and of Thr⁴⁶⁵ with Ala (T465A), increased the K_m value for L-carnitine by 326-fold and 68-fold, respectively. Each of these two mutations is expected to eliminate a hydrogen-bonding interaction between the enzyme and the carboxylic group of L-carnitine. Mutation of the charged Arg⁵¹⁸ to an uncharged residue (R518Q) increased the L-carnitine K_m value by 232-fold. This substitution is expected to disrupt the electrostatic interaction between the enzyme and the carboxylate group of carnitine. This result is consistent with the previous study conducted with COT (Cronin, 1997a).

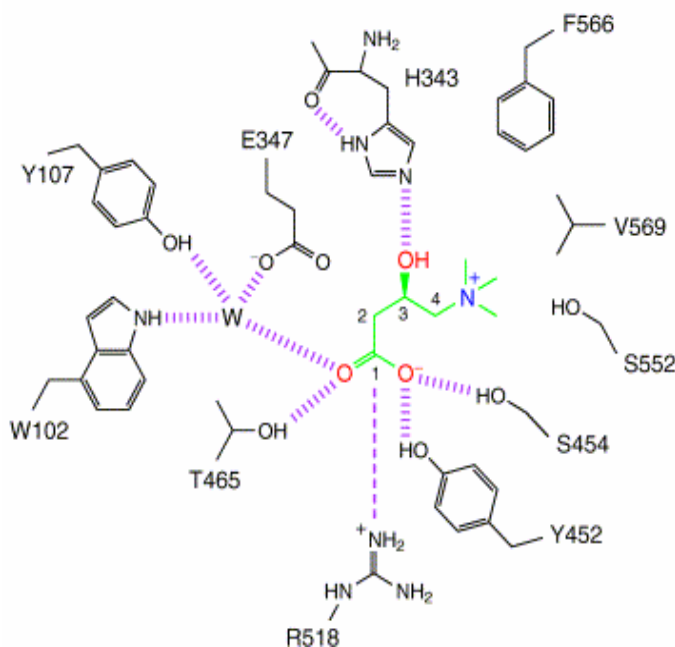


Fig.10. **The carnitine binding site of mouse CrAT.** Schematic drawing of the interactions between carnitine (green) and CrAT. Hydrogen-bonding interactions are shown as thick dashed lines and the electrostatic interaction between the carboxylate group and Arg⁵¹⁸ is shown as a thin dashed line. Reproduced from Ramsay (2003).

Finally, Phe⁵⁶⁶ was replaced by Ala and Tyr. The mutation to Ala caused an 18-fold increase in the K_m for L-carnitine, whereas replacement with Tyr, an amino acid that conserves the aromatic ring, caused only minor changes in the kinetic parameters (Govindasamy, 2004a). These results demonstrate the importance of the presence of an aromatic group at this position in order to interact with the quaternary nitrogen of L-carnitine through π -cation interactions (Fig. 10). The Phe at this position is conserved in all carnitine acyltransferases except for COT, where the equivalent residue is a valine (Val⁵⁵⁵). In COT, the Phe⁵⁶⁶ has a compensatory effect and helps to maintain the overall shape and the hydrophobic nature of the trimethylammonium binding site (Fig. 12A).

7.3. THE FATTY ACID BINDING SITE

7.3.1. The binding site for short-chain acyl groups

In CrAT, the acetyl group of acetylcarnitine, modeled based on the structure of the carnitine complex, points towards a shallow hydrophobic pocket that is enclosed by the intersection of the two β sheets in the enzyme (Fig. 11). This pocket is partly occupied by the bulky side chain of Met⁵⁶⁴, which may be part of the molecular basis of the preference for short-chain acyl groups by CrAT.

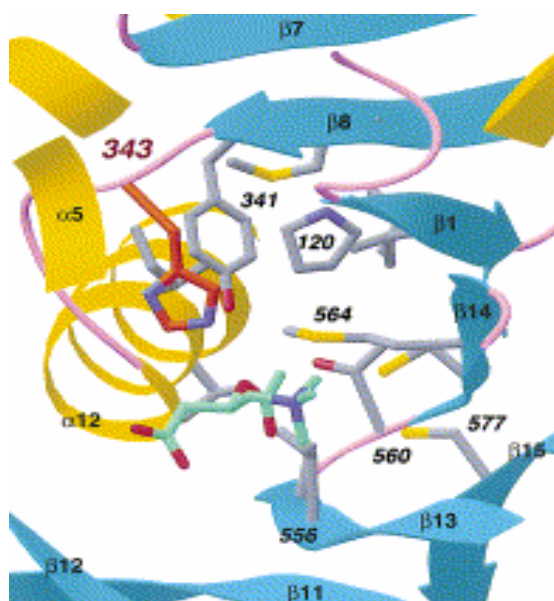


Fig. 11. **The possible fatty acid binding site of carnitine acetyltransferases.** A model for the binding mode of acetylcarnitine is shown. The catalytic histidine is shown in red and the acetylcarnitine molecule is shown in green. Reproduced from Jogl (2003).

7.3.2. The binding site for medium-chain acyl groups

The crystallization of COT in complex with octanoylcarnitine revealed for the first time the structure of the acyl group binding site in the carnitine acyltransferase family (Jogl, 2005). The fatty acid binding pocket in COT is lined throughout its entire length with hydrophobic residues. These define a narrow, cylindrical binding site, which represents a good fit to the contour of acyl groups (Fig. 12B). With the exception of Met³³⁵ and Cys³²³, residues in this binding site show little conformational change between the free enzyme and the octanoylcarnitine complex, suggesting that the acyl group binding site is essentially preformed in the structure of COT. The side chains of Met³³⁵ and Cys³²³ interact with each other and with the C8 atom of the octanoyl group, and they assume dual conformations in the complex with octanoylcarnitine (Fig. 12A and B). The comparison of COT crystal structures in complex with carnitine and with octanoylcarnitine shows that the presence of the acyl group does not disturb the binding of carnitine.

