

The role of carnitine acetyltransferases in the metabolism of *Saccharomyces cerevisiae*

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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20/02/2003

Date

SUMMARY

L-carnitine is a compound with a long history in biochemistry. It plays an important role in mammals, where many functions have been attributed to it. Those functions include the β -oxidation of long-chain fatty acids, the regulation of the free CoA-SH/Acyl-CoA ratio and the translocation of acetyl units into mitochondria. Carnitine is also found in lower eukaryotic organisms. However, in contrast to the multiple roles it plays in mammalian cells, its action appears to be restricted to the transport of activated acyl residues across intracellular membranes in the lower eukaryotes. In the yeast *Saccharomyces cerevisiae*, the role of carnitine consists mainly of the transfer of activated acetyl residues from the peroxisome and cytoplasm to the mitochondria. This process is referred to as the carnitine shuttle. This system involves the transfer of the acetyl moiety of acetyl-CoA, which cannot cross organellar membranes, to a molecule of carnitine. Subsequently, the acetylcarnitine is transported across membranes into the mitochondria, where the reverse transfer of the acetyl group to a molecule of free CoA occurs for further metabolism. Carnitine acetyl transferases (CATs) are the enzymes responsible for catalysing the transfer of the activated acetyl group of acetyl-CoA to carnitine as well as for the reverse reaction.

In the yeast *S. cerevisiae*, three CAT enzymes, encoded by the genes *CAT2*, *YAT1* and *YAT2*, have been identified. Genetic data suggest, that despite the high sequence similarity, each of the genes encodes for a highly specific activity that is part of the carnitine shuttle. So far, the specific function of any of the three CAT enzymes has been elucidated only partially.

The literature review focuses mainly on the importance of the carnitine system in mammals. After discussing the discovery and biosyntheses of carnitine, the enzymatic background of and molecular studies on the carnitine acyltransferases are described.

The experimental section focuses on elucidating the physiological roles and cellular localisation of the three carnitine acetyltransferase of *S. cerevisiae*. We developed a novel enzymatic assay to study CAT activity *in vivo*. By C-terminal tagging with a green fluorescent protein, we localised the three CAT enzymes.

However, all our genetic attempts to reveal specific roles for and functions of these enzymes were unsuccessful. The overexpression of any of the CAT genes could not cross-complement the growth defect of other CAT mutant strains. No phenotypical difference could be observed between strains carrying single, double and triple deletions of the CAT genes. Furthermore, the expression of the *Schizosaccharomyces pombe* dicarboxylic acid transporter can complement the deletion of the peroxisomal citrate synthase, but has no effect on the carnitine shuttle per se. Our data nevertheless suggest that *Cat2p* is the enzyme mainly responsible for the forward reaction, e.g. the formation of acetylcarnitine and free CoA-SH from

acetyl-CoA and carnitine, whereas Yat1p and Yat2p may be required mainly for the reverse reaction.

OPSOMMING

L-karnitien is 'n verbinding met 'n lang geskiedenis in die biochemie-veld. Dit speel 'n belangrike rol in soogdiere, waar verskeie funksies daaraan toegeskryf word. Dié funksies sluit in die β -oksidasie van lang-ketting-vetsure, die regulering van die vrye KoA-SH-tot-asiel-KoA-verhouding en die oordrag van asetieleenhede na die mitochondria. Karnitien word ook in laer eukariotiese organismes gevind. In teenstelling met die verskeidenheid rolle wat dit in soogdierselle vervul, is die funksie in laer eukariote tot die transport van geaktiveerde asetielderivate oor intrasellulêre membrane beperk. In die gis *Saccharomyces cerevisiae* is die funksie van karnitien meestal beperk tot die vervoer van geaktiveerde asetielresidu's vanaf die sitoplasma en piroksisome na mitochondria, 'n proses wat as die "karnitiensiklus" bekend staan. Die proses behels die oordrag van die asetielgedeelte van asetiel-KoA, wat nie oor organelmembrane kan beweeg nie, na 'n molekule van karnitien. Gevolglik word die asetielkarnitien oor die membraan na die mitochondria vervoer, waar - met die oog op verdere metabolisme - die omgekeerde oordrag van die asetielgroep na 'n vrye molekule van KoA plaasvind. Karnitienasetiel-transferases (KAT's) is die ensieme wat verantwoordelik is vir die katalisering van die oordrag van die geaktiveerde asetielgroepe van asetiel-KoA na karnitien, sowel as vir die omgekeerde reaksie.

In die gis *S. cerevisiae* is drie KAT-ensieme geïdentifiseer wat deur die gene *CAT2*, *YAT1* en *YAT2* gekodeer word. Genetiese data dui daarop dat, ten spyte van die hoë mate van homologie van die DNA-volgordes, elke geen vir 'n hoogs spesifieke aktiwiteit, wat deel van die karnitiensiklus is, kodeer. Tot dusver is die spesifieke funksie van die drie individuele KAT-ensieme net gedeeltelik ontrafel.

Die literatuurstudie fokus hoofsaaklik op die belangrikheid van karnitiensisteme in soogdiere. Na 'n bespreking van die ontdekking en biosintese van karnitien, word die ensimatiese agtergrond en molekulêre studies van KAT's beskryf.

Die eksperimentele deel konsentreer op die ontrafelling van die fisiologiese rol en intrasellulêre lokalisering van die drie KAT-ensieme van *S. cerevisiae*. Eerstens is 'n nuwe ensimatiese toets ontwikkel om KAT-aktiwiteit *in vivo* te bestudeer. Deur C-terminale aanhegting van 'n groen fluoreserende proteïen kon die drie KAT-ensieme gelokaliseer word.

Daar kon egter nie met behulp van genetiese studies verder lig gewerp word op die spesifieke rolle en funksies van hierdie KAT-ensieme nie. Die ooruitdrukking van enige van die KAT-gene kon nie die groeidefek van ander KAT-mutantrasse kruiskomplementeer nie. Geen fenotipiese verskil tussen rasse wat 'n enkel, dubbel of trippel delesie van die KAT-gene bevat, kon waargeneem word nie. Verder kon die uitdrukking van *Schizosaccharomyces pombe* se dikarboksielsuurtransporter die delesie van die peroksisomale sitraatsintetase komplementeer, maar het dit as sulks geen effek op die karnitiensiklus gehad nie. Die data wat deur hierdie studie verkry

is, dui nogtans daarop dat Cat2p die ensiem is wat hoofsaaklik verantwoordelik is vir die voorwaartse reaksie, met ander woorde die vorming van asetielkarnitien en vrye KoH-SH van asetiel-KoA en karnitien, terwyl Yat1p en Yat2p hoofsaaklik vir die omgekeerde reaksie benodig word.

This thesis is dedicated to my parents and to the spirits and forces
behind us.

BIOGRAPHICAL SKETCH

Sven Kroppenstedt was born in Gross Umstadt, Germany on 22 August 1975. He matriculated at the Bertolt Brecht Gymnasium in 1994.

After completing his vocational training as a viticulturist in 1996, he enrolled at the University for Applied Science in Geisenheim and obtained a Diploma in Viticulture and Oenology in 2000.

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PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and is written according to the style of the journal *Analytical Biochemistry*, to which a modified version of Chapter 3 was submitted for publication.

Chapter 1 General Introduction and Project Aims

Chapter 2 Literature Review

Carnitine biosynthesis and molecular enzymology of carnitine acyl transferases

Chapter 3 Research Results

The role of carnitine acetyltransferases in *S. cerevisiae*
The determination of carnitine acetyltransferase activity in
Saccharomyces cerevisiae By HPLC-electrospray mass
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Chapter 4 General Discussion and Conclusions

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Chapter 1

INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

The compound β -hydroxy- γ -trimethylammonium butyrate, also referred to as L-carnitine (carnitine), was discovered almost a hundred years ago by Guleweitsch and Krimberg (1905). For a long time it was classified as vitamin B_T (Carter *et al.*, 1952) because it is required for the survival of *Tenebrio molitor* larvae. This requirement of the larvae for carnitine was used as an assay to determine the levels of carnitine in a variety of biological materials (Fraenkel, 1953). Carnitine plays an important role in mammalian cells, where many functions have been attributed to it. These functions include contributions to (a) the β -oxidation of long-chain fatty acids; (b) the elimination of selective acyl residues for detoxification; (c) the modulation of the free CoA-SH/Acyl-CoA ratio; (d) the storage of activated acetyl units and (e) the translocation of acetyl units into mitochondria (reviewed in Bieber, 1988). Carnitine is given in high doses to patients for the treatment of primary carnitine deficiency, a disease caused by metabolic and genetic disorders resulting in low levels of carnitine in the tissue (Pons and De Vivo, 1995). In addition, carnitine has been shown to have a positive therapeutic effect when administered to patients with diseases such as diabetes, Alzheimer's and AIDS (Carta *et al.*, 1999; De Simone *et al.*, 1993; Keller *et al.*, 1993). However, the mechanism underlying these therapeutic effects is not clear.

L-carnitine is found in all eukaryotic organisms. However, in contrast to the multiple roles it plays in mammalian cells, in lower eukaryotes its action appears to be restricted to the transport of activated acyl residues across intracellular membranes. In the yeast *Saccharomyces cerevisiae*, the role of carnitine lies mainly in the transfer of activated acetyl residues from the peroxisome and cytoplasm to the mitochondria (Van Roermund *et al.*, 1995; Schmalix and Bandlow, 1993). This process is referred to as the carnitine shuttle. This system involves the transfer of the acetyl moiety of acetyl-CoA, which cannot cross organellar membranes, to a molecule of carnitine. Subsequently, the acetylcarnitine is transported across membranes into the mitochondria, where the reverse transfer of the acetyl group to a molecule of free CoA-SH occurs for further metabolism. Carnitine acetyl transferases (CATs) are the enzymes responsible for catalysing the transfer of the activated acetyl group, acetyl-CoA, to carnitine, as well as for the reverse reaction (reviewed in Bieber, 1988).

No clearly identifiable peroxisomal structures can be detected when yeast grows on fermentable carbon sources, but when it is grown on fatty acids, such as oleic acid, a proliferation of peroxisomes is observed (Veenhuis *et al.*, 1987). Peroxisomes are required for yeast to catabolise fatty acids, since they are the sole site for β -oxidation, the fatty acid degradation pathway that results in the formation of acetyl-CoA in the peroxisome (Kunau *et al.*, 1988).

Van Roermund *et al.* (1995) showed that *S. cerevisiae* uses two pathways to further utilise this product. The first pathway is the previously described carnitine shuttle. Another pathway utilising peroxisomally-produced acetyl-CoA is the glyoxylate cycle, a modified version of the tricarboxylic acid cycle (TCA), which, at least in part, takes place in the peroxisome. This cycle results in the net synthesis of C₄ compounds, in particular succinate, from two molecules of acetyl-CoA, which can then be further utilised for anabolic or catabolic purposes (Van Roermund *et al.*, 1995).

The existence of two pathways to further utilise peroxisomally-produced acetyl-CoA explains the absence of growth defects on oleic acid media of strains lacking either the *CIT2* gene, which encodes the first enzyme of the glyoxylate cycle (citrate synthesis), or the *CAT2* gene, which encodes the peroxisomal and inner mitochondrial carnitine acetyltransferase (Van Roermund *et al.*, 1995). On the other hand, double mutants are unable to grow in these conditions, implying that the two pathways can complement each other.

Besides its importance for the acetyl shuttle mechanism between peroxisomes and mitochondria during growth on fatty acids, carnitine also plays a role when yeast cells are grown on other non-fermentable carbon sources, such as acetate and ethanol. The catabolism of these compounds results in the production of acetyl-CoA in the cytoplasm, which needs to be transferred to the mitochondria for energy production. The transfer can take place through the formation of acetylcarnitine and the subsequent transfer thereof to this cellular compartment where the reverse reaction takes place (Swiegers *et al.*, 2001; Stemple *et al.*, 1998; Schmalix and Bandlow, 1993).

To date, three genes coding for carnitine acetyltransferases have been identified in *S. cerevisiae*. The first gene, *CAT2*, codes for a CAT found in the peroxisomes and mitochondria and contributes >99% of the total CAT activity in galactose-grown cells (Kispal *et al.*, 1993). The second gene, *YAT1*, codes for a CAT presumably associated with the outer surface of the mitochondria and contributes an estimated 5% of total CAT activity in acetate- and ethanol-grown cells (Schmalix and Bandlow, 1993). The third gene, *YAT2*, codes for a CAT that presumably, is cytosolic, with a high contribution to total CAT activity in ethanol-grown cells (Swiegers *et al.*, 2001).

Swiegers *et al.* (2001) showed that the independent deletion of any of the three CAT genes in a $\Delta cit2$ background results in an *S. cerevisiae* strain that is unable to grow on acetate, ethanol, glycerol or oleic acid as sole carbon sources. Interestingly, no phenotypical differences could be observed between the double mutant strains $\Delta cit2/\Delta cat2$, $\Delta cit2/\Delta yat1$ and $\Delta cit2/\Delta yat2$. All strains were unable to grow on all the non-fermentable carbon sources tested. This indicates that, in the absence of the glyoxylate cycle citrate synthetase, all carnitine acetyl transferases are essential for growth. Transformation of the double mutants with plasmids carrying either *CAT2*, *YAT1* or *YAT2* did not lead to any cross-complementation, indicating that, despite the high sequence similarity, each of the genes encodes for a highly specific activity. So

far, the specific function for any of the three CAT enzymes in *S. cerevisiae* has been established only partially or is unknown.

Our current understanding of the shuttle system does not require the existence of three independent CATs to ensure the survival on all non-fermentable carbon sources. Besides the shuttle system, carnitine has been suggested to play an important role in the regulation of the availability of free CoA-SH. Considering the highly compartmentalised distribution of CoA-SH, this regulation might require specifically compartmentalised and independently regulated CAT activities. Furthermore, the proper channelling of activated acetyl groups into either anabolic pathways or catabolic energy metabolism is essential during growth on non-fermentable carbon sources, and might require the presence of three independently regulated CATs.

1.2 PROJECT AIMS

This study was conducted in order to gain a better understanding of the specific role of each CAT enzyme in the cellular metabolism of *S. cerevisiae*.

The specific aims and approaches of this study were as follows:

- (i) to establish a reliable and cheap method to assess CAT activity *in vivo*
- (ii) to determine the cellular localisation of each of the three CAT enzymes
- (iii) to investigate the phenotypical effect of single, double and triple deletion mutants of CAT genes, by:
 - (a) investigating the effect of CAT gene deletion on cellular metabolism, in particular on the balance between carnitine, acetylcarnitine, free CoA-SH and acetyl-CoA *in vivo*.
 - (b) investigating the effect of CAT gene deletion on the potentially associated pathways, in particular the formation of acetyl esters and malate utilisation.
 - (c) assessing the effect of overexpression of one of the CAT genes in a double mutant $\Delta cit2$ background.
 - (d) investigating the effect of CAT gene deletion on growth speed under various conditions.