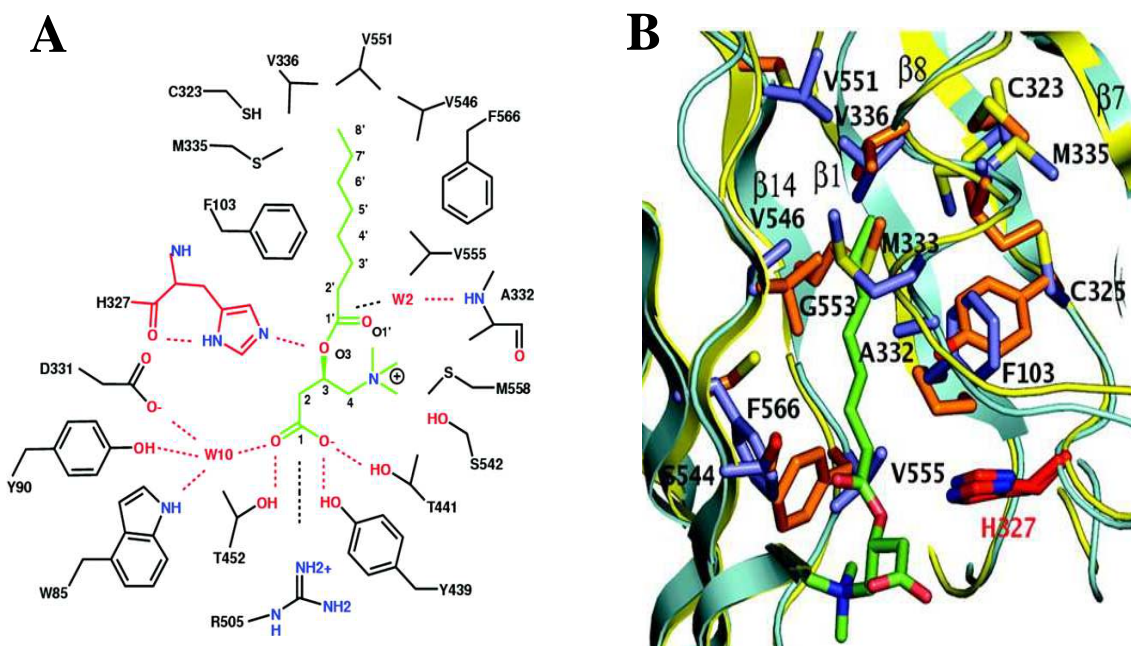


Fig. 12. **The binding mode of octanoylcarnitine** A) Schematic drawing of the interactions between octanoylcarnitine and COT. Hydrogen bonding interactions are shown by dashed red lines. The electrostatic interaction between the carboxylate group and Arg⁵⁰⁵ is shown as a dashed black line. B) Stereo representation of the octanoylcarnitine binding site in COT. His³²⁷ is shown in red. The octanoylcarnitine molecule is shown in green. Reproduced from Jogl (2005).

Sequence and structural variations between COT and CrAT in the acyl group binding site determine their substrate preferences. Remarkably, only three of the residues in this binding site are conserved between CrAT and COT enzymes (Val³³⁶, Ser⁵⁴⁴, and Val⁵⁴⁶ in COT coordinates). Sequence variations in the other residues in the binding site probably play a larger role in determining the substrate preference of the two enzymes. The most important difference between COT and CrAT is the replacement of Gly⁵⁵³ in COT with Met⁵⁶⁴ in CrAT. This residue is also a Gly in CPTs (Gly⁷¹⁰). In CrAT, the bulky side chain of Met⁵⁶⁴ would block the access of medium and long-chain fatty acid to the hydrophobic pocket. In COT, the smaller Gly creates additional space for the binding of medium-chain acyl-CoAs. Interestingly, in L-CPT I, mutation of the orthologous Gly to Glu (G710E) can cause L-CPT I deficiency (Prip-Buus, 2001). This mutation replaces the small glycine in the fatty acid binding pocket with a charged group, which can be expected to be detrimental for substrate binding. Interestingly, Met⁵⁶⁴ is part of a cluster of three residues (⁵⁶³VMS⁵⁶⁵ in rat CrAT) that are replaced by a glycine in the CPTs. These residues are located in strand β 14, which helps enclose the acyl binding site.

Another residue that may be involved in fatty-acyl chain-length specificity is the Met³³⁵ in COT. The side chain of Met³³⁵ assumes dual conformations in the complex with octanoylcarnitine. In one of the conformations, the Met³³⁵ side chain is swung away from the C8 atom, creating a larger acyl group binding pocket. Interestingly, this Met residue is replaced by the smaller Val in the CPTs, an observation which also suggests that this residue might control the access of long-chain substrates. The mutation of Met³³⁵ to Val or Ala in COT produced no changes in substrate preference, but instead showed that this residue may be important for catalysis (Jogl, 2005).

Finally, structural variations among the carnitine acyltransferase classes may also represent the underlying molecular basis for selectivity of fatty acid chain length. The most striking difference is the presence in the CPT I enzymes of a 13-amino acid insertion between two β -strands (strands 13 and 14 in the mouse CrAT structure) that runs directly through the active site. These changes in CPT may form a flexible binding pocket that can accommodate long chain acyl groups that CrAT cannot utilize as substrates (Wu, 2003).

In the present work we addressed whether sequence variations between CrAT and COT are important in determining the substrate preference of both enzymes.

7.4. THE CATALYTIC MECHANISM OF CARNITINE ACYL TRANSFERASES

7.4.1. The catalytic mechanism: substrate-assisted catalysis

For catalysis by the carnitine acyltransferases, the catalytic His acts as a general base to extract the proton from the 3-hydroxyl group of carnitine or the thiol group of CoA, depending on the direction of the reaction (McGarry, 1997). In the structures of the carnitine and CoA complexes, the reactive groups of both substrates are directly hydrogen-bonded to the His³⁴³ side chain (Jogl, 2003). The activated hydroxyl or thiol group can then directly attack the carbonyl carbon in acyl-CoA or acylcarnitine, resulting in the formation of a tetrahedral intermediate (Fig. 13). Finally, this intermediate collapses to release acetylcarnitine and free CoA (Wu, 2003).

Structural analysis suggests that the oxyanion in the tetrahedral intermediate of the reaction may be stabilized by the positive charge of the trimethylammonium group of carnitine itself. This is supported by kinetic data showing that the positive charge of carnitine is not critical for binding, but is absolutely required for catalysis (Saeed, 1993). Thus, carnitine acyltransferases are an example of substrate-assisted catalysis.

The oxyanion should also be able to hydrogen-bond with the side-chain hydroxyl of Ser⁵⁵⁴, the last serine in the strictly conserved STS motif. However, Ser⁵⁵⁴ probably makes only a minor contribution to the catalysis by the carnitine acyltransferases as mutation of this residue in bovine COT produced only a 10-fold decrease in k_{cat} , while having little impact on the K_m for carnitine (Cronin, 1997b).

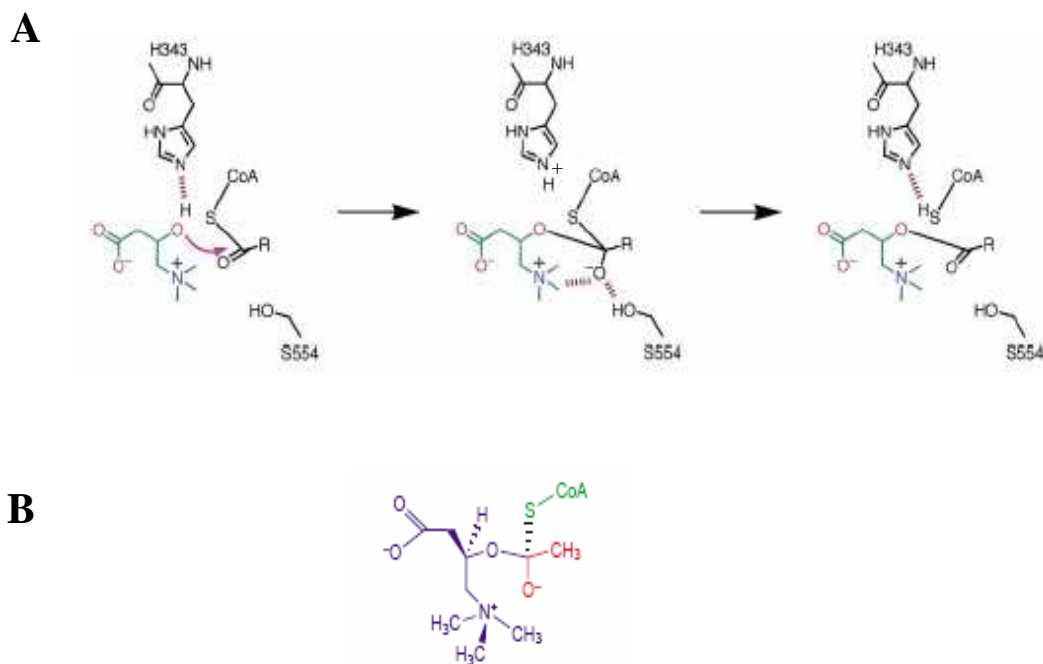


Fig. 13. **The catalytic mechanism of carnitine acyltransferases.** A) The catalytic His³⁴³ residue can extract the proton from either carnitine or CoA. The oxyanion in the tetrahedral intermediate is stabilized by interactions with carnitine and the side-chain hydroxyl of Ser⁵⁵⁴. B) Structure of the tetrahedral intermediate anion. The conformation shown illustrates how the trimethylammonium ion can stabilize the intermediate anion (termed substrate-assisted catalysis). Reproduced from Jogl (2003).

7.4.2. A catalytic dyad?

The use of a Ser-His-Asp catalytic triad to perform catalysis has been observed in a variety of enzymes, including proteases, lipases and acetylcholine esterase (Sussman, 1991). In the case of the serine proteases, particularly chymotrypsin (Steitz, 1982), deprotonation of a Ser by His facilitates nucleophilic attack of the activated Ser on the substrate. The transfer of a proton from the Ser to the His is facilitated by a charge relay system involving an Asp residue.

Some authors have speculated on the existence of a catalytic mechanism similar to a catalytic triad in carnitine/choline acyltransferases, but one in which the hydroxyl group of carnitine replaces the hydroxyl of Ser to act as the nucleophile toward the acyl-CoA substrate (Brown, 1994). This model is supported by the results of kinetic experiments (a´Bhaird, 1993; Chase, 1966) and mutagenesis studies (Brown, 1994).

In the structure of murine CrAT in the absence of ligands, His³⁴³ is hydrogen bonded to Glu³⁴⁷ (which is either conserved or conservatively substituted to Asp), suggesting the presence of a charge relay system (Jogl, 2003). This interaction is also shown in the human CrAT crystal (Wu, 2003), and probably potentiates the activity of His³⁴³ and stabilizes the positive charge that would develop after substrate deprotonation. In rat CPT II, the substitution of Asp³⁷⁶ (homologous to Glu³⁴⁷) with Ala produced a completely inactive protein (Brown, 1994). Furthermore, in rat L-CPT I substitution of the homologous Asp⁴⁷⁷ with Ala nearly abolished its catalytic activity (Morillas, 2004). Thus, it appears that carnitine acyltransferases might utilize a His-Glu/Asp dyad to carry out catalysis (Fig. 14).