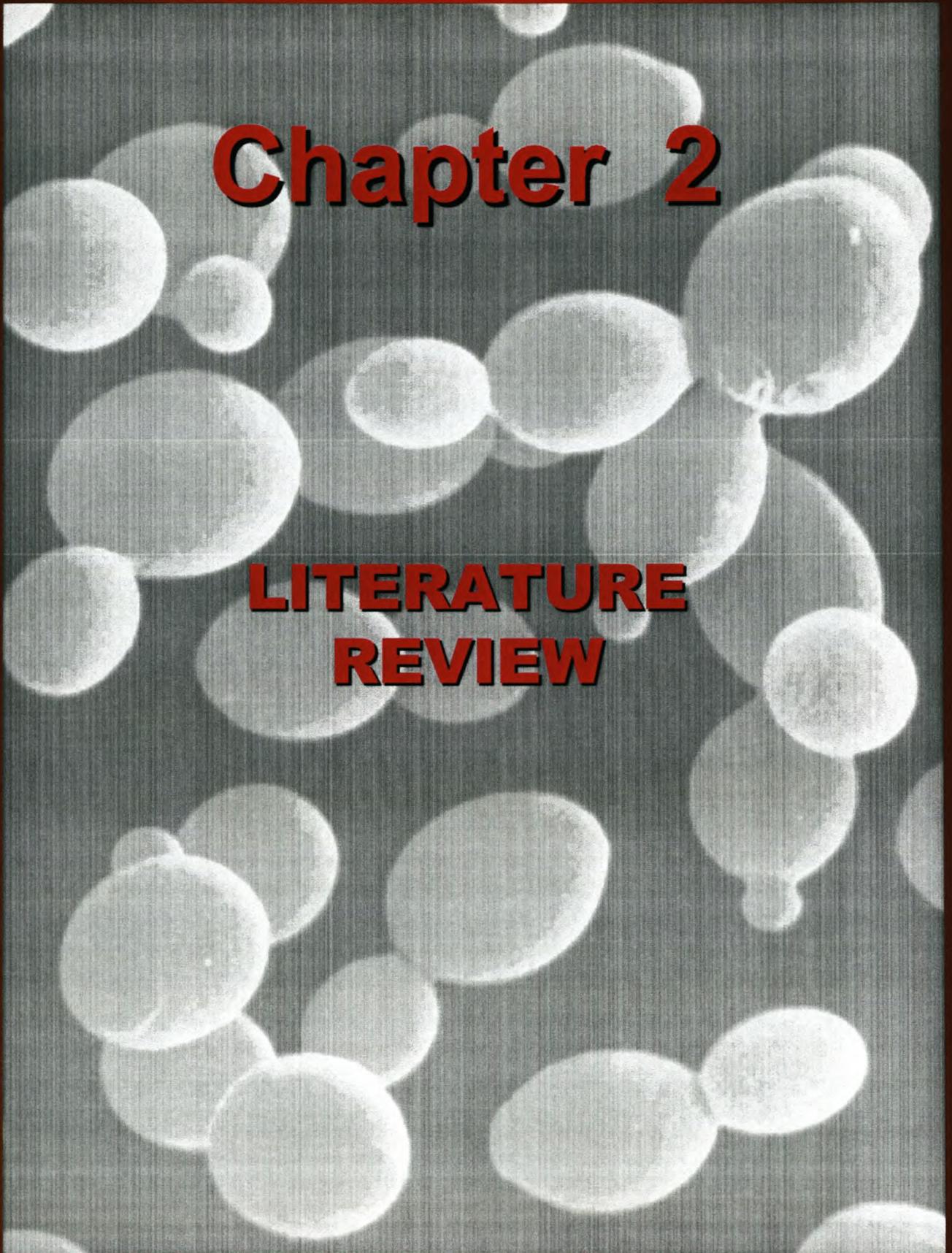
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Chapter 2

LITERATURE REVIEW



CARNITINE BIOSYNTHESIS AND MOLECULAR ENZYMOLOGY OF CARNITINE ACYL TRANSFERASES

2.1 INTRODUCTION

The mammalian cell relies on the carnitine system (**Figure 2.1**) to regulate localised, limited pools of CoA derivatives. Acyl-CoA pools provide activated substrates for many key metabolic pathways, such as the TCA cycle and lipid and cholesterol synthesis, for posttranslational modification of proteins and for detoxification mechanisms. The reversible transfer of activated acyl groups from limited pools of membrane impermeable CoA-SH to the mobile carnitine provides transport between compartments, a considerable reservoir of activated acyl groups and a possibility for excretion of excess acyl moieties. Transport occurs in the import of fatty acids for energy production in mammalian mitochondria (reviewed in Bremer, 1983) and in yeast peroxisomes (Van Roermund *et al.*, 1999). The reservoir function refers to the acetyl-L-carnitine pool in the heart and sperm (reviewed in Bremer, 1983) and to the long-chain acylcarnitine pool, which is used by cells for membrane repair when they lack energy to activate fatty acids (Arduini *et al.*, 1992). The excretion of carnitine derivatives occurs via the urine, in which they provide a marker for clinical measurements, and in bile, in which it recently was demonstrated that long-chain acyl derivatives accumulate (Rashed *et al.*, 1995). As a whole, the carnitine system both connects the various acyl-CoA pools and damps fluctuations in their acylation state that would be detrimental to cell homeostasis.

The carnitine system consists of carrier proteins that transport carnitine across the membranes and of enzymes, carnitine acyltransferases, that catalyse the reversible equilibrium:

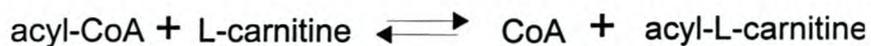


Figure 2.1 gives a schematic presentation of a mammalian cell and its compartments. It shows the localisation of proteins that are involved in the carnitine system.

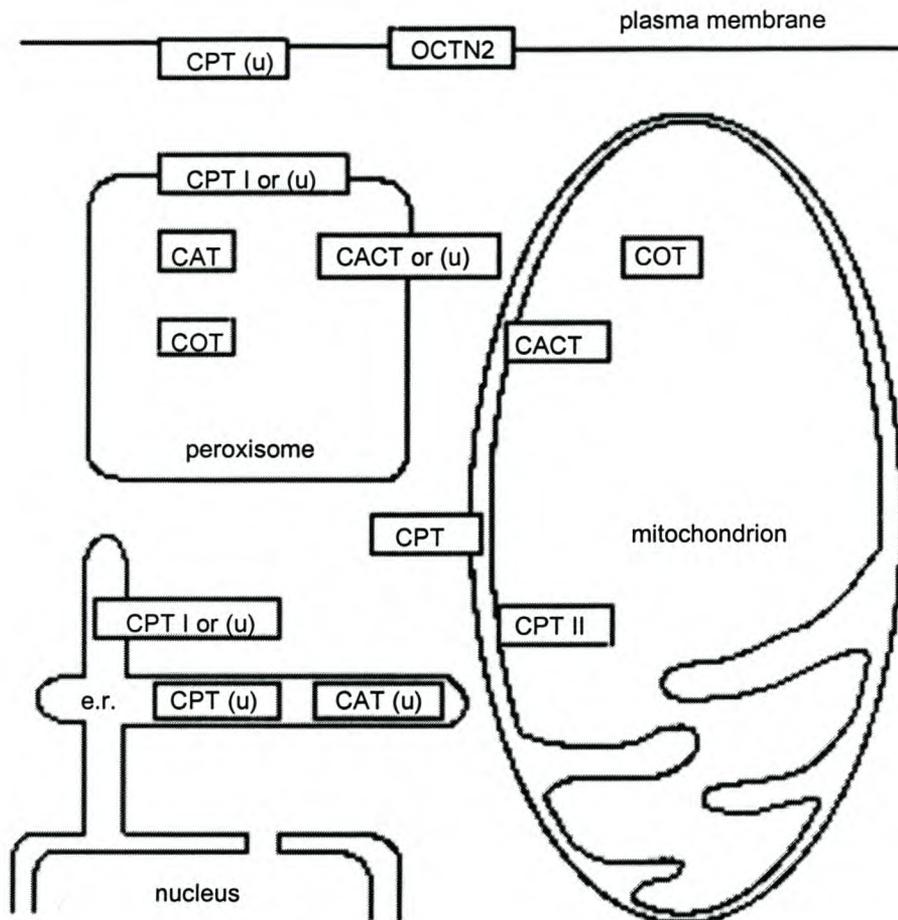


Figure 2.1 Locations of the proteins that are involved in the carnitine system in a mammalian cell. u, unknown; e.r., endoplasmic reticulum; OCTN2, mammalian plasma membrane transporter; CACT, mitochondrial carnitine carrier; CAT, carnitine acetyltransferase; COT, carnitine octanoyltransferase; CPT I and CPT II, carnitine palmitoyltransferase (adapted from Ramsay *et al.*, 2001).

Here we briefly review the discovery and biosynthesis of carnitine, as well as the molecular genetics and enzymology of proteins that play key roles in activated acyl group homeostasis and transport in *S. cerevisiae* and mammalian systems. If not stated otherwise, the default mammalian system for discussion is humans.

2.2 CARNITINE

2.2.1 DISCOVERY AND PRIMARY FUNCTIONS

Carnitine, a name derived from the Latin *caro* (flesh), was first discovered in muscle extracts in 1905 (Guleweitsch and Krimberg, 1905; Kutscher, 1905). Soon thereafter, the chemical formula $C_7H_{15}NO_3$ was established and its structure, a trimethylbetaine of 4-amino-3-hydroxybutyric acid, was resolved (Tomita and Sendju, 1927) (**Figure 2.2**). In 1962, the configuration of the physiological enantiomer was determined as L(-) or R(-)- β -hydroxy- γ -trimethylammonium butyrate (Kaneko and Yoshida, 1962).

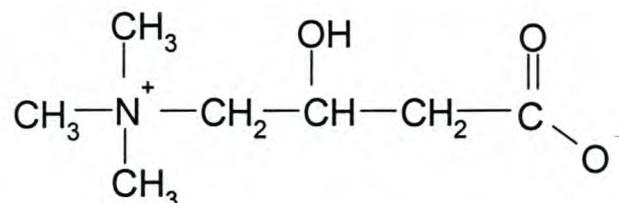


Figure 2.2 The chemical structure of carnitine: β -hydroxy- γ -trimethylammonium butyrate, a zwitterionic quaternary amine with a molecular mass of 161.2 g/mol (inner salt).

A role for carnitine in metabolism was established, almost 50 years after its discovery, by Fraenkel and colleagues (1953), when they demonstrated that carnitine is an essential growth factor for the larvae of the mealworm, *Tenebrio molitor*, and therefore named it vitamin B_T (T for *Tenebrio*). Parallel investigations by Bremer and colleagues and Fritz and colleagues established a role for carnitine in the β -oxidation of long-chain fatty acids (Bremer, 1983; Fritz, 1963). As interest in the function of carnitine increased, it became apparent that carnitine had more than one role in intermediary metabolism, especially when the multiorganelle and multienzyme distribution of carnitine acyltransferases was considered (Bieber *et al.*, 1982). During the past three decades, several roles for carnitine in mammalian metabolism have been proposed, both direct and indirect, most of which involve conjugation of the acyl residues to the β -hydroxyl group, with subsequent translocation from one cellular compartment to another (Bremer, 1983; Bieber *et al.*, 1982). This process affects both the availability of activated acyl residues and the availability of free coenzyme A (CoA-SH).

In mammals, carnitine is involved in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix, where β -oxidation takes place (**Figure 2.3**). Cytosolic long-chain fatty acids, which are present as CoA esters, are transesterified to L-carnitine in a reaction catalysed by carnitine palmitoyltransferase I (CPT I) at the outer membrane of the mitochondria. In this reaction, the acyl-moiety of the long-chain fatty acid is transferred from the CoA to the hydroxyl group of carnitine. The resulting long-chain acyl-carnitine esters are transported over the inner mitochondrial membrane via a specific carrier, carnitine-acylcarnitine translocase (CACT). At the matrix side of the mitochondrial membrane, the long-chain fatty acids are transesterified to intramitochondrial CoA, a reaction that is catalysed by carnitine palmitoyltransferase II (CPT II). The released carnitine can then leave the mitochondria via CACT for another round of transport (reviewed in McGarry and Brown, 1997). In the mitochondrial matrix, the enzyme carnitine acetyl-transferase (CAT) is able to reconvert short- and medium-chain acyl-CoAs into acyl-carnitine using intramitochondrial carnitine. These acyl-carnitines can then leave the mitochondria via CACT.

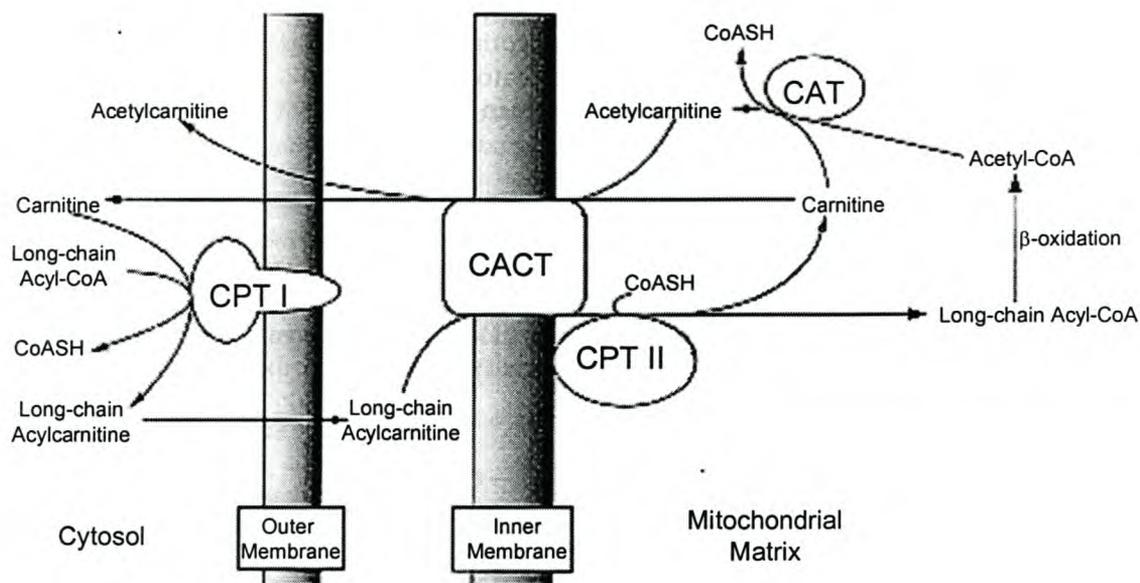


Figure 2.3 Carnitine functions in the transport of mitochondrial long-chain fatty acid oxidation and the regulation of the innermitochondrial acylCoA/Coa ratio (adapted from Vaz and Wanders, 2002).

Through this mechanism of reversible acylation, carnitine as co-factor is able to modulate the intracellular concentrations of acyl-CoA and free CoA. It has been suggested that this mechanism is also used to store acetyl units as acetyl-carnitine, which can be converted into acetyl-CoA upon energy shortage (Bremer, 1983). Carnitine is also involved in the transport of products of the peroxisomal β -oxidation system from peroxisomes to mitochondria (Verhoeven *et al.*, 1998). Very long-chain fatty acids ($>C_{22}$) and certain branched-chain fatty acids are partially degraded in this organelle, and the resulting products are transported as carnitine esters to the mitochondrion for further degradation. For this purpose, peroxisomes also contain carnitine acyl transferases, including CAT, and carnitine octanoyltransferase (COT). Like the mitochondrial enzyme, peroxisomal CAT converts short- and medium-chain acyl-CoAs into acyl-carnitines for transport to the mitochondria. The peroxisomal and mitochondrial CATs are encoded by a single gene (reviewed in Ramsay *et al.*, 2001).