In the rat ChAT crystal, the same hydrogen-bonding interaction has been observed between the catalytic histidine (His³³⁴) and a highly conserved glutamate (Glu³³⁸), a finding which suggests a similar catalytic mechanism (Govindasamy, 2004b).

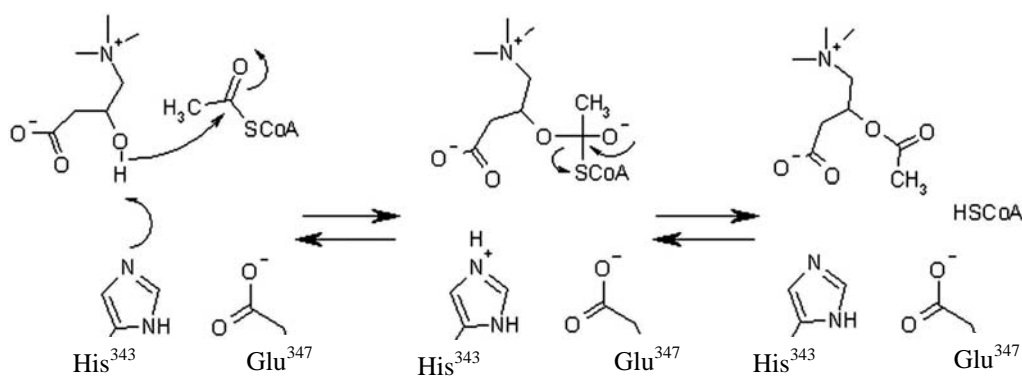


Fig.14. **Carnitine acetyltransferase catalytic mechanism involving a catalytic dyad.** In the forward reaction, His³⁴³ acts as a general base to deprotonate the primary alcohol of carnitine, resulting in a positively charged histidine. Glu³⁴⁷ serves to polarize the histidine to increase catalytic activity while also stabilizing the positive charge that develops on the histidine ring. The catalytic residues His³⁴³ and Glu³⁴⁷ as well as carnitine, acetyl-CoA, CoA, and acetylcarnitine are indicated. Reproduced from Wu (2003).

Table 6 summarizes the amino acid residues that are important for catalysis and binding of acyl-CoA and carnitine substrates in the carnitine acyltransferase family of proteins.

Introduction

Residue (CrAT)	Role	Substitution (Enzyme)	Effect	References
<i>Catalysis</i>				
His ³⁴³	Catalytic histidine	H473A (CPT I)	Inactive protein	Morillas, 2001
		H372A (CPT II)	Inactive protein	Brown, 1994
		H327A (COT)	Inactive protein	Morillas, 2000
		H343A (CrAT)	Inactive protein	Wu, 2003
Glu ³⁴⁷	Hydrogen bond with His ³⁴³	D376A (CPT II)	Inactive protein	Brown, 1994
		D477A (CPT I)	Loss 98% activity	Morillas, 2004
Ser ⁵⁵⁴	Stabilization of reaction intermediate	S544A (COT)	Decrease in k_{cat}	Cronin, 1997b
		S687A (CPT I)	Inactive protein	Morillas, 2004
<i>Carnitine binding</i>				
Tyr ⁴⁵²	Hydrogen bond with carboxylate group	Y452F (CrAT)	Increase in K_m carnitine	Govindasamy, 2004
Ser ⁴⁵⁴	Hydrogen bond with carboxylate group			
Thr ⁴⁶⁵	Hydrogen bond with carboxylate group	T465A (CrAT)	Increase in K_m carnitine	Govindasamy, 2004
Arg ⁵¹⁸	Electrostatic interaction with carboxylate group	R505N (COT)	Increase in K_m carnitine	Cronin, 1997a
		R518Q (CrAT)	Increase in K_m carnitine	Govindasamy, 2004
Ser ⁵⁵²	Close to the trimethylammonium group	S542A (COT)	Increase in K_m carnitine	Cronin, 1997b
Thr ⁵⁵³	Hydrogen bond with Tyr ⁴⁵²	T543A (COT)	Increase in K_m carnitine	Cronin, 1997b
		T686A (CPT I)	Inactive protein	Morillas, 2004
Phe ⁵⁶⁶	π -cation interaction with the trimethylammonium group	P566A (CrAT)	Increase in K_m carnitine	Govindasamy, 2004
		P566Y (CrAT)	No change K_m carnitine	Govindasamy, 2004
Val ⁵⁶⁹	Hydrophobic interaction with trimethylammonium group			
<i>Fatty acid binding site</i>				
Met ⁵⁶⁴	Blocks entry of fatty acid binding site	M564G (CrAT)	Increased activity with MCFA-CoA	Hsiao, 2004
		G710E (CPT I)	Inactive protein	Prip-Buus, 2001
		G553M (COT)	Loss of activity with MCFA-CoA	Jogl, 2005

Table 6 .Summary of amino acid residues that are important for catalysis and binding of the acyl-CoA and carnitine substrates in carnitine acyltransferases. MCFA-CoA: medium-chain acyl-CoAs.