2.2.2 OCCURRENCE AND DISTRIBUTION

Carnitine is most likely present in all animal species, in numerous microorganisms and in many plants (Rebouche and Seim, 1998; Kleber, 1997; Panter and Mudd, 1969). A similar, nearly ubiquitous distribution of carnitine palmitoyltransferase has been found (Crabtree and Newsholm, 1972; Norum and Bremer, 1966). The general occurrence of carnitine and of its accompanying carnitine acyltransferases shows that it must have evolved at a phylogenetically early stage, probably during a time closely associated with the development of mitochondria (reviewed in Bremer 1983). The concentration of carnitine varies greatly in different tissues and in different species. The highest concentrations reported have been found in horseshoe crab

muscle and in rat epididymal fluid, in which carnitine can reach a concentration of 60 mM (Brooks *et al.*, 1974). Animal tissues contain relatively high amounts of carnitine, varying between 0.2 and 6 $\mu\text{mol/g}$, with the highest concentrations in heart and skeletal muscle (Tein *et al.*, 1996; Bremer, 1983; Brooks *et al.*, 1974). Certain bacteria, including strains of *Pseudomonas* and *Acinobacter*, can use carnitine as sole carbon and nitrogen source (Rebouche and Seim, 1998; Kleber, 1997). Other bacteria use carnitine and its derivatives as osmoprotectants (Rebouche and Seim, 1998). Plants contain very little carnitine, except avocados and asparagus, which contain some carnitine, but not nearly as much as animal tissues (Rebouche, 1992; Panter and Mudd, 1969). Although animals acquire carnitine primarily from the diet, most mammals are capable of synthesising carnitine endogenously.

2.2.3 BIOSYNTHESIS OF CARNITINE

Carnitine is synthesised from the essential amino acids lysine (Tanphaichitr *et al.*, 1971) and methionine (Bremer, 1961). In some proteins, lysine residues are trimethylated by protein-dependent methyltransferases that use S-adenosyl methionine as the methyl group donor (Paik and Kim, 1971). Free lysine cannot be methylated. When the proteins are degraded, the trimethyllysine that is released cannot be used for the synthesis of new proteins due to the absence of a transfer RNA for trimethyllysine. Its levels therefore are sufficient for carnitine biosynthesis.

The biosynthetic pathway (**Figure 2.4**) of carnitine from 6-N-trimethyllysine (TML) involves several enzymes and cofactors. The first enzyme is 6-N-trimethyllysine dioxygenase, which hydroxylates 6-N-trimethyllysine at the three position (Hulse *et al.*, 1978). This is the only mitochondrial enzyme in the pathway. It has an activity that is similar in function to proline hydroxylase and requires 2-oxoglutarate, ascorbate and Fe^{2+} . However, the enzyme has been proven to be difficult to isolate and has therefore not been studied in detail.

The second enzyme, 3-hydroxy-6-N-trimethyllysine aldolase, catalyses the cleavage of glycine from 3-hydroxy-6N-trimethyllysine (HTML), leaving the 4-N-trimethylaminobutyraldehyde (TMABA) (Rebouche, 1992). This enzyme is reported to be similar to serine hydroxymethyltransferase (Hulse *et al.*, 1978) and requires pyridoxal 5'-phosphate (PLP) as a cofactor. Although the K_M for trimethyllysine is much higher than that for other substrates, e.g. serine and threonine, no other enzyme has been implicated in this reaction (Rebouche, 1992).

The next enzyme, 4-N-trimethylaminobutyraldehyde dehydrogenase, is a cytosolic enzyme that catalyses the production of 4-N-trimethylaminobutyrate (butyrobetaine) from 4-N-trimethylaminobutyraldehyde (Hulse *et al.*, 1980) with the transfer of the hydrogen ions to oxidised nicotinamide adenine dinucleotide. The synthesis of 4-N-trimethylaminobutyrate can occur in most cells (Rebouche, 1982). Trimethyllysine and butyrobetaine are found in blood and urine (Zaspel *et al.*, 1980).

The last enzyme in the carnitine pathway, 4-N-trimethylaminobutyrate dioxygenase, is similar to 6-N-trimethyllysine dioxygenase in that it requires 2-

oxogluterate, ascorbate and Fe^{2+} (Rebouche, 1991). It catalyses the conversion of 4-N-trimethylaminobutyrate to carnitine. It is a cytosolic enzyme that is found in only a few tissues. In humans, the enzyme is found in the kidney, liver and possibly the brain. The highest specific activity is found in the kidney (Rebouche and Engel, 1980). This enzyme is missing in rat kidney, which means that the liver becomes the main site of synthesis. In the rat, it has been shown to be induced by thyroxine (Pande and Parvin, 1980a). 4-N-trimethylaminobutyrate dioxygenase is difficult to isolate, due, in part, to its instability in dilute solutions.

This carnitine biosynthetic pathway also requires ferrous ions and a number of vitamins: ascorbate, niacin and pyridoxine. The net effect of this pathway is the removal of the amino acid glycine from trimethyllysine for re-utilisation and the production of one molecule of reduced nicotinamide adenine dinucleotide. The Regulation of carnitine biosynthesis is currently not well defined (reviewed in Carter *et al.*, 1995).

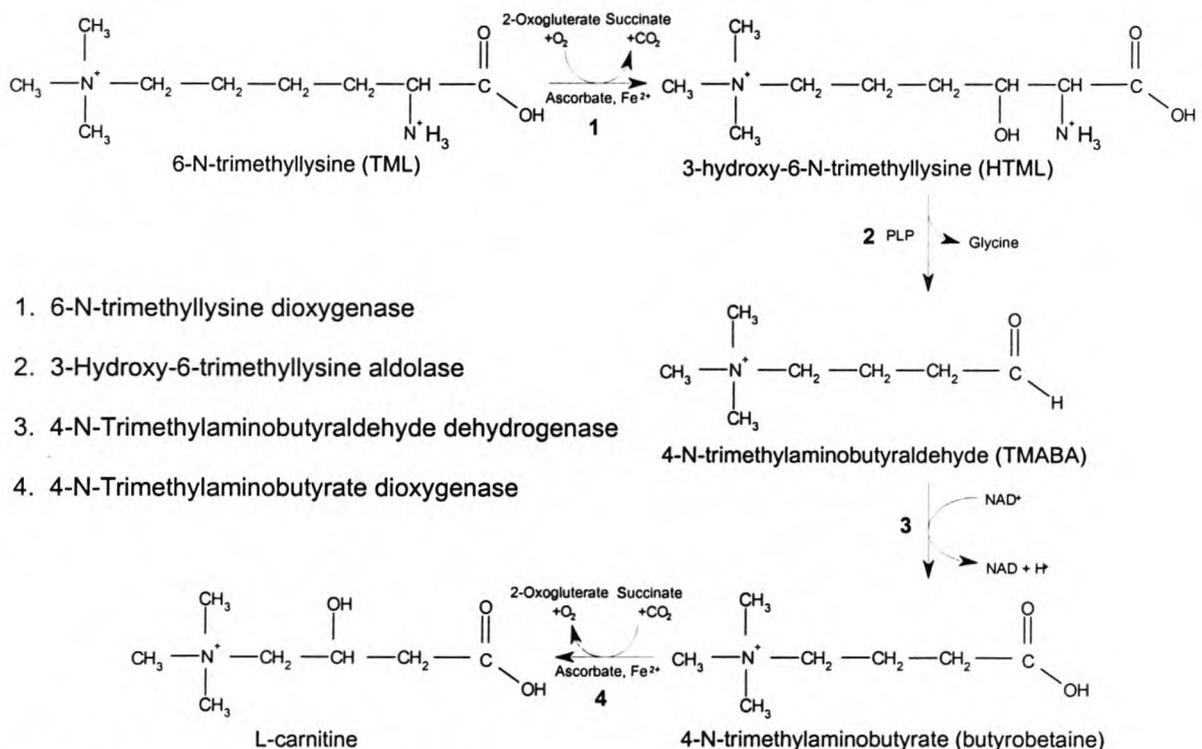


Figure 2.4 Carnitine biosynthesis from 6-N-trimethyllysine (TML); see text for description

2.3 UPTAKE AND TRANSPORT

2.3.1 INTRODUCTION

Most tissues have a carnitine concentration that is >10-fold higher than that of blood plasma, therefore an active uptake of carnitine must take place. Recent research provides a very good picture on the uptake of carnitine. In mammalian systems, a plasmalemmal carnitine transporter is encoded by the OCTN2 gene family (Schömig *et al.*, 1998; Tamai *et al.*, 1998; Wu *et al.*, 1998; Sekine *et al.*, 1998).

In *Saccharomyces cerevisiae*, the *AGP2* gene, coding for a protein that belongs to the family of amino acid permeases, has been proven to fulfil similar functions (Van Roermund *et al.*, 1999).

After uptake, intracellular transport must take place to supply carnitine to the different cellular compartments, where it is required for the transport of acyl residues across membranes. In mammalian cells, the carnitine/acylcarnitine translocase (CACT) catalyses the exchange of acylcarnitine of various lengths for free carnitine in the mitochondrial matrix (Indiveri *et al.*, 1990).

Closely related to the human CACT gene, with 29% identity, is the *CRC1* gene that is found in the genome of *S. cerevisiae*. Recent studies showed that *Crc1p* is a mitochondrial carnitine carrier (Palmieri *et al.*, 1999; Van Roermund *et al.*, 1999).

2.3.2 THE MAMMALIAN PLASMA MEMBRANE TRANSPORTERS (OCTN2)

Carnitine and acetylcarnitine can cross the membranes of mammalian cells, indicating that a non-saturable component to uptake exists in cells (Shennan *et al.*, 1998). However, proteins mediate both the cellular accumulation of carnitine across the plasma membrane, from about 50 μM in plasma to millimolar levels in cells, and the rapid flux across the mitochondrial inner membrane required for β -oxidation (Bremer, 1983). The discovery of this plasma membrane transport of carnitine dates to the recognition in the 1970s that liver, the major site of the final step of carnitine biosynthesis, has a low affinity ($K_M = 5.6 \text{ mM}$) (Christiansen and Bremer, 1976) and that other tissues have a high affinity ($K_M = 4\text{-}20 \mu\text{M}$) (Bohmer *et al.*, 1977) for carnitine. Presumably, OCTN2 is responsible for the high-affinity activities measured in this early work (reviewed in Ramsay *et al.*, 2001).

The human gene for OCTN2, *SLC22a5* (i.e. member 5 of solute carrier family 22), contains 10 exons and maps to chromosome region 5q31, a region that researchers identified using linkage analysis of families with inherited systemic carnitine deficiency (Wu *et al.*, 1998; Sekine *et al.*, 1998; Shoji *et al.*, 1998). The mouse gene, known from the *juvenile visceral steatosis (jvs)* mutation (Koizumi *et al.*, 1988), a missense mutation, maps to chromosome 11 (Nikaido *et al.*, 1995). This protein of 557 amino acids contains 12 putative transmembrane domains; however, there is some disagreement about predictions of these domains (Wu *et al.*, 1998; Nezu *et al.*, 1999; Lu *et al.*, 1998). Identified motives include three putative N-glycosylation sites in the first extracellular loop, several putative phosphorylation sites in the intracellular loops (Tamai *et al.*, 1998; Wu *et al.*, 1998), a sugar transporter protein signature motif (Tamai *et al.*, 1998), and an ATP/GTP binding motif (**Figure 2.5 and Figure 2.6**) (Wu *et al.*, 1998). Unique among organic cation transporters is that the transport of carnitine by OCTN2 is sodium dependent, but independent of any other cations (Tamai *et al.*, 1998; Seth *et al.*, 1999). OCTN2 most closely resembles OCTN1 and OCTN3, the latter known only from mouse (Tamai *et al.*, 2000).

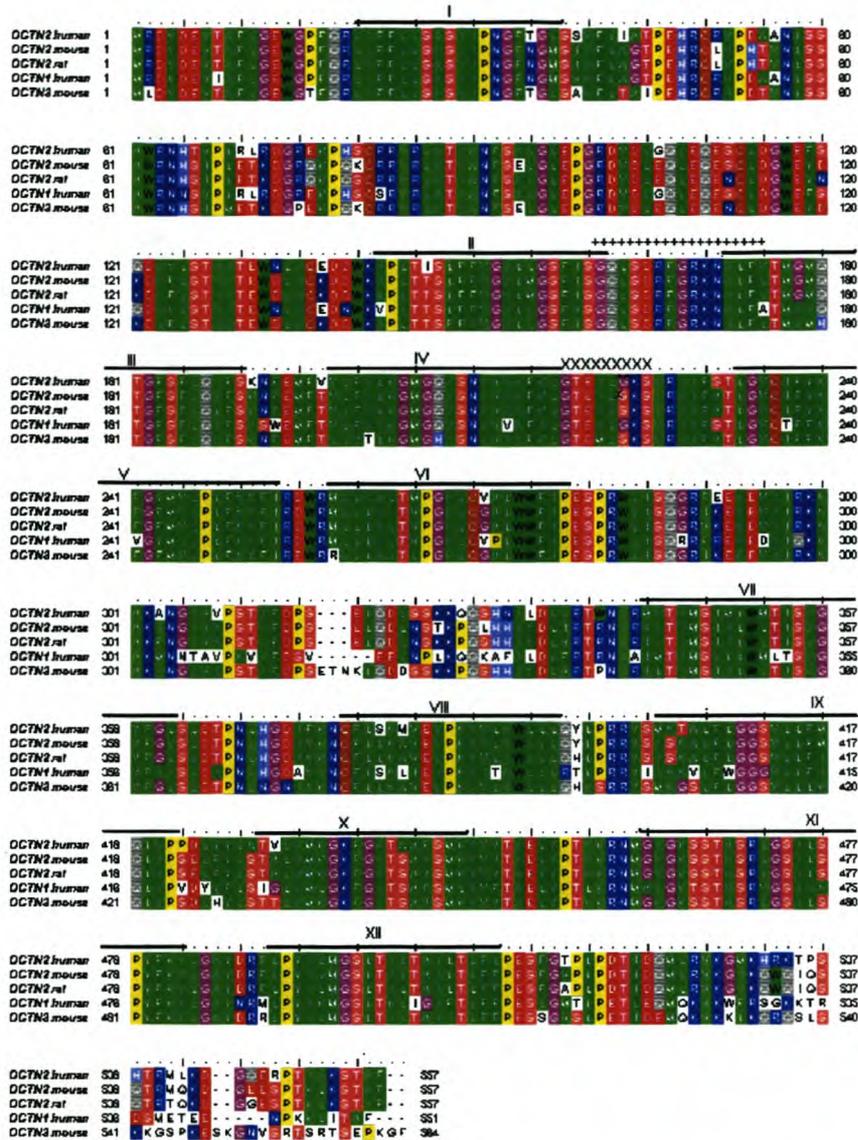


Figure 2.5 Amino acid sequence alignment of human, mouse and rat OCTN2 and related proteins from the solute carrier superfamily 22. Twelve putative transmembrane domains are indicated with roman numbered bars above the sequence alignment. The sugar transporter motif is indicated by + symbols and the AGP/GTP binding signature motif by X symbols.

An ancestral OCTN gene must have been duplicated as a direct repeat, as the genes for OCTN1, a low-affinity, sodium-independent carnitine transporter and OCTN2 are in tandem (Zhu *et al.*, 2000; Yabuuchi *et al.*, 1999). Deletion of both genes causes carnitine deficiency in mice (Zhu *et al.*, 2000), with all the characteristics of the *jvs* mutation (Kuwajima *et al.*, 1999; Koizumi *et al.*, 1988), including cardiomyopathy and fatty liver. Complementation with a genomic fragment that encodes human OCTN2, but not human OCTN1, rescues the phenotype (Zhu *et al.*, 2000).

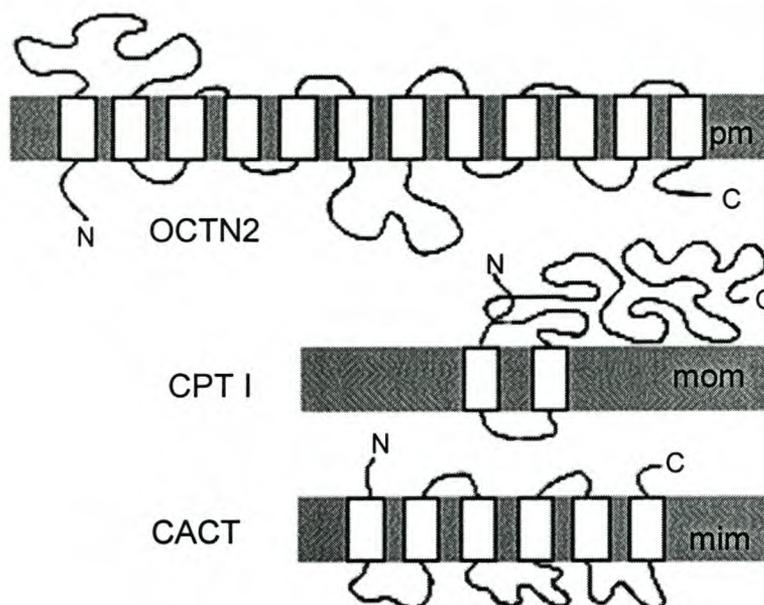


Figure 2.6 Schematic representation of the plasmalemmal carnitine transporter OCTN2, carnitine palmitoyltransferase I (CPT-I) and carnitine acylcarnitine translocase (CACT). Bilayer phospholipid membranes are depicted in grey: pm, plasmalemma; mom, mitochondrial outer membrane; mim, mitochondrial inner membrane. Hydrophilic parts of the protein (black lines), membrane-spanning protein domains (white boxes), the amino-terminus (N), and the carboxy-terminus (C) of each protein are indicated. The orientation of the proteins is in accordance with the layout of a cell. Crossing of the lines indicates the close interaction between the CPT-I N-terminus and the much larger C-terminal part of the protein (adapted from Ramsay *et al.*, 2001).

2.3.3 THE YEAST PLASMA MEMBRANE TRANSPORTERS (AGP2)

In *Saccharomyces cerevisiae*, the uptake of carnitine takes place via a non-specific transporter. Van Roermund *et al.* (1999) showed that the *AGP2* gene (ORF YBR132C), which encodes Agp2p, a protein of 596 amino acids, is required for the uptake of carnitine. Agp2p contains 12 potential transmembrane domains and is related to Put4p, Alp1p, Lyp1p, Can1p and Gap1p, which are all members of the family of amino acid permeases. Proteins belonging to this family are assumed to function as plasma membrane proton-symporters. Based on sequence similarity, Agp2p was described as one of the 18 members of this family with an unknown function (Nelissen *et al.*, 1997; Andre, 1995). Members of this family initially are inserted into the membrane of the endoplasmic reticulum (ER) and subsequently translocated to the plasma membrane via the yeast secretory pathway (Green and Walter, 1992; Green *et al.*, 1989). Immunogold electron microscopy studies confirmed that Agp2p is located primarily in the plasma membrane, but also in the ER and the vacuole (Van Roermund *et al.*, 1999; Ljungdahl *et al.*, 1992).

Inspection of the 5' region of the *AGP2* gene revealed the presence of a putative oleate response element (ORE) (Karpichev and Small, 1998). OREs are found in a number of oleate-inducible yeast genes, including the genes coding for β -oxidation, suggesting that the protein product plays a role in fatty acid degradation.

2.3.4 THE INTRACELLULAR TRANSPORTERS

2.3.4.1 MOLECULAR GENETICS OF THE MITOCHONDRIAL CARNITINE TRANSPORTER (CACT)

Mitochondria are impermeable to acyl-CoAs of any length. To traverse the inner mitochondrial membrane, acyl residues are transiently transferred to L-carnitine by specific carnitine acyltransferases. The carnitine/acylcarnitine translocase (CACT) from mammalian mitochondria catalyses the entry of acylcarnitines of various lengths, in exchange for free carnitine, into the mitochondrial matrix, where the acyl moieties are oxidised by the enzymes of the β -oxidation pathway and the TCA cycle (Iacobazzi *et al.*, 1998; Indiveri *et al.*, 1990). The identification and characterisation of mitochondrial carnitine/acylcarnitine exchange carrier function date to the 1970s (Ramsay and Tubbs, 1975; Pande, 1975). The same gene product may also provide carnitine transport in peroxisomes, since both organelles contain a protein that reacts with the same CACT peptide antibody (Fraser and Zammit, 1999). The rat (Indiveri *et al.*, 1997) and human (Huitzing *et al.*, 1997) CACT-encoding genes have been cloned. CACT is a member of the mitochondrial carrier family, which includes ornithine, ADP/ATP, phosphate, 2-oxoglutarate and citrate carriers, as well as proton carriers (uncoupling proteins). All these transporters have a molecular weight of about 30 kDa, with rat CACT at 32.5 kDa (Palmieri *et al.*, 1999). Mammalian CACT proteins contain 301 amino acid residues. These proteins have a triple repeat structure; each repeat contains two membrane-spanning domains. CACT proteins are anchored in the mitochondrial inner membrane, with the N-terminus, two small hydrophilic loops and the C-terminus facing the inner membrane space; three larger hydrophilic loops face the matrix side (**Figure 2.6**). CACT proteins appear to have a carnitine-specific binding sequence near the C-terminus. All analyses of CACT mutations (deletions, frameshifts) have revealed drastic effects on protein structure (reviewed in Ramsay *et al.*, 2001).

The kinetic parameters of CACT reveal the asymmetric nature of the electrically neutral and pH-independent exchange. In a purified system (Indiveri *et al.*, 1994; Indiveri *et al.*, 1990), the V_{\max} is $1.7 \mu\text{mol min}^{-1} \text{mg}^{-1}$, with K_M values of 0.5 mM and 8.7 mM on the internal and external faces respectively. The transport of acyl-L-carnitine homologues (acyl chain lengths of 12-16 carbons) is as rapid as carnitine transport, but with K_M values of $\sim 5 \mu\text{M}$. The pingpong mechanism for the carnitine carrier contrasts with other mitochondrial carriers. This mechanism allows the carrier to function as a uniporter to adjust the organelle concentration of carnitine (Indiveri *et al.*, 1994; Pande and Parvin, 1980b).

The activity of the carrier requires thiol groups. N-Ethylmaleimide and mersalyl inhibit (IC_{50} $5 \mu\text{M}$ and $0.05 \mu\text{M}$ respectively) both the exchange and uniport activities (Indiveri *et al.*, 1994). However, at high concentrations of mercurials, passive efflux of carnitine occurs; the opening of a nonspecific channel, as seen for some other mitochondrial carriers, might be the reason. The thiol groups modified to result in the

loss of specificity seem to differ from those modified for inhibition of exchange (Indiveri *et al.*, 1992).

Other inhibitors include acyl-L-carnitine analogues with a charged group at the ω end (Ramsay and Tubbs, 1975). Acyl-D-carnitines bind as well as acyl-L-carnitines (the K_i value for decanoyl-D-carnitine inhibition of L-carnitine exchange is 12 μ M), but are transported slowly or not at all (Ramsay, 1978). A very recent study demonstrates convincingly that acyl-L-carnitines target CACT for the inhibition of fatty acid oxidation (Baillet *et al.*, 2000).

2.3.4.2 THE YEAST MITOCHONDRIAL CARNITINE CARRIER (CRC1)

Saccaromyces cerevisiae encodes 35 members of the mitochondrial carrier family (Palmieri *et al.*, 1996). The transport functions of most family members are unknown. Among them, the *CRC1* (ORF YOR100C) is the most closely related to CACT, with 29% identity. This gene is the only one in *S. cerevisiae* encoding a member of the mitochondrial carrier family that has a promoter region containing an ORE, and its transcription has been shown to be induced by oleate (Karpichev *et al.*, 1998). Palmieri *et al.* (1999) and Van Roermund *et al.* (1999) identified and characterised the gene product Crc1p as the mitochondrial carrier protein for carnitine in *S. cerevisiae*. It has been shown to be a carnitine carrier that also transports acetylcarnitine, propionylcarnitine and, much less efficiently, medium- and long-chain acylcarnitines. The protein has an apparent molecular mass of about 40 kDa. It has inhibition characteristics similar to those previously described for the mammalian carnitine carrier.

2.4 THE CARNITINE ACYLTRANSFERASES

2.4.1 INTRODUCTION

Carnitine acyltransferases are enzymes that catalyse the reversible reaction between carnitine and acyl-CoA to free CoA-SH and acylcarnitine. In mammalian cells four different enzymes have been identified that can use carnitine as co-factor for the shuttling of acyl residues across intracellular membranes. All the identified enzymes catalyse the same reaction, but vary in their chain length specificity and cellular location. Carnitine acetyltransferase (CAT) catalyses the carnitine exchange of acetate, carnitine octanoyltransferase the exchange for medium chain lengths (C_{6-10}) and carnitine palmitoyltransferases I and II the exchange for long chain fatty acids (C_{12-20}) (reviewed in Bremer, 1983). In *S. cerevisiae*, only the activity of carnitine acetyltransferase can be detected.

2.4.2 CARNITINE ACETYLTRANSFERASE (CAT)

In mammalian cells, the activity of carnitine acetyltransferases has been found in mitochondria, peroxisomes and the endoplasmic reticulum (ER) (Markwell *et al.*, 1973). In rat heart cells, CAT is indeed not a cytosolic enzyme (Abbas *et al.*, 1998). Translated as a precursor of 626 amino acids, human mitochondrial CAT contains a 28 or 29 residue N-terminal mitochondrial targeting signal (MTS), which is clipped off during translocation through the mitochondrial inner membrane (Corti *et al.*, 1994a; Corti *et al.*, 1994b). Corti *et al.* (1994b) have discussed the possibility that cleavage of the MTS probably occurs between residues 28 and 29 of the precursor. However, their experimental data suggest that cleavage probably occurs between residues 29 and 30, where the sequence more closely resembles the well-conserved cleavage site consensus RXY↓S/A (Gavel and Van Heijne, 1990). At the C-terminus, the precursor contains a peroxisomal sorting signal (AKL), which functions predominantly when the MTS is not translated. Translation of the peroxisomal form of CAT starts at the second start codon and, for human CAT, produces a protein of 605 amino acid residues. The different use of start codons is the result of differential splicing that produces two mRNAs with and without the exon for the mitochondrial targeting sequence (Corti *et al.*, 1994b). The second or third exon contains the start of translation of the peroxisomal form (Van der Leij *et al.*, 2000). However, the differential splicing might result from the aberrant splice donor dinucleotide GC instead of GU in the mitochondrial first intron (Corti *et al.*, 1994b).

In mammals, it is not known whether the ER form of CAT comes from the same gene that encodes mitochondrial and peroxisomal CAT. The sequence KVEL in CAT (pos. 492-495) could possibly function as an ER retention signal, KDEL (Corti *et al.*, 1994b), which is normally found at the C-terminus.

In yeast, a single CAT gene also enables the translation of both a mitochondrial and peroxisomal isoform (Kispal *et al.*, 1996; Elgersma *et al.*, 1995), but the underlying mechanism does not involve splicing. In both *S. cerevisiae* and *Candida tropicalis*, the CAT mRNA are differentially translated because the translational machinery has a differential preference for the first or second start codon (Ueda *et al.*, 1998). Elgersma *et al.* (1995) showed that, in addition to the C-terminal AKL sequence, the *CAT2* gene of *S. cerevisiae* has an internal peroxisomal targeting signal (PTS). When both the mitochondrial targeting sequence and the C-terminal AKL are deleted, the majority of the gene product is still sorted to the peroxisomes. Depending on the carbon source used for the growth of the cells, the targeting of the protein might vary. In mammals, translation of the ORF takes place from the second methionine (residue 24) when cells are grown on oleic acid. Therefore, the N-terminal mitochondrial targeting signal would not be present.

Yeasts have two additional CAT genes, *YAT1*, which encodes a cytosolic form that is attached to the outer mitochondrial membrane and contributes an estimated 5% to the total CAT activity in acetate and ethanol grown cells (Schmalix and

Bandlow, 1993). The *YAT2* gene of *S. cerevisiae* encodes a third CAT with a significant homology to the cytosolic carnitine acetyltransferase of *A. nidulans*, FacC. The absence of distinct signal sequences in the protein and the high percentage of total CAT activity in ethanol media suggest that this enzyme could be a cytosolic carnitine acetyltransferase (Swiegers *et al.*, 2001). **Figure 2.7** shows the amino acid sequence alignment of the three carnitine acetyltransferases that are encoded in *S. cerevisiae*.