8. STRUCTURE OF CHOLINE ACETYLTRANSFERASE

ChAT (E.C. 2.3.1.6) is the fourth member of the carnitine/choline acyltransferase family. CrAT and ChAT catalyze a similar reaction, with the difference that in ChAT the acetyl group from acetyl-CoA is transferred to choline instead of carnitine. ChAT is responsible for catalyzing the biosynthesis of the neurotransmitter acetylcholine (ACh) from its precursors acetyl-CoA and choline (Fig. 15)

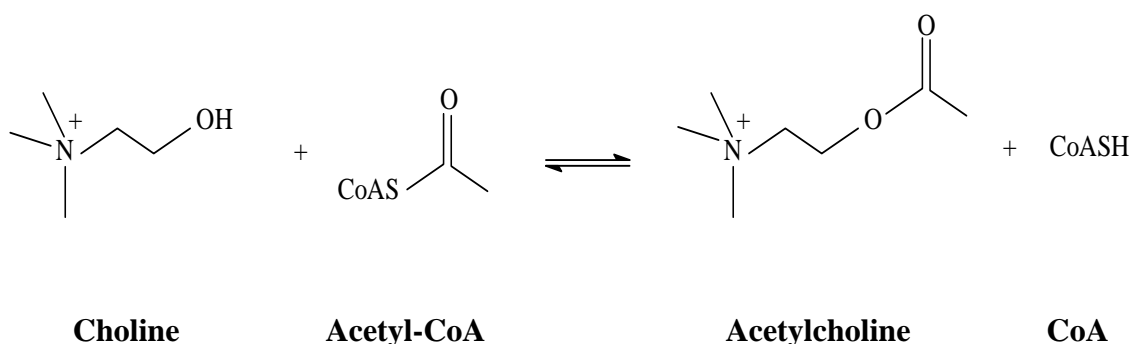


Fig. 15. The reaction catalyzed by choline acyltransferase.

ACh functions in the cholinergic neurons of the peripheral and central nervous systems. In the peripheral nervous system, ACh stimulates muscle contraction, while in the central nervous system it facilitates learning and short-term memory formation. Diminished ChAT activity signals damage to cholinergic neurons in a number of neurodegenerative disorders, including Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis. Schizophrenia and sudden infant death syndrome are also correlated with decreased ChAT activity (Oda, 1999).

The crystal structure of rat ChAT can be divided into two structural domains and the active site is located in a solvent-accessible tunnel at the domain interface. As expected from the 42% sequence identity, ChAT and CrAT share similar backbone structures, and the overall structure of the active site region is similar in both enzymes (Cai, 2004).

8.1. MODEL FOR CHOLINE BINDING AND SUBSTRATE DISCRIMINATION

The model for choline binding in ChAT shows that ChAT binding interactions with choline mimic the interactions between CrAT and the choline-like portion of carnitine. As in CrAT, the active site histidine (His³³⁴) could hydrogen bond to the hydroxyl group of choline. The positively charged trimethylammonium group of choline would form a cation- π interaction with the side chain of an aromatic residue, in this case Tyr⁵⁶² rather than the Phe⁵⁶⁶ in CrAT, and has hydrophobic interactions with the side-chain residues Ser⁵⁴⁸ and Val⁴⁶⁵ (Fig. 16).

Despite the similarity of the ChAT and CrAT choline-binding sites, ChAT shows a strong preference for choline rather than carnitine as a substrate, with a greater than 1,000,000-fold difference in catalytic efficiency values (Cronin, 1998). The difference between these two substrates is that carnitine has an additional carboxymethyl group that replaces a hydrogen at C₁ of the choline (Fig.16). Thus, the presence of the extra carboxymethyl group in carnitine greatly diminishes its affinity for ChAT.

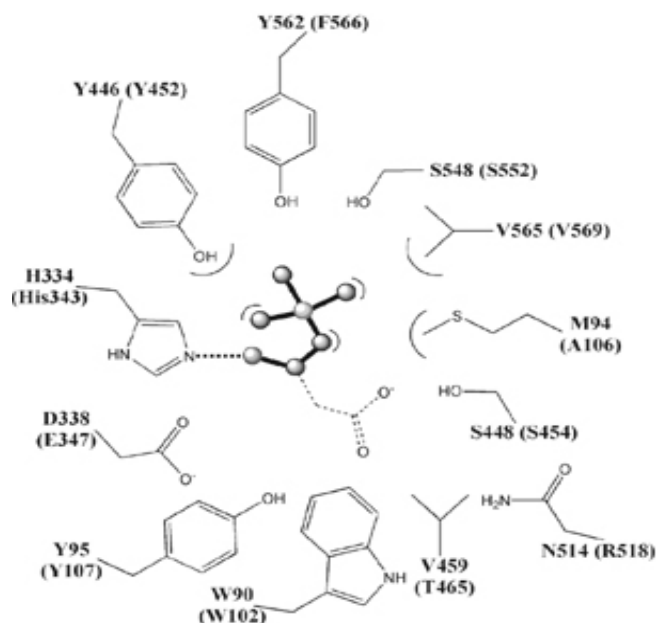


Fig. 16. **Diagram of choline and side chains in ChAT binding site.** The additional carboxylate group in carnitine is indicated by dashed lines. The equivalent residues in CrAT are indicated in brackets. Reproduced from Cai (2004).

There are three sequence differences between CrAT and ChAT near the carboxymethyl group that are important in establishing this level of substrate discrimination (Table 7).

Two of the residues that play critical roles in rat CrAT by interacting electrostatically with the carboxylate group of carnitine, Thr⁴⁶⁵ and Arg⁵¹⁸, are substituted in rat ChAT by neutral amino acids (Val⁴⁵⁹ and Asn⁵¹⁴). When both residues were mutated in rat ChAT to their counterparts in CrAT (V459T and N514R), along with the change N461T, the resulting ChAT mutant showed an increase in catalytic efficiency towards carnitine (Cronin, 1998). In addition to these electrostatic interactions, steric factors may also contribute to carnitine/choline discrimination in ChAT. The bulky Met⁹⁴ in ChAT is substituted by the smaller Ala¹⁰⁶ in CrAT. The voluminous side chain of Met⁹⁴ in ChAT would sterically overlap with the position that would be occupied by the charged carboxylate group of carnitine, impeding the correct positioning and catalysis of this larger substrate.