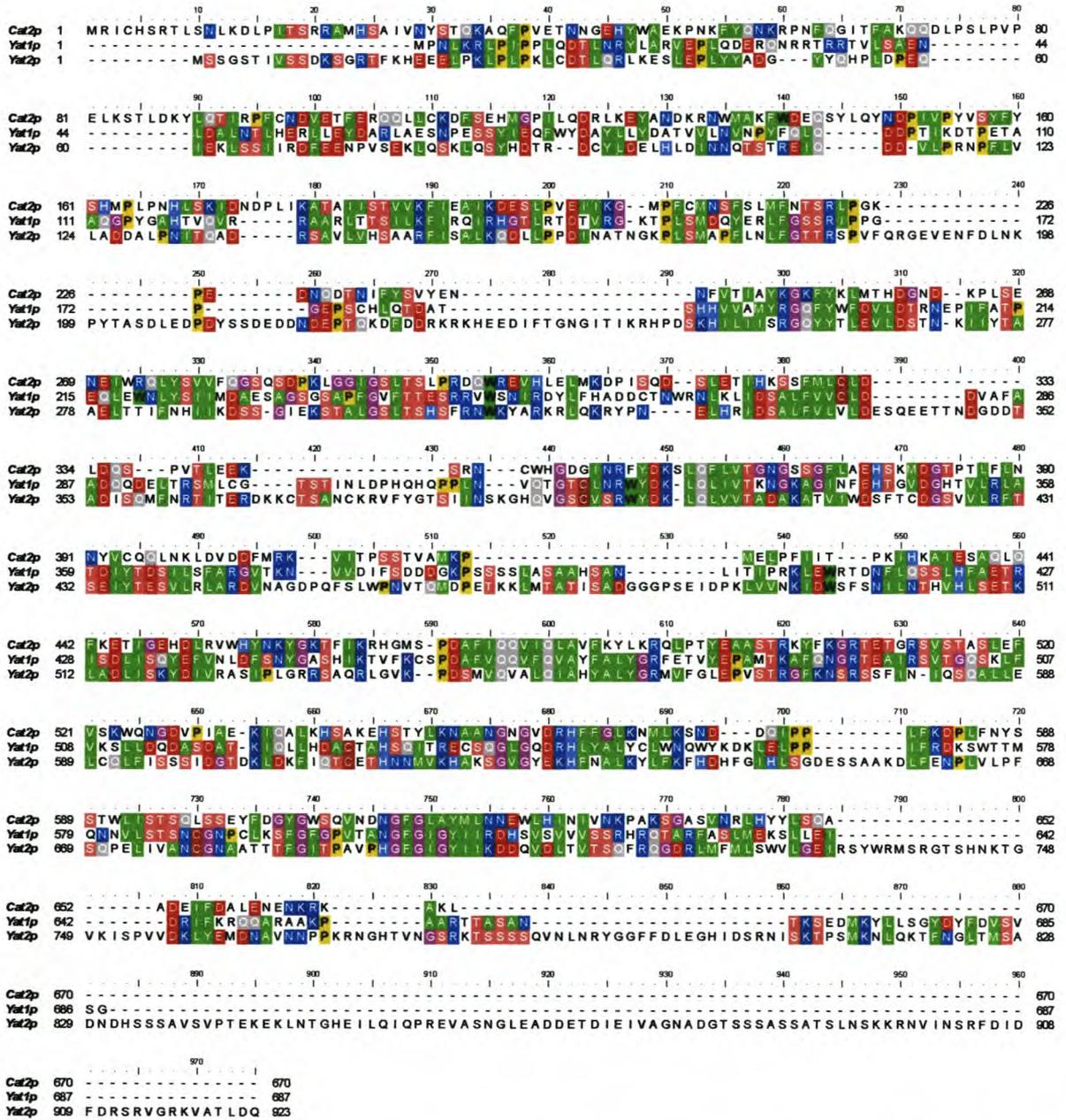


Figure 2.7 Amino acid alignment of the three carnitine acetyltransferases in *S. cerevisiae*, respectively Cat2p, Yat1p and Yat2p.

2.4.3 CARNITINE OCTANOYLTRANSFERASES (COT)

The human (Ferdinandusse *et al.*, 1999), rat (Choi *et al.*, 1995) and bovine (Cronin, 1997) mRNA sequences of peroxisomal COT encode proteins of 612 amino acids (about 70 kDa) that contain different C-terminal sequences (THL, AHL and PHL respectively) thought to serve as PTSs. However, as in CAT, other PTSs may be involved or the folded state of the protein may contain specific information for sorting. Expression of the rat gene for COT is subject to trans-splicing, i.e. splicing of different primary transcripts to one mRNA (Caudevilla *et al.*, 1998). In the case of rat liver COT, the different mRNAs that result from trans-splicing contain either a direct repeat of exon 2 or a direct repeat of exons 2 and 3. Therefore, including the simple transcript, the three mRNA sequences result in the expression of two COT proteins: one of normal size, i.e. 69 kDa, and one of 79 kDa (Caudevilla *et al.*, 1998). The transcript that contains the repeat of exon 2 does not produce a COT protein, due to a frameshift, because the number of nucleotides in exon 2 is not a multiple of three. The human gene, *CROT*, contains an additional intron at the start of the coding region (Van der Leij *et al.*, 2000).

2.4.4 CARNITINE PALMITOYLTRANSFERASES I (CPT-I)

Different genes, called *CPT1A* and *CPT1B*, encode the two known mammalian isoforms of CPT-I, liver-type carnitine palmitoyltransferase (L-CPT-I) and muscle-type carnitine palmitoyltransferase (M-CPT-I) respectively (reviewed in McGarry and Brown, 1997). The L-CPT-I isoforms are proteins of 773 amino acids with a predicted and apparent size of about 88 kDa. The M-CPT-I isoforms are proteins of 772 amino acids with a predicted size of about 88 kDa, but apparent sizes of about 82 kDa in SDS-PAGE analysis. mRNA sequences of the genes for L-CPT-I are known for rat, man, mouse and sheep (reviewed in Ramsay *et al.*, 2001). Protein sequencing of rat L-CPT-I reveals that the mature enzyme retains the N-terminus (Kolodziej and Zammit, 1993). A mitochondrial targeting sequence at residues 123-147 immediately behind the second transmembrane region has also been shown to act as a stop-transfer sequence to anchor CPT-I in the outer membrane (Cohen *et al.*, 2001). After ATP-dependent integration in the membrane (Cohen *et al.*, 1998), the majority of the enzymes are on the cytosolic face of the mitochondria, anchored by two transmembrane domains (**Figure 2.6 and Figure 2.8**). The initial biochemical studies (Fraser *et al.*, 1997) for rat L-CPT-I and the recent cytological studies on human M-CPT-I with green fluorescent protein (Van der Leij *et al.*, 1999) support these conclusions.

The rat M-CPT-I cDNA, obtained by screening a brown adipose cDNA library with a white adipose cDNA probe, encodes a protein that was called CPT-I-like protein (Yamazaki *et al.*, 1995). The strong cardiac expression of this gene (Esser *et al.*, 1996; Yamazaki *et al.*, 1995) and of its human orthologue (Yamazaki *et al.*, 1996) is

consistent with the identification of the encoded protein as M-CPT-I (Esser *et al.*, 1996).

In another approach to clone human *CPT1B*, part of the cDNA sequence was assembled after database screening of human-expressed sequence tags with the rat L-CPT-I cDNA sequence as query (Van der Leij *et al.*, 1997). This *in silico* cloning effort permitted the isolation of several cDNAs from human heart and skeletal muscle.

An analysis of these cDNAs shows that the last intron of human *CPT1B* sometimes is not removed from the primary transcript because of competition between splicing and polyadenylation. The first exon is untranslated and alternative first exons exist, as noted by Zhu *et al.* (1997). Further analysis of the alternative splicing of the *CPT1B* gene in human (Yu *et al.*, 1998a) and rat (Yu *et al.*, 1998b) reveals that the two first exons (exon 1a and 1b, also called U and M) do not co-exist in one transcript and that two promoters drive the expression of human *CPT1B*. Consequently, the expression of the 5' untranslated mRNA for human M-CPT-I differs from the alternative splicing of the transcript for L-CPT-I in rats, during which the second untranslated region is sometimes skipped (Park *et al.*, 1998).

Human *CPT1B*, which was sequenced as the middle gene of seven from a 180 kb BAC clone, is located closely downstream of a choline/ethanolamine kinase gene (Van der Leij *et al.*, 1997; Yamazaki *et al.*, 1997). *CPT1B* is even co-expressed with the upstream gene (Yamazaki *et al.*, 2000). The gene synteny is conserved in rats (Wang *et al.*, 1998) and mice (Cox *et al.*, 1998). In *Drosophila*, only one gene for CPT-I appears to be present (Jackson *et al.*, 1999). The protein is slightly larger than its mammalian counterparts (782 amino acids) and, when expressed in yeast, shows characteristics typical of a CPT-I enzyme.

2.4.5 CARNITINE PALMITOYLTRANSFERASES II (CPT-II)

CPT-II is a mitochondrial matrix protein associated with the inner mitochondrial membrane and is translated as a precursor of 658 amino acids in both human (Finocchiaro *et al.*, 1991) and rat (Woeltje *et al.*, 1990). The mature protein has a molecular weight of 70 kDa after the N-terminal MTS of 25 amino acids is removed during mitochondrial import (Finocchiaro *et al.*, 1991; Brown *et al.*, 1991). The genomic structure of human CPT2 (Verderio *et al.*, 1995) is similar to the mouse gene (Gelb, 1993), which also encodes a precursor of 658 amino acids.

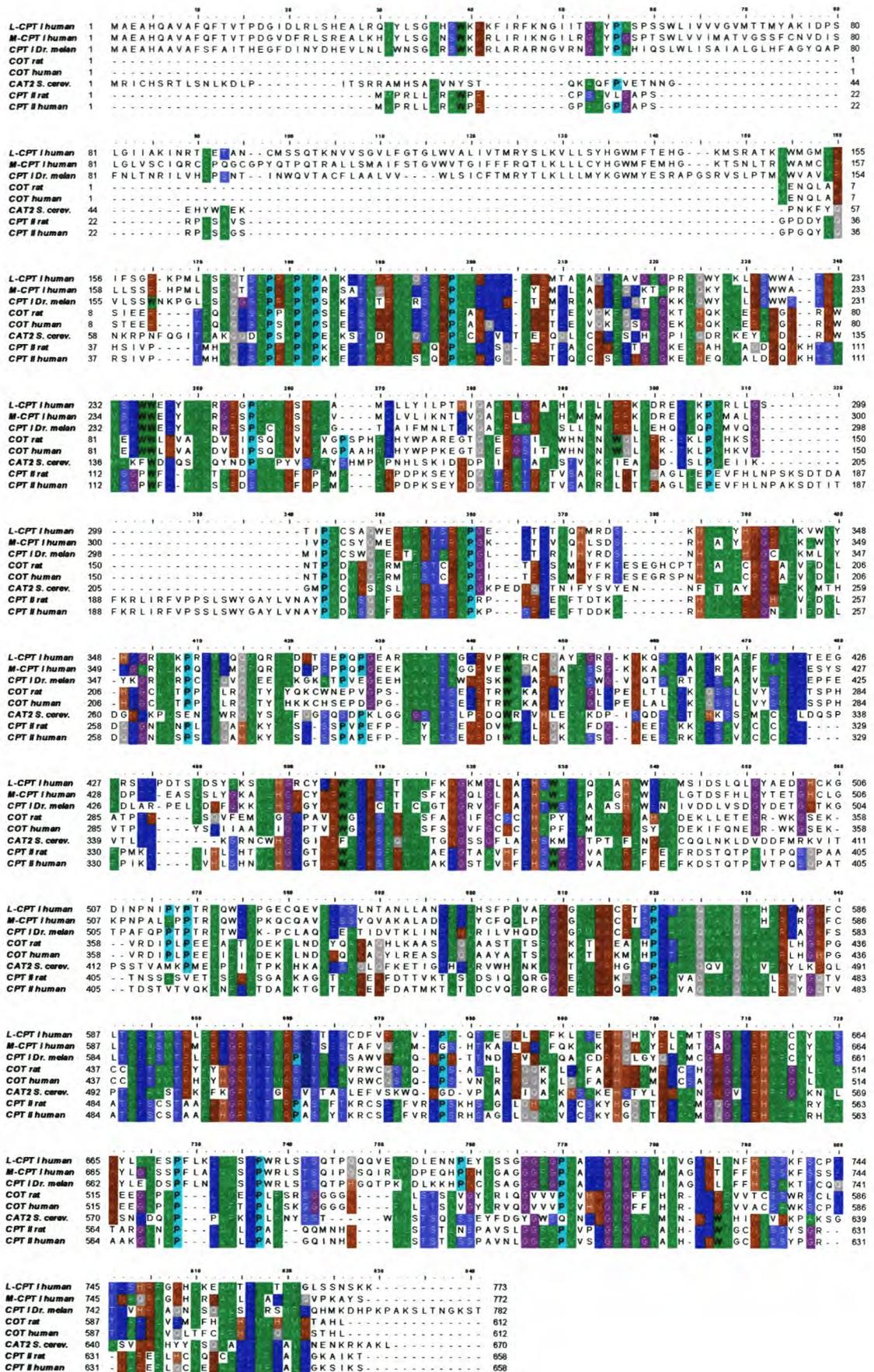


Figure 2.8 Amino acid sequence alignment of eight carnitine acyltransferases.

2.4.6 CARNITINE ACYLTRANSFERASES IN THE ENDOPLASMIC RETICULUM

A recent proposal, based on common immunoreactivity, suggests that all the cytoplasmic-facing CPT enzymes are the same (Fraser *et al.*, 1999). Although kinetic data support this proposal for mitochondrial and peroxisomal enzymes, the overt microsomal activity shows subtle kinetic differences (Abo-Hashema *et al.*, 1999). The only known candidate gene for an endoplasmic carnitine acyltransferase in humans, unrelated to the transferase family discussed above, is GRP58 (Murthy and Pande, 1994), also known as Erp57, Erp60 or Erp61. This protein is thought to have various functions; whether it is also a carnitine acyltransferase remains controversial (reviewed in Zammit, 1999).

2.4.7 MUTATION IN CARNITINE ACYLTRANSFERASES

Mutations at more than 30 positions in the acyltransferase protein family have been diagnosed or made through site-directed mutagenesis. Of the naturally occurring human mutations, the most frequent occur in CPT-II. CPT-II deficiency, the most common muscular lipid metabolism disorder (Taroni *et al.*, 1992), can be present with different times of onset and with different phenotypes. The adult form, with muscular or hepatomuscular presentation, and the neonatal form, with haptatocardiomyopathy presentation, represent the most extreme phenotypes of this recessive disorder. Infantile and intermediate phenotypes occur as well (Bonfont *et al.*, 1999; Taggart *et al.*, 1999).

The S113L mutation, which was among the first identified, is the most common in CPT-II (Taroni *et al.*, 1992). Reported in several studies of homozygotic or compound heterozygotic CPT-II deficiency, this mutation has an allele frequency of 60% of the affected genes (Bonfont *et al.*, 1999; Handig *et al.*, 1996; Verderio *et al.*, 1995). The affected serine residue at position 113 in human CPT-II is conserved within CPT-I, CPT-II and "vertebrate" CAT (human, mouse, pigeon), but not in any of the COT proteins or in yeast CAT (**Figure 2.8**). S113L is a "mild" mutation and is associated only with the adult form of CPT-II deficiency. A strict correlation between the severity of each mutation and the onset and phenotype, however, cannot be made that clearly, as exceptions exist. For example, a homozygous R631C mutation results in different expression of the disorder in unrelated patients (Bonfont *et al.*, 1999). Therefore, genotypic differences outside the CPT2 gene may have major influences in certain cases.

The presence of the two polymorphisms of the mutant allele in CPT-II appears to have no significant effect on CPT-II enzyme function (reviewed in Bonfont *et al.*, 1999; McGarry and Brown, 1997). S113L, P50H (Taggart *et al.*, 1999; Handig *et al.*, 1996) and E174K (Wataya *et al.*, 1998; Yamamoto *et al.*, 1996), which have mild consequences, are associated with the adult form of CPT-II deficiency. P50H defines a change in the first fully conserved proline of an LPXLP motif (pos. 49-53 of CPT-II). The glutamate at position 174 is less conserved, although other transferases share glutamate in the vicinity (**Figure 2.8**). Severe consequences for CPT-II function result

from F383Y (Wataya *et al.*, 1998) and Y628S (Bonfont *et al.*, 1996). Both affect residues that are barely conserved outside CPT-II.

In contrast to CPT-II deficiency, deficiencies in the other transferases are still rare and only known at the sequence level for *CPT1A* (Ijlst *et al.*, 1998). A *CPT1A* mutation analysis has identified G710E, which is adjacent to a GFG pattern that is associated with carnitine binding in rat CPT-II (Brown *et al.*, 1994).

2.4.8 FUNCTIONAL PROPERTIES OF CARNITINE ACYLTRANSFERASES

Recent reviews have summarised the locations and functions of CATs in cells (Zammit, 1999; Ramsay, 1999). The native structures of CAT and COT are monomers (Ramsay, 1987). For CPT-II, only aggregates (Clarke and Bieber, 1981) are isolated after detergent solubilisation, although a homotetramer has been suggested (Finocchiaro *et al.*, 1990). Inactivation of CPT-I by radiation of the outer membranes has produced a target molecular weight that is close to the monomer molecular weight, suggesting that the normal form in the membrane could be a monomer (Kolodziej and Zammit, 1990).

The sigmoidal kinetics often observed for CPT-I (Fiol *et al.*, 1987; Saggerson and Carpenter, 1982) could indicate multisubunit cooperation. However, artefactual sigmoidicity or non-linear time courses caused by interactions of palmitoyl-CoA with albumin or micelles must first be excluded. Acyl-CoA-binding protein (ACBP) prevents these technical problems (Abo-Hashema, 1999). Sigmoidicity could still arise via the malonyl-CoA regulatory site, which could also explain the high substrate inhibition observed for palmitoyl-CoA (Murthy and Pande, 1987). Sigmoidicity with palmitoyl-CoA is not changed by malonyl-CoA inhibition (Fiol *et al.*, 1987), but disappears after treatment with the thiol reagent DTNB (Saggerson and Carpenter, 1982). Consequently, the sigmoidicity arises from the protein and not from the artefact. This implicates a thiol group in the reversible alteration of sensitivity to malonyl-CoA (Zammit, 1983). These observations may be explained by (a) the flexible interaction between the N-terminal region and the main part of the enzyme, (b) the complex responses of malonyl-CoA inhibition to membrane environment or (c) both.

The effect of detergents on activity and membrane attachment generated many early controversies about the CPTs. The use of Triton X-100, which inactivates CPT-I completely but activates and solubilises CPT-II, delayed the identification of CPT-I as a separate protein (Esser *et al.*, 1993). In one laboratory, octylglucoside solubilised active CPT-I (Murthy and Pande, 1987), whereas, in another, it inhibited and failed to solubilise it (Woeltje *et al.*, 1990). In a third, it failed to solubilise either CPT-I or the peroxisomal malonyl-CoA-sensitive enzyme, but, of the five detergents studied, octylglucoside had the least effect on the activity of COT, CPT-I and CPT-II (reviewed in Ramsay *et al.*, 2001). Others found that octylglucoside produces complex effects on extracted CPT-II (Fiol and Bieber, 1988).

Despite numerous publications on acyltransferase activity, its measurement remains problematic. In particular, the susceptibility of the assay to artefacts, complicated by both high substrate and strong product inhibition, has produced data that are only suitable for internal comparisons. Kinetic studies of carnitine acyltransferases have to take into account numerous variables, i.e. micelle concentrations of the long-chain acyl derivatives (Goni *et al.*, 1996), the presence (or absence) of albumin (Pauly and McMillin, 1988; Bartlett *et al.*, 1985), the fact that membranes bind palmitoyl-CoA (Murthy and Pande, 1987; Goni *et al.*, 1996), and the concentrations of the lipid or detergent or both (Woeltje *et al.*, 1990; Fiol and Bieber, 1988; Murthy and Pande, 1987), all of which influence acyl-CoA availability (Pauly and McMillin, 1988). As mentioned above, the recent use of recombinant ACBP overcomes some of the problems inherent to experiments with long-chain acyl-CoA substrates (Abo-Hashema *et al.*, 1999). **Table 2.1** gives true kinetic constants for purified CAT, COT and CPT-II (Nic a' Bhaird *et al.*, 1993; Chung and Bieber, 1993; McGarry *et al.*, 1983; Chase and Tubbs, 1966), but it is difficult to discuss the level of saturation of these enzymes with acyl-CoA substrates in the cell. The cytoplasmic CoA-SH content is low and represents less than 10% of the cellular pool in heart cells (Brass and Ruff, 1992). The concentration of free acyl-CoA is essentially zero, because all the cytoplasmic long-chain acyl-CoA will be bound to ACBP. It has been suggested that CPT-I directly uses the pool of acyl-CoA bound to ACBP (Bhuiyan and Pande, 1994).

From the earliest studies on the first purified transferase, CAT from pigeon breast muscle (Chase, 1967), to the more recent studies, the results show that the V_{\max} changes little in the pH range from 7-8 (reviewed in Bremer, 1983). **Table 2.1** gives examples of the values obtained for each transferase. The V_{\max} for acyl-carnitine formation (removal of acyl-CoA, the forward reaction) is generally higher than that for the reverse reaction. However, as noted above, local concentrations of the substrates will determine the net flux.

Table 2.1 also gives information about the different kinetic properties of the isoforms of CPT-I (muscle and liver). The enzymes have the same K_M for palmitoyl-CoA, but the K_M for carnitine of the muscle form is ten-fold higher than of the liver form. This correlates with the higher carnitine concentration in heart than in liver (Idell-Wenger *et al.*, 1978). Indeed, McGarry *et al.* (1983), who surveyed a range of tissues and species, have shown an inverse correlation between the K_M for carnitine and the IC_{50} of malonyl-CoA. Jackson *et al.* (2000a) have now demonstrated that the inverse relationship of carnitine K_M and malonyl-CoA sensitivity is caused by modulation of the catabolic domain by the N-terminal residues.

Table 2.1 Comparison of the kinetic constants for the various carnitine acyltransferases

Gene	<i>CPT1A</i>	<i>CPT1B</i>	<i>CPT2</i>	<i>CROT</i>	<i>CRAT</i>	?
Enzyme	L-CPT-I	M-CPT-I	CPT-II	COT _p	CAT	CPT _{ER}
MW (kDa)	88	82	68	71	67.5	54
K_M (μ M)						
Acetyl-CoA	^a	^a	< 5	0.6	34	< 4
Carnitine	30	500	1500	108	120	600
Acy-Car			46	7.4	350	1000
CoA	40		112	16	37	300
V (U/mg)						
Forward			32	46	500	100
Reverse			15	37	396	13
References	Rat liver	Rat skeletal muscle	Beef liver	Beef liver	Beef liver	Rat liver

^a 30 – 60 μ M in the presence of bovine serum albumin

The first structural information on CPT-I and studies of the effect of mutations of the N-terminal residues make it easier to understand some observations. The data suggest that interaction of the N-terminal with the large C-terminal domain helps to stabilise the catalytic site and to modulate malonyl-CoA sensitivity (Shi *et al.*, 2000; Fraser *et al.*, 1997). As shown by Jackson *et al.* (2000b), by using a set of six chimeric proteins as well as the parental forms, interactions between the two cytosolic parts of the protein (see **Figure 2.6**) contribute to interaction with the first substrate, palmitoyl-CoA. The pairing of the transmembrane domains influences the interaction with carnitine, but removal of the N-terminal transmembrane domain still leaves an active protein (Jackson *et al.*, 2000a). From their study of both the M and L isoforms of the enzyme, it can be seen that the C-terminal portion of the protein gives rise to a 100-fold difference in sensitivity to malonyl-CoA, with the M form being more sensitive. The work by Jackson *et al.* (2000a; 2000b) suggest that the catalytic core contains the malonyl-CoA binding site. Modulation of the malonyl-CoA sensitivity depends, however, on the N-terminal section.

The same studies (Jackson *et al.*, 2000b) imply that the membrane anchor helices could transmit the influence of membrane changes. Recent observations *ex vivo* (Fraser and Zammit, 1998) emphasise microenvironmental influences on the kinetics of CPT I from rat liver cells. More CPT-I is found at the contact sites between the outer and inner membrane (Fraser and Zammit, 1998), and its kinetic properties

are changed dramatically. In the outer membrane, malonyl-CoA inhibition affects the V_{\max} but not the K_M for palmitoyl-CoA, whereas, in the contact sites, V_{\max} is unchanged, but the apparent K_M for palmitoyl-CoA is greatly increased. Consequently, the mode of inhibition by malonyl-CoA is competitive in the contact sites, where channelling to β -oxidation seems likely, so that excess long-chain acyl-CoA could overcome the inhibition and prevent accumulation. In the outer membrane, uncompetitive inhibition ensures that the accumulating acyl-CoA required for other cytosolic functions is not converted into acylcarnitine (reviewed in Ramsay *et al.*, 2001).

The main target for designing inhibitors of this enzymatic family is CPT-I, since it influences the rate of mitochondrial fatty acid oxidation. However, the kinetics are difficult to study because they have to be determined in the mitochondrial outer membrane. Problems include the influence of the membrane fluidity on activity, the sensitivity of malonyl-CoA inhibition to the fluidity, the binding of palmitoyl-CoA and palmitoylcarnitine to the membrane, sensitivity to proteolysis and the differences in mitochondria prepared from animals in different physiological states (starvation, diabetes, hormonal changes, the presence of proliferators, etc.). The reconstitution of the protein into artificial membranes (Zhu *et al.*, 1997) and its expression in yeast free of endogenous activity (Jackson *et al.*, 2000b) should permit detailed kinetic studies.

2.5 ACYL GROUP TRANSFER – ENZYMATIC REGULATION AND CHEMICAL MECHANISM

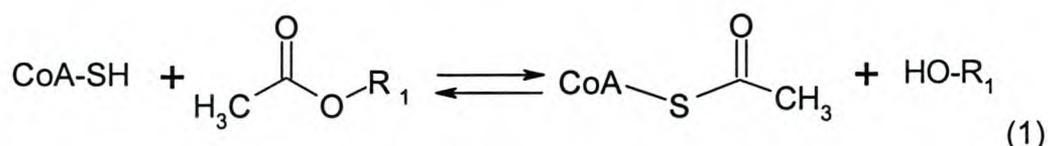
2.5.1 ENZYMATIC REGULATION

As previously mentioned, kinetic parameters have been established for CAT (Chase and Tubbs, 1966), COT and CPT-II (Nic a' Bhaird *et al.*, 1993;) (see **Table 2.1**). From primary plots and product inhibition studies, CAT and COT show rapid equilibrium random order kinetics, indicating that all four substrates bind well to the free enzyme. In contrast, CPT-II follows a compulsory order ternary complex mechanism in which CoA must bind first. CPT-II has a very low affinity for L-carnitine, which has a K_i value of 11.7 mM. In contrast, COT has a 100-fold greater affinity for L-carnitine and has a K_M value (0.1 mM) equivalent to the dissociation constant. The observation that CPT-II binds acylcarnitine analogues whereas COT binds carnitine analogues reflects the difference.

The ordered mechanism of CPT-II, in which acyl-CoA binds first, ensures that this reversible enzyme responds to the acylation state of the CoA pool in the mitochondrial matrix. The matrix concentrations of both CoA-SH and acyl-CoA (Idell-Wenger *et al.*, 1978) exceed the measured K_M values (Nic a' Bhaird *et al.*, 1993), so that most CPT-II will have the first (CoA-SH) substrate bound. Certainly, CPT-II in normal mitochondria does not appear to limit the rate of fatty acid oxidation. The enzyme that appears to control the access of fatty acid acyl-CoA to the matrix for β -oxidation is CPT-I on the outer mitochondrial membrane (Gandour *et al.*, 1993).

2.5.2 CHEMICAL MECHANISM

In the past decade, our understanding of the basic chemistry of carnitine acyltransferases has progressed considerably. Computational studies of acyl transfer equilibria explain why acylcarnitines have a large acyl group transfer potential; sequence studies and site-directed mutagenesis provide further information for elaborating a chemical mechanism; and measurements of K_M and K_i values for substrate and putative transition state analogues provide additional data for developing a picture of the active site. Despite the absence of a crystal structure of a carnitine acyltransferase or detailed chemical mechanistic studies, one can define the mechanistic possibilities for the reaction shown below.



2.5.2.1 REACTION ENERGETICS

Acyl-CoA, a thioester, has a high acyl transfer potential. Acylcarnitine, an oxyester, must have similar potential to avoid coupling the acylcarnitine-to-CoA transfer to an energy-releasing reaction. The equilibria between acetyl-CoA and various oxyesters (see Eq. 1) are calculated from the free energies of hydrolysis ($\Delta G^\circ_{\text{hyd}}$ at 25° C) and are given in **Table 2.2**.

Table 2.2 Equilibria between acetyl-CoA and various oxyesters, calculated from the free energy of hydrolysis

compound	KJ/mol	References
acetyl-CoA	- 34.3	Burton, 1955
acetylcarnitine	- 33.0	Fritz <i>et al.</i> , 1963
acetylcholine	- 27.1	Müller and Strack, 1973
ethyl acetate	- 24.7	Jencks <i>et al.</i> , 1960

Acetylcarnitine has the most favourable equilibrium for the transfer of CoA-SH; in fact, acetyl transfer occurs readily in both directions (Fritz *et al.*, 1963). For acetylcholine and ethyl acetate, acetyl transfer to CoA costs energy.

2.5.2.2 PROPOSED MECHANISM

Most suggested mechanisms for acyl transfer involve a tetrahedral intermediate. However, no direct evidence supports or disputes this mechanism for carnitine acyltransferases. A role for histidine in the mechanism was suggested by Chase and Tubbes (1970), who used both chemical modification and substrate analogues. Site-

directed mutagenesis identified the residue as H372 in CPT-II (Brown *et al.*, 1994) and as H327 in COT (Morillas *et al.*, 2000). The loss of activity of the D376A and D464A mutants of CPT-II suggests a role for aspartate, and recent chemical modification studies suggest that a lysine contributes to catalysis (Brown *et al.*, 1994). A catalytic role for an acyl intermediate with serine is unlikely (Nic a' Bhaird *et al.*, 1993; Cronin, 1997). A direct transfer between carnitine and CoA-SH appears logical. The simplest mechanism proposes that the histidine serves as a general base – removing a proton from carnitine or CoA-SH, depending on the direction of transfer – to promote the formation of a tetrahedral intermediate. An aspartate may potentiate the histidine function. The lysine stabilises the putative oxyanion. The protonated histidine donates a proton for the departing group, either carnitine or CoA-SH.

Other residues contribute to binding, but not directly to catalysis. An arginine probably forms a strong salt bridge with the carboxylate of carnitine. A cleft similar to the aromatic residue-rich pocket in acetylcholinesterase for recognising the trimethylammonio group of choline (Sussman *et al.*, 1991) is likely for carnitine. A serine-threonine-serine (STS) triad conserved in all the carnitine acyltransferases (and in choline acyltransferases) contributes, perhaps by providing an aqueous-like microenvironment, to the binding of carnitine (Cronin, 1997), which is strongly solvated. Substitution of all three residues of the STS triad results in a 1000-fold increase in K_M for carnitine. In CPT-II, three substitutions – V605A, G609A and G611A – resulted in a higher K_M for carnitine (Brown *et al.*, 1994).