Group	Residue			Intermolecular interaction
	Mouse CrAT	Mouse COT	Rat ChAT	
<i>Hydroxyl</i>	His ³⁴³	His ³²⁷	His ³³⁴	Hydrogen bond
<i>Trimethylammonium</i>	Ser ⁵⁵²	Ser ⁵⁴²	Ser ⁵⁴⁸	π -cation
	Phe ⁵⁶⁶	^a Val ⁵⁵⁵	^b Tyr ⁵⁶²	
	Val ⁵⁶⁹	^b Met ⁵⁵⁸	Val ⁵⁶⁵	
<i>Carboxylate</i>	Tyr ⁴⁵²	Tyr ⁴³⁹	Tyr ⁴⁴⁶	Hydrogen bond
	Ser ⁴⁵⁴	^b Thr ⁴⁴¹	Ser ⁴⁴⁸	Hydrogen bond
	Thr ⁴⁶⁵	Thr ⁴⁵²	^a Val ⁴⁵⁹	Hydrogen bond
	Arg ⁵¹⁸	Arg ⁵⁰⁵	^a Asn ⁵¹⁴	Electrostatic

Table 7. **Choline and CoA substrate binding sites for CrAT, COT and ChAT.** Non-conservative and conservative substitutions are shown in red and blue, respectively. ^aIn COT, the amino acid Phe⁵⁶⁶ (CrAT Cys⁵⁷⁷) compensates for the loss of an aromatic ring at this position. ^bIn COT, Val⁵⁵⁵ would compensate for the substitution of the Val⁵⁶⁹ in CrAT with Met.

9. BIOTECHNOLOGICAL APPLICATIONS OF CARNITINE ACETYLTRANSFERASE IN YEAST

9.1. ROLE OF CARNITINE AND CARNITINE ACETYLTRANSFERASE IN YEAST

Unlike most eukaryotic organisms, the yeast *Saccharomyces cerevisiae* is unable to synthesise carnitine *de novo*. Furthermore, wild-type strains appear not to require carnitine under any of the conditions investigated thus far (van Roermund 1995, van Roermund, 1999). However, enzymatic activities that require carnitine, in particular carnitine acetyltransferases, are present in *S. cerevisiae*, and genes encoding a plasma membrane transport protein (*AGP2*) and a mitochondrial carnitine:acetylcarnitine translocase (*CRC1*) have also been identified (van Roermund, 1999) (Fig. 17).

In mammalian cells, the role of carnitine and carnitine acyltransferases entails the transfer of long-chain fatty acids to the mitochondria for β -oxidation via the CPT system, as well as the transport of medium- and short-chain organic acids from the peroxisomes to the mitochondria, via COT and CrAT activities, respectively. In *S. cerevisiae*, the role of carnitine is more limited, and only consists in the transfer of activated acetyl groups of peroxisomal or cytoplasmic origin to the mitochondria (van Roermund 1995; Swiegers, 2001).

Unlike in mammals, degradation of fatty acids in yeast takes place exclusively in the peroxisomes, where they are oxidized completely to acetyl-CoA (Kunau, 1995). The acetyl-CoA produced has to be transported from the peroxisomes to the mitochondria for complete oxidation to CO₂ and H₂O. In yeast, there are two pathways for the transport of acetyl units out of the peroxisomes that can complement each other (van Roermund, 1995) (Fig. 17). The first pathway is the well-known carnitine shuttle, where peroxisomal CrAT catalyses the transfer of the activated acetyl group from CoA to carnitine. The acetylcarnitine is transferred to the mitochondria, where mitochondrial CrAT catalyses the reverse transfer of the acetyl group to free CoA for further metabolism. The second pathway involves peroxisomal conversion of acetyl-CoA into glyoxylate cycle intermediates, followed by the transport of these intermediates to the

mitochondria. Besides its importance when yeast cells are grown on fatty acids, CrAT also plays a role during growth on other non-fermentable sources, such as acetate or ethanol. Catabolism of these compounds results in the cytoplasmatic production of acetyl-CoA, which needs to be transferred to the mitochondria for the production of energy. The transfer of the activated acetyl group can take place through the formation of acetylcarnitine in the cytosol and the subsequent transfer to the mitochondria where it combines with CoA to form acetyl-CoA (Schmalix, 1993, van Roermund, 1995).

9.2. MOLECULAR GENETICS OF YEAST CARNITINE ACETYLTRANSFERASE

In contrast to mammals, which only contain one carnitine acetyltransferase gene, *S. cerevisiae* contains three CrAT-encoding genes. The first gene, *CAT2*, resembles the mammalian *CRAT* gene since it enables the translation of both the mitochondrial and the peroxisomal isoforms (Elgersma, 1995). In yeast, both isoforms are generated by alternative initiation of translation (Ueda, 1998; Elgersma, 1995), whereas in mammals the mechanism involves splicing (Corti, 1994b). Cat2 protein (Cat2p) accounts for more than 95% of the total CrAT activity in yeast cells grown on oleate and galactose (Elgersma, 1995; Kispal, 1993).

CAT2 encodes a protein with an MTS at the N-terminus and a PTS at the C-terminus (the tripeptide AKL). Since expression of the full-length gene produces a protein which is targeted exclusively to the mitochondria, the MTS overrules the PTS when both targeting signals are present. Deletion of the first 22 amino acids in *CAT2*, resulting in a *CAT2* starting at the second in-frame ATG codon (just behind the MTS), produces a protein that is directed exclusively to peroxisomes. Deletion of both motifs, results in a protein that localises to peroxisomes and cytosol, a finding which indicates the presence of an internal PTS in *CAT2* (Elgersma, 1995). The second gene, *YAT1*, codes for a CrAT presumably associated with the outer mitochondrial membrane and Yat1p accounts for an estimated 5% of the total CrAT activity in acetate- and ethanol-grown cells (Schmalix, 1993). Finally, the *YAT2* gene encodes a third CrAT that could be a cytosolic carnitine acetyltransferase (Swiegers, 2001). Yat2p accounts for 50% of the total CrAT activity when cells are grown on ethanol (Fig. 17).