2.6 REGULATION OF CPT ACTIVITY

2.6.1 INTRODUCTION

In the past few years, several studies on the transcription of *CPT1* and *CPT2* have revealed a picture of the regulatory mechanism of these enzymes (McGarry and Brown, 1997; Brunner *et al.*, 1997). CPT-I activity increases during starvation (Grantham and Zammit, 1987) and decreases in patients with hypothyroidism (Mynatt *et al.*, 1994). Insulin regulates CPT-I via the insulin growth factor I receptor (Park *et al.*, 1995).

CPT-II mRNA and activity are likewise increased during starvation and diabetes and also by peroxisomal proliferators. Peroxisomal COT increases in parallel with CPT-II after induction by peroxisomal proliferators, suggesting a parallel mechanism. These peroxisomal proliferators have been identified as a major transcriptional element and will be discussed in the following section. Fish oil diets also induce peroxisomal COT in rats, in keeping with the role of the peroxisomes in shortening very-long-chain fatty acids for transfer as medium-chain acylcarnitines to the mitochondria. In yeast, where β -oxidation occurs only in peroxisomes, CAT and the enzymes of fatty acid oxidation are induced during growth on fatty acid (reviewed in Brady *et al.*, 1993).

2.6.2 TRANSCRIPTIONAL REGULATION

The tissue-specific and temporal expression of *CPT1A* and *CPT1B* as compared to the body-wide and relatively steady expression of *CPT2* has been reviewed (McGarry and Brown, 1997). *CPT1A* is expressed in the liver and many other tissues, whereas *CPT1B* is expressed significantly in skeletal and cardiac muscle and in the testes. However, adipocytes show species-specific differences in the expression of *CPT1B*, e.g. mouse versus man and rat (Brown *et al.*, 1997). However, the *CPT1B* promoter sequences are remarkably conserved among man, mouse, rat and sheep, indicating a similar triggering mechanism for expression.

The expression of several enzymes needed for fatty acid transport and oxidation is regulated at the transcriptional level via the peroxisome proliferator-activated receptor α (PPAR α) (Aoyama *et al.*, 1998). Like many nuclear membrane receptors, PPAR α can form heterodimers with related receptors, e.g. the retinoid X receptor (Brandt *et al.*, 1998), to mediate the signalling between ligand and target gene. The promoters of these target genes contain specific sequences that are binding sites for the receptor dimers. The PPAR α binding sites are known as peroxisome proliferator response elements and those responsive to fat have also been called fat-activated response elements (FARE). Studies with a knockout mouse model provide direct evidence that PPAR α participates in the transcription of *CPT1B*, but that it is less prominent in that of *CPT1A* and *CPT2* (Leone *et al.*, 1999; Lee *et al.*, 1995).

The *CPT1B* gene responds to accumulating intracellular fatty acid intermediates (presumably long-chain acyl-CoAs) via the presence of a FARE in the promoter (Mascaro *et al.*, 1998). Hence, *CPT1B* is strongly activated when etomoxir inhibits CPT-I (Brandt *et al.*, 1998) or under fasting conditions (Leone *et al.*, 1999). A PPAR α -mediated response to fasting might also contribute to activating *CPT1B* transcription in the liver (Yu *et al.*, 1998b). Furthermore, a mammalian orthologue of a chicken ovalbumin upstream promoter transcription factor (COUP-TF) counteracts the FARE and competes with PPAR α (Yu *et al.*, 1998b). This has special interest, because COUP-TF and other transcription factors, such as SP1, play a role in cardiac development and in hypertrophic responses of the heart associated with metabolic foetal gene re-expression programmes (Sack *et al.*, 1997).

The *CPT1B* gene is also induced by fasting and etomoxir treatment. *CPT1A* promoter studies have revealed roles for transcription factors such as SP1 and SRY (Steffen *et al.*, 1999), but the molecular mechanism of the responses to fatty acids and cyclic AMP (Brunner *et al.*, 1997) remains unknown. *CPT1A* is expressed in the heart when cardiac carnitine levels are low (Brown *et al.*, 1995), and the juvenile steatosis mouse, which lacks a functional OCTN2, shows increased cardiac *CPT1A* expression (Uenaka *et al.*, 1996). Administering carnitine represses and reverses the effect (Uenaka *et al.*, 1996), thus bypassing the active step in cardiac carnitine uptake through the action of low-affinity carnitine transporters or through diffusion. Although the effect of carnitine on cardiac *CPT1A* expression might suggest suppression by carnitine, a direct role for carnitine in gene transcription has not been

shown (Brunner *et al.*, 1997), and the relieving effect of carnitine on long-chain acyl-CoA accumulation may well explain the consequences of carnitine supply in the juvenile steatosis mouse.

Intriguingly, the *CPT2* gene promoter does not contain a FARE similar to the known one of *CPT1B*, but *CPT2* does respond to specific induction through PPAR α , at least in the liver (Aoyama *et al.*, 1998). This *CPT2* response is seen only with fibrates and not with (long-chain) fatty acids, pointing to activator-specific causes of *CPT2* transcription induction.

The transcription of *CPT1* and *CPT2* has been studied relatively extensively compared to the expression of COT and CAT (Brunner *et al.*, 1997) and no promoter studies of the genes for CAT, CACT and OCTN2 have been reported.

2.6.3 REGULATION BY MALONYL-COA

Malonyl-CoA, a substrate analogue, is, as would be expected, a competitive inhibitor of CAT, COT and CPT-II. The K_i value for malonyl-CoA of COT is similar to the values for other short-chain CoA esters (Nic a' Bhaird and Ramsay, 1992). The inhibition involves the interaction of malonyl-CoA, presumably in the active site, and differs from the regulatory sensitivity of CPT-I to malonyl-CoA.

The observation of the extreme sensitivity of L-CPT-I to malonyl-CoA dates back to 1978 (McGarry *et al.*, 1978). Malonyl-CoA, a fatty acid synthesis intermediate, enables the inhibition of fatty acid oxidation under conditions in which synthesis is required. The understanding of the molecular basis for inhibition of L-CPT-I by malonyl-CoA has led to controversy and can be summarised as follows:

- Both the L and M form contain a malonyl-CoA binding site and a catalytic site in the protein. Contrary to earlier conclusions (summarised in McGarry and Brown, 1997), the malonyl-CoA binding site and the active site are on the same side of the membrane (Fraser *et al.*, 1997).
- Isoforms L and M differ in malonyl-CoA sensitivity (Chase and Tubbs, 1966; Saggerson and Carpenter, 1982).
- The N-terminus, which is not essential for catalytic activity, modulates the response to malonyl-CoA, but the large C-terminus domain is responsible for sensitivity (Jackson *et al.*, 2000b; Shi *et al.*, 2000; Jackson *et al.*, 2000a). Deleting various portions of the N-terminus both negatively and positively affects the sensitivity (Jackson *et al.*, 2000a).
- Malonyl-CoA sensitivity decreases as pH increases (for attenuation of fatty acid oxidation during acidosis) (Stephens *et al.*, 1983).
- Inhibition by malonyl-CoA is non-competitive with palmitoyl-CoA in the outer membrane, but is purely competitive in the contact sites (Ramsay *et al.*, 2001).

- Membrane insertion contributes to the modulation of malonyl-CoA sensitivity, as demonstrated by reconstitution into liposomes at different temperatures (McGarry and Brown, 2000). The alterations in the kinetics of L- and M-CPT-I when each transmembrane domain is exchanged demonstrate how these effects are transmitted (Jackson *et al.*, 2000b).

The altered kinetics of malonyl-CoA inhibition of L-CPT-I in response to the environment (temperature, lipids, membrane) suggests flexibility in the protein. Arguments for an allosteric site for malonyl-CoA have been summarised (McGarry and Brown, 1997). However, protection by malonyl-CoA against inactivation by the covalent suicide substrate etomoxiryl-CoA or bromoacetyl-CoA suggests either that the two sites overlap, or that they affect each other strongly enough to mutually influence binding. The recent revelation (Jackson *et al.*, 2000a) that the N-terminus affects both malonyl-CoA inhibition and carnitine binding demonstrates that malonyl-CoA acts at the active site.

In addition, novel data by Guzmán *et al.* (2000) show that, in concert with malonyl-CoA, cytoskeletal components regulate CPT-I. However, this recent model of regulation needs further exploration.

2.6.4 PHARMACEUTICAL IMPORTANCE AND DRUG DEVELOPMENT

Anderson (1998) points out that L-CPT-I is a better target for drug development against diabetes than M-CPT-I or CPT-II. Therefore, one approach to formulating an effective drug for type II (non-insulin-dependent) diabetes mellitus (NIDDM) is to design selective inhibitors of L-CPT-I. L-CPT-I catalyses a key step in supplying fatty acyl groups for fatty acid oxidation. Controlling fatty acid oxidation can regulate blood glucose levels and ameliorate some symptoms of NIDDM, a condition that accounts for over 90% of diabetes cases. Inhibitors of L-CPT-I can decrease fatty acid oxidation and, therefore, serve as adjuvant therapeutic agents to help manage NIDDM (Anderson, 1998). Isozyme-selective inhibitors offer the potential of minimising undesirable side effects.

In order to treat NIDDM, these inhibitors must overcome the challenges described by Anderson (1998). In cynomolgus monkeys, SDZ-CPI-975, a reversible inhibitor of CPT-I, lowers blood glucose without inducing the cardiac hypertrophy caused by etomoxir, an irreversible inhibitor (Deems *et al.*, 1998). SDZ-CPI-975 causes "hepatic mitochondrial aberrations" and the development of this drug has slowed down (Anderson, 1998). Anderson rightfully concludes that "major issues would need to be more critically examined before committing to full development" of an L-CPT-I inhibitor as a drug.

Irreversible inhibitors are being developed cautiously because of myocardial hypertrophy. Etomoxir has been used in two recent studies on humans. In a dietary study, etomoxir stimulated appetite in subjects who had a high dietary intake of fat (Kahler *et al.*, 1999). Inhibiting fatty acid oxidation signals a desire to eat in those

subjects who consume fat in large quantities. In a limited study with patients who suffered from chronic heart failure, etomoxir improved the clinical status of the patients and showed no side effects after long-term (three months) administration (Schmidt-Schweda and Holubarsch, 2000). In congestive heart failure, calcium homeostasis is impaired and certain contractile proteins are altered. Through gene expression, etomoxir enhances the levels of sarcoplasmic reticulum Ca^{2+} -ATPase and of α -myosin heavy chain protein. These clinical observations may encourage clinical studies of other CPT-I inhibitors.

Developing isozyme-selective inhibitors of CPT remains a viable goal for the following reasons: (1) CPTs have functions beyond the liver and (2) acylcarnitines can modulate the activity of other enzymes. For example, L- and M-CPT-I have roles in sperm maturation (Adams *et al.*, 1998), so a selective CPT inhibitor might serve as a male contraceptive agent.

Other potential applications aim on higher lever of palmitoylcarnitine. For example, palmitoylcarnitine inhibits protein kinase C in neuroblastoma NB-2a cells (Nalecz *et al.*, 1997), and a palmitoylcarnitine analogue might therefore inhibit tumour cell proliferation. In a second application, palmitoylcarnitine, a lysophospholipase transacylase inhibitor (Gross and Sobel, 1983), interferes with *Candida* adherence to lysophospholipids and the Hep-2 cell line (Prakobphol *et al.*, 1997). A palmitoylcarnitine analogue therefore might serve as an antimicrobial agent (Savle *et al.*, 1999). Consequently, continued development of acylcarnitine analogues offers benefits beyond controlling NIDDM.

2.7 CONCLUSION

This review has focussed mainly on the carnitine system in mammals. However, the carnitine system is being studied in species other than mammals. For example, metabolic studies in fish indicate that the relationship between fatty acid oxidation and CPT levels and the sensitivity of CPT-I to malonyl-CoA are similar to mammalian ones (Froyland *et al.*, 1998). Plants have carnitine and carnitine acyltransferase activities, but the proteins are unidentified so far. Although CPT activity exists on either side of the pea leaf chloroplast inner membrane, only a 20 kDa protein cross-reacted with antiserum to beef heart CPT-II (Masterson and Wood, 2000). A sequence from the *Arabidopsis thaliana* genome sequencing project is annotated as homologues to the transporter, CACT, but phylogenetic analyses point to a closer relationship with ornithine translocators (Van der Leij *et al.*, 2000). Some researchers dispute the physiological significance of the carnitine system in plants, yet plants, like animals, use limited pools of CoA. Carnitine in plants most likely facilitates the transport of activated fatty acids during desaturation, elongation and lipid synthesis – e.g., during periods of rapid membrane synthesis – but also during lipid mobilisation and transport to the glyoxysome.

The sequencing of genomes and transcripts of various organisms continues to reveal genes and proteins that belong to the family of carnitine/choline acyltransferases. Gene phylogeny studies have now been carried out on about 50 genes of this family (Van der Leij *et al.*, 2000). The first results of maximum likelihood and parsimony analyses of human and yeast genes, known to encode active transferases, are given by Van der Leij *et al.* (2000). For *CPT1A* and *CPT1B*, the general picture is that an ancestral *CPT1* gene duplicated probably when, or even before, vertebrates evolved. *CPT1* genes are the closest relatives, a conclusion supported by conservation of their exon junction positions. Human genes for COT, CAT and CPT-II are more closely related to each other than the genes for CPT-I. Apparently, CPT-I and CPT-II are the most distant members of the family. This distance between CPT-I and CPT-II is independent of the differences in the C- and N-termini. The relation of the human genes to the yeast *CAT2* gene for mitochondrial/peroxisomal CAT (Cat2p) and the *YAT1* gene for Yat1p points to separate branches for human CAT and Cat2p. However, the distance between CAT and Cat2p is less than the intraspecies distances between Cat2p and Yat1p or between CPT-I and CPT-II. Therefore, although the root of the tree has not been defined, it is likely that a common ancestor of the carnitine/choline acyltransferase family is a CAT-like enzyme. Furthermore, *YAT1* shares an ancestral gene with CPT2 that is not shared by other genes, suggesting the possible conservation of protein functions despite their differences in subcellular localisation and substrate specificity (reviewed in Ramsay *et al.*, 2001).

The sequence and molecular genetics information generated in the last ten years has opened up the molecular studies of carnitine proteins and facilitated dissection of their multiple roles and intracellular locations. Molecular probing of CPT-I begins to give a picture of CPT-I and its physiological regulation by malonyl-CoA for the control of fatty acid oxidation. The next big advance will be the resolution of the protein structure of these enzymes, which will lead to a better understanding of molecular mechanisms and act as a tool in drug design.