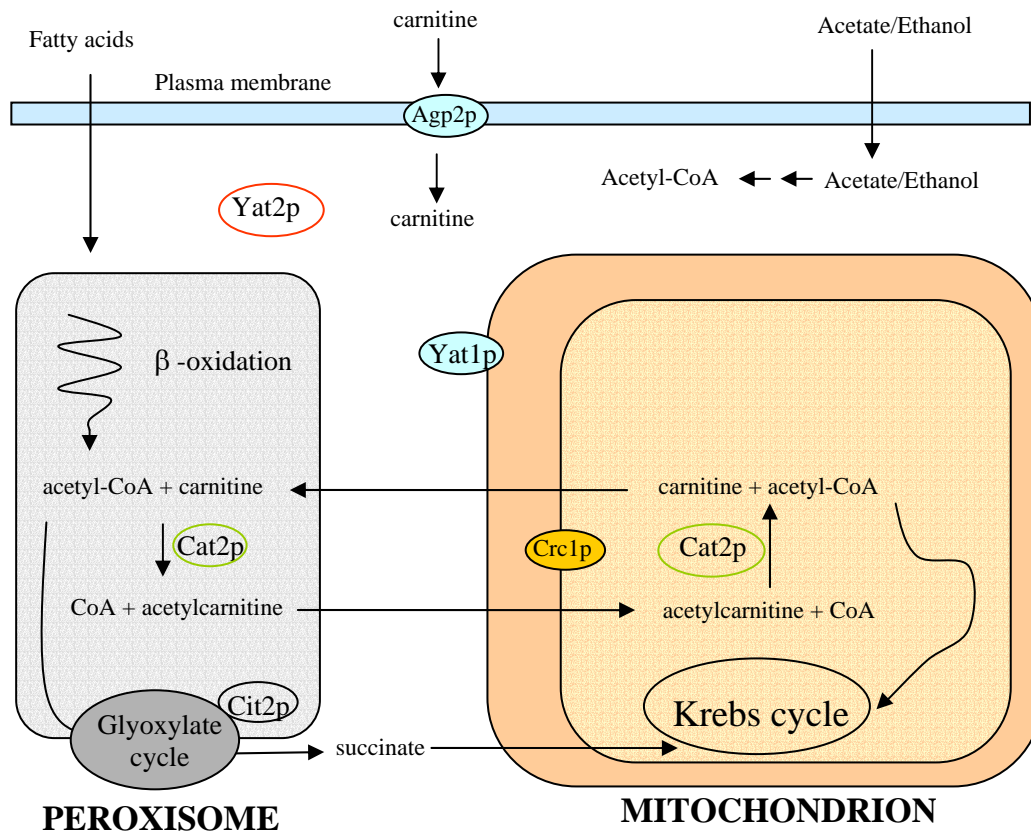


Fig. 17. **Cellular localisation of the proteins involved in carnitine-dependent metabolic activities.** In an *S. cerevisiae* strain lacking the citrate synthase gene ($\Delta cit2$), the cell depends on the carnitine shuttle to transfer acetyl groups to the mitochondria for energy production. Exogenous carnitine, as well as Agp2p, Cat2p, Crc1p, Yat1p and Yat2p, are all essential in a $\Delta cit2$ strain for growth on fatty acids. Cit2p: citrate synthase.

9.2.1. Phenotypes of *CRAT*-deleted strains

Disruption of any of the three CrAT-encoding genes, *CAT2*, *YAT1* and *YAT2*, does not lead to a distinct phenotype compared to wild type: in fact, the deleted strains grow similarly to the wild-type strain on non-fermentable carbon sources. However, when any of these genes is disrupted in an *S. cerevisiae* strain lacking the citrate synthase-encoding gene *CIT2*, which encodes the first enzyme of the glyoxylate cycle, the double mutant strains do not grow on non-fermentable carbon sources, but grow normally on glucose media. Therefore, all CrATs are essential for growth on non-fermentable carbon sources in the absence of the glyoxylate cycle enzyme citrate synthase (Swiegers, 2001). Interestingly, all three CrATs are unable to cross-complement each other, suggesting that each of the proteins has a highly specific activity or they are limited by their cellular localisation.

9.3. MODULATION OF WINE FLAVOUR: A POSSIBLE BIOTECHNOLOGICAL APPLICATION OF CrAT IN YEAST

9.3.1. Volatile esters and wine flavour

During fermentation processes, yeast cells produce a broad range of aroma-active substances which greatly affect the complex flavour of fermented alcoholic beverages such as wine or beer. While these secondary metabolites are often formed only in trace amounts, their concentrations determine the distinct aroma of these beverages. Flavour-active substances produced by fermenting yeast cells can be divided into five main groups: sulphur-containing molecules, organic acids, higher alcohols, carbonyl compounds and volatile esters (Nykänen, 1986; Pisarnitskii, 2001). Of these categories, volatile esters represent the largest and most important group. Alcohol acetates and C₄ to C₁₀ fatty acid ethyl esters are found in the highest concentrations in wine and brandy (Schreier, 1979). The characteristic fruity odours of wine, brandy and other grape-derived alcoholic beverages are primarily due to a mixture of hexyl acetate, ethyl caproate and ethyl caprylate (apple-like aroma), isoamyl acetate (banana-like aroma) and 2-phenylethyl acetate (fruity, flowery favour with a honey note) (Swiegers, 2005).

Esters are formed intracellularly in an enzyme-catalysed reaction between a higher alcohol and an activated acyl-CoA molecule (Fig.18). Hence, the ester production rate is influenced by two primary factors, namely, the concentrations of the two cosubstrates and the total ester synthase activity (Verstrepen, 2003a). Although the physiological role of ester biosynthesis has not yet been demonstrated, it has been suggested that it is the result of the detoxification of toxic long-chain fatty acids and a metabolic process to balance the acetyl-CoA/CoA ratio (Mason, 2000).

The best-known enzymes involved in ester synthesis are the so-called alcohol acetyltransferases (AATases; E.C. 2.3.1.84). These enzymes catalyze the formation of acetate esters from an alcohol and acetyl-CoA (Fig. 18). Three AATases have been identified: AATase I (or Atf1p), Lg-AATase I, and AATase II (or Atf2p). These AATases are encoded by *ATF1*, *Lg-ATF1* and *ATF2*, respectively (Yoshioka, 1981; Yoshimoto, 1998). These enzymes have a low alcohol substrate specificity and high specificity towards acetyl-CoA. Atf1p is known to be located within lipid particles in

the cytosol (Verstrepen, 2004) and plays a major role in the production of isoamyl acetate and to a lesser extent ethyl acetate. In addition to the three known AATases, a possible alcohol acyltransferase, Eht1p (ethanol hexanoyl transferase) has been described (Mason, 2000). This ester synthase is involved in the formation of medium-chain fatty acid esters (C₆-C₁₀).

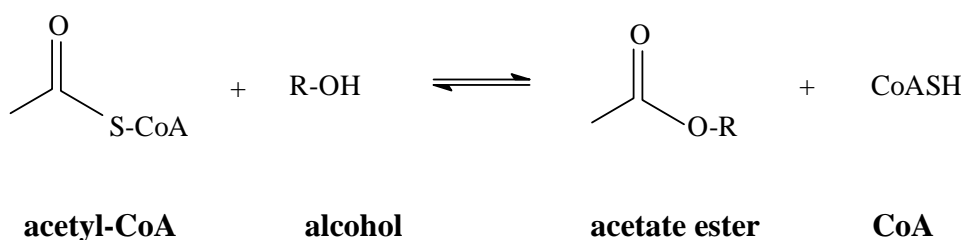


Fig. 18. Reaction catalyzed by alcohol acetyltransferases.

9.3.2. Effect of gene technology on ester production

The food industry is interested in the production of esters for use as flavouring compounds. The ability to create large quantities of an ester or to genetically alter a host for the production of a stronger or altered ester scent would have many industrial applications.

Much attention has been focused on the effect of yeast alcohol acetyltransferase activity on ester production and on the sensory quality of wines (Lilly, 2000; Verstrepen, 2003b). Overexpression of *ATF1* during fermentation in yeast results in a more than 180-fold increase in isoamyl acetate production, as well as a 10 to 200-fold increase in the production of other esters, such as ethyl acetate or phenylethyl acetate (Verstrepen, 2003b). The drastic increases in the levels of these esters have a pronounced effect on the aroma of the fermented products (Lilly, 2000). Overexpression of *ATF2* produces a more modest increase in ethyl acetate and isoamyl acetate levels (Verstrepen, 2003b). These observations could lead to the development of a variety of wine strains for the improvement of the flavour profiles of different types and styles of wine and distillates.

Studies have been performed in *E. coli* on the possible applicability of a CoA/acetyl-CoA manipulation system to increase the productivity of industrially useful compounds derived from acetyl-CoA, such as esters. Overexpression of pantothenate kinase (*panK*), the key regulatory enzyme in the CoA biosynthetic pathway, with simultaneous supplementation of the CoA precursor pantothenic acid leads to an increase in the intracellular levels of CoA and acetyl-CoA (10-fold and 5-fold, respectively) (Vadali, 2004a). In another study, a genetically engineered *E. coli* strain expressing both the yeast *ATF2* gene and the *panK* gene produced 6-fold more isoamyl acetate than the control strain expressing only the *ATF2* gene (Vadali, 2004b). These experiments demonstrate that increasing intracellular CoA/Acetyl-CoA levels leads to an increase in isoamyl acetate production (Fig. 19).

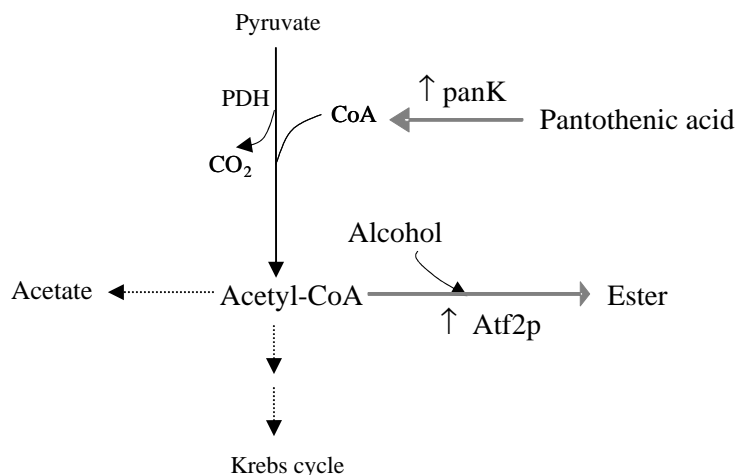


Fig. 19. **Central metabolic pathway of *E. coli* around the acetyl-CoA node, including the newly added ester production pathway.** PDH indicates pyruvate dehydrogenase; *panK*, pantothenate kinase; Atf2p, alcohol acetyltransferase II.

In the present work we have attempted to study the effect of CrAT overexpression on ester production during fermentation in yeast and the biotechnological applications of carnitine acyltransferases.