2.8 LITERATURE CITED

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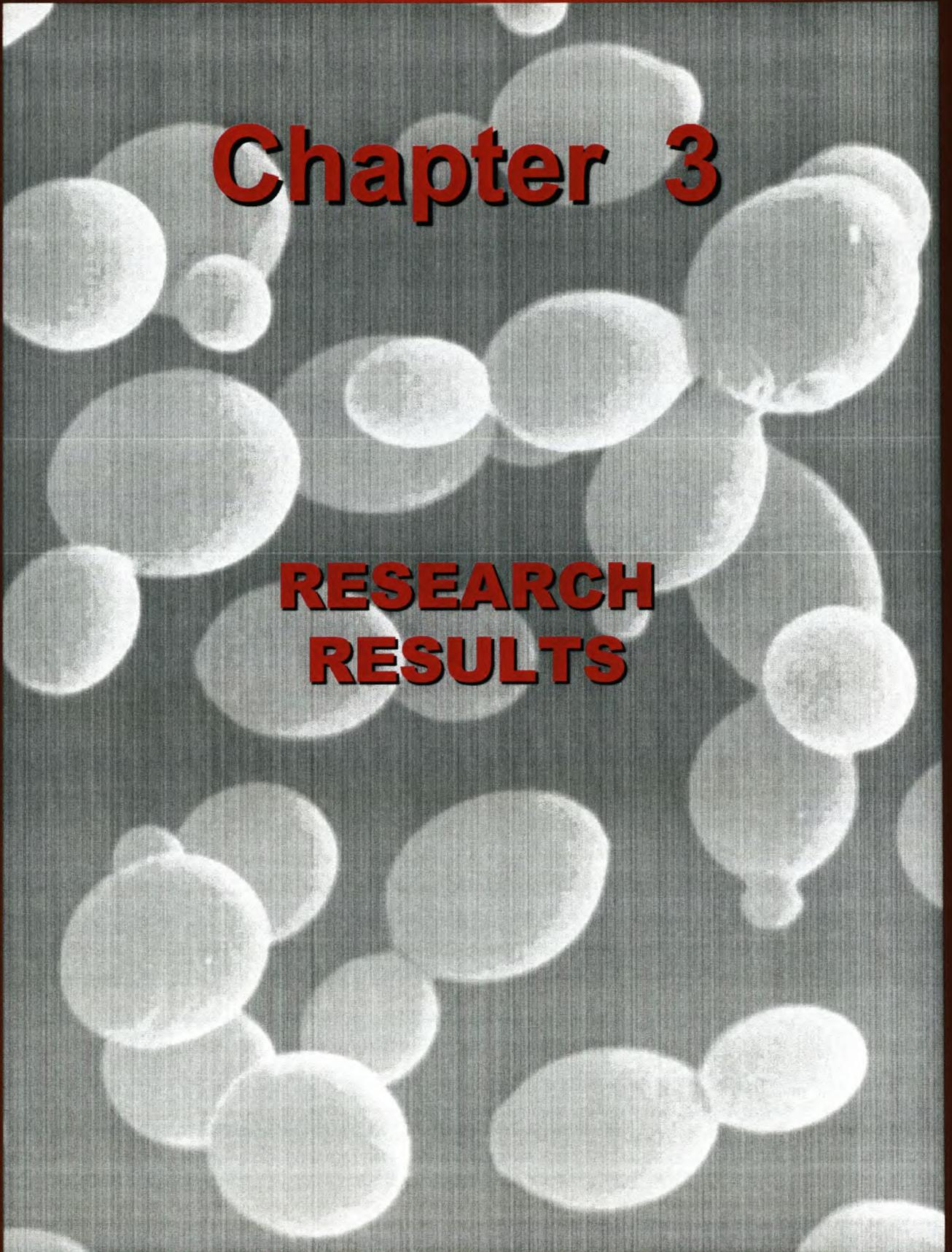
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Chapter 3

RESEARCH RESULTS



RESEARCH RESULTS

3.1 INTRODUCTION

The major aim of this study was to determine the contribution of each carnitine acetyltransferase in *S. cerevisiae* to the carnitine shuttle and to investigate other possible carnitine-associated metabolic pathways. It therefore was necessary to develop an accurate and precise method to measure the formation of acetylcarnitine from acetyl-CoA and carnitine. The assay should allow the measurement of these compounds in homogenised yeast crude extract. This enzymatic assay was developed in this study and submitted for publication. It therefore corresponds to the first part of the following section.

The second part of this section summarises the preliminary results obtained by our attempts to identify the specific roles of the three *S. cerevisiae* CAT enzymes that are encoded by the yeast genome. Beyond the data obtained by measuring CAT activity in different knockout strains, we investigated the phenotypical effects of single, double and triple gene deletions on growth speed in fermentable and non-fermentable carbon sources. We show that no phenotypical differences can be observed between the various strains, even in the presence of the *Schizosaccharomyces pombe* dicarboxylic acid transporter. We furthermore assessed the effect of the overexpression of any of the three CAT genes in $\Delta cit2$ double mutant strains carrying deletions of *YAT1*, *YAT2* or *CAT2* respectively. In addition, we show that the expression of the *Schizosaccharomyces pombe* malate permease gene (*MAE1*) can complement a $\Delta cit2$ mutation when malate is added to the growth substrate. Finally, we show the intracellular localisation of the three CAT enzymes.

CHAPTER 3

RESEARCH RESULTS

The Determination of Carnitine Acetyltransferase Activity in *Saccharomyces cerevisiae* by HPLC- Electrospray Mass Spectrometry

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The Determination of Carnitine Acetyltransferase Activity in *Saccharomyces cerevisiae* by HPLC-Electrospray Mass Spectrometry

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3.2.1 SUMMARY

Carnitine acetyltransferases (CATs) catalyse the transfer of activated acetyl groups between the acetylated and free forms of coenzyme A and carnitine. This reaction is part of the carnitine shuttle, a metabolic pathway that is required for the exchange of activated acetyl groups between cellular compartments. Indeed, only acetyl-carnitine, and not acetyl-CoA, can be transported across intracellular membranes. Three CAT enzymes, encoded by the genes *CAT2*, *YAT1* and *YAT2*, have been identified in the model organism *Saccharomyces cerevisiae*. While genetic evidence suggests that each CAT plays a unique role in cellular metabolism, our current understanding of the metabolic role of CATs does not explain why three independent enzymes are required. To investigate the specific role of each CAT, an accurate method for the determination of CAT activity is required. Methods employing spectrophotometric measurements are unreliable, particularly when crude homogenate is used. A radiolabeled forward exchange assay can be used to eliminate this problem, but safety concerns make this method undesirable. Here we have developed an HPLC tandem electrospray mass spectrometric method for the measurement of CAT activity in crude cellular homogenate. In this method, the conversion of carnitine to acetylcarnitine is measured quantitatively over a period of time by monitoring the precursor ion containing the $m/z = 85$ fragment, which is unique to the acetylcarnitine molecule. Crude extract from a mutant strain with all three CAT-encoding genes deleted showed no CAT activity when this method was employed, indicating that there are only three CATs present in *S. cerevisiae*.

3.2.2 INTRODUCTION

Carnitine acetyltransferases (EC 2.3.1.7) are present in eukaryotic organisms and have been described in yeast, fungi, plants and mammals (Burgess and Thomas, 1986; Masterson and Wood, 2000; Jernejc and Legisa, 1996; Kozulic *et al.*, 1987; Stemple *et al.*, 1998; Bieber, 1988). They catalyse the transfer of activated acetyl groups from acetyl-CoA to L-carnitine or from acetylcarnitine to CoA-SH. This reaction is required because organellar membranes are impermeable to acetyl-CoA (Van Roermund *et al.*, 1995), while a carnitine-acetylcarnitine antiport system can

ensure the transfer of the activated acetyl group without loss of energy (Palmieri *et al.*, 1999; Van Roermund *et al.*, 1999).

In the model organism *Saccharomyces cerevisiae*, three genes code for carnitine acetyltransferases, *CAT2*, *YAT1* and *YAT2* (Stemple *et al.*, 1998; Bieber, 1988; Schmalix and Bandlow, 1993; Kispal *et al.*, 1991; Swiegers *et al.*, 2001). The three enzymes have unique metabolic functions, since all three are essential in some genetic backgrounds and no cross-complementation can be observed (Swiegers *et al.*, 2001; Van Roermund *et al.*, 1995). In order to obtain a better understanding of the metabolic role of CAT enzymes in yeast, the activity of these enzymes has to be measured collectively and individually in cells grown in various conditions and an accurate method to measure CAT activity in crude cellular extracts is required. Existing methods to measure CAT activity depend on spectrophotometric measurements of either the formation or disappearance of acetyl-CoA derivatives at 232 nm, or the indirect measurement of the release of CoA-SH with sulfhydryl reagents. These methods are inaccurate when crude cellular homogenates are used, due to the high background absorbance (Colowick and Kaplan, 1986). The problem associated with the spectrophotometric assays has lead researchers to centrifuge crude cellular homogenate, using the clear, cell-free supernatant for the assay (Schmalix and Bandlow, 1993; Kispal *et al.*, 1991; Swiegers *et al.*, 2001). This practice, however, is not optimal, as some enzyme inevitably will be lost. A possible alternative is the use of a radioisotope exchange forward assay, but the need for specialised reagents, working space and equipment, as well as safety concerns when working with radioactive reagents, makes this method undesirable for routine assays (Demaugre *et al.*, 1988; McGarry *et al.*, 1983; Stakkestad and Bremer, 1982).

Previously, electrospray mass spectrometry was used by Nolte *et al.* (1998) to measure the activity of carnitine palmitoyltransferase I in leucocytes. The method was shown to be accurate and the specific activity of the enzymes compared well when other methods of determination were used. Here we present a method based on tandem mass spectrometry, in which the precursor ions containing the $m/z = 85$ fragment, which is unique to acetylcarnitine molecules, can be detected. Separation of the components in the reaction mixture by reverse phase high performance liquid chromatography (HPLC) was combined with tandem electrospray mass spectrometry (MSMS). The detection mode employed was multiple reaction monitoring, in which the first analyser of a triple quadrupole mass spectrometer was set to pass the precursor ion (acetylcarnitine) into the fragmentation cell, where collision-induced dissociation created the $m/z = 85$ fragment, which was detected by the second analyser. The combination of both HPLC and MS/MS provides a highly selective and specific method for the detection and quantification of acetylcarnitine in the presence of high concentrations of L-carnitine. No extraction steps are required in this assay, contributing to the simplicity and accuracy of the method.

3.2.3 MATERIAL AND METHODS

3.2.3.1 YEAST STRAINS AND PLASMIDS

All strains used in this study derive from FY23 and are listed together with the plasmids in Table 1. For the cloning and disruption of the genes, standard DNA techniques were used (Sambrook *et al.*, 1989).

Table 1 Strains and plasmids used in this study

Strains and plasmids	Genotype	Source or reference
Strains:		
FY23	<i>MATa leu2 trp1 ura3</i>	Winston <i>et al.</i> 1995
FY23 Δ <i>yat2</i>	<i>MATa trp1 ura3 yat2::LEU2</i>	Swiegers <i>et al.</i> 2001
FY23 Δ <i>yat2</i> Δ <i>cat2</i> Δ <i>yat1</i>	<i>MATa yat2::LEU2 cat2::URA3 yat1::TRP1</i>	This study
Plasmids:		
YDp-W	<i>TRP1</i>	Berben <i>et al.</i> 1991
Ydp-U	<i>URA3</i>	Berben <i>et al.</i> 1991
PGEM-T-easy		Promega
PGEM-T-easy-CAT2		This study
p Δ <i>cat2</i>	Δ <i>cat2::URA3</i>	This study
p Δ <i>yat1</i>	Δ <i>yat1::LEU2</i>	Swiegers <i>et al.</i> 2001
p Δ <i>yat1</i>	Δ <i>yat1::TRP1</i>	This study

3.2.3.2 CLONING AND DISRUPTIONS OF GENES

To create a yeast strain without any known CAT-encoding genes, the *CAT2* and *YAT1* genes were deleted from strain FY23 Δ *yat2* (Swiegers *et al.*, 2001). A 595 bp *Bam*HI/*Bgl*II fragment internal to the *CAT2* gene of the plasmid PGEM-T-easy-CAT2 was replaced by a 1.1 kb *URA3* gene from plasmid YDp-U. The resulting construct, p Δ *cat2::URA3*, was used to isolate a 2.9 kb disruption cassette containing the *URA3* gene plus *CAT2* flanking regions, which was then transformed into FY23 Δ *yat2*. Transformants were isolated on selective media and the disruption was verified using the primers CAT2F1 and CATR1. The Δ *yat1* disruption construct was created using the plasmid p Δ *yat1::LEU2* and replacing the *LEU2* gene by removing it as a 1.6 kb *Bam*HI fragment and replacing it with a 0.8 kb *TRP1* fragment from plasmid YDp-W. The resulting disruption construct, p Δ *yat1::TRP1*, was used to isolate a 1.9 kb fragment containing the *TRP1* gene and *YAT1* flanking regions, which was transformed into strain FY23 Δ *yat2::LEU2* Δ *cat2::URA3*. The disruptions were verified using primers YAT1F1 and YAT1R1.

3.2.3.3 CULTIVATION AND HOMOGENISATION OF YEAST

Strain FY23 wild-type was grown in YND medium [0.67% (w/v) YNB without amino acids (Difco) and 2% (w/v) glucose] to an optical density (OD_{600}) of 3.0, corresponding to ca. 1.2×10^8 cells/ml. From this preculture, YNE medium [0.67% (w/v) YNB without amino acids and 2% (v/v) ethanol] was inoculated to an initial OD_{600} of 0.15 and the cells were grown to an OD_{600} of 1.0. A 40 ml sample of this culture was centrifuged at 5000 rpm for 5 min and the cells were washed with 40 ml of water and again centrifuged at 5000 rpm for 5 min. After resuspension in 1 ml of 15 mM KCl and 5 mM TRIS buffer (pH 7.4), the cells were transferred into 1.5 ml microcentrifuge tubes and centrifuged for 3 min at 5000 rpm. The excess water was removed and the pellet was resuspended in 200 μ l ice-cold 0.05% (w/v) Triton X-100. Glass beads (0.16 g) were added to the suspension and the cells were vortexed for 10 min at 8°C. 100 μ l of the crude extract were transferred to new microcentrifuge tubes and buffered with ice-cold 900 μ l 15 mM KCl and 5 mM TRIS buffer (pH 7.4). The crude extract was kept on ice and used for the enzyme assay the same day. The total protein content was measured using the bicinchonic acid (BCA) kit.

3.2.3.4 CARNITINE ACETYLTRANSFERASE ASSAY

The assay mixture consisted of a buffer containing 13.5 mM KCl and 4.5 mM TRIS (pH 7.4), complemented with 0.049 mM acetyl-CoA and 2.47 mM L-carnitine. After a preincubation of 10 min at 30°C, the reaction was initiated by the addition of 10 μ l of cell extract with a concentration of 1.3 mg/l total proteins. To measure the formation of acetylcarnitine, samples were taken at 10 min intervals by removing 100 μ l aliquots from the sample mixture. These were added to 900 μ l acetonitrile, thereby stopping the reaction. The samples were kept on ice and then deproteinised by centrifugation at 12000 rpm for 5 minutes. The supernatants were transferred to new tubes and analysed by HPLC/MS/MS.

3.2.3.5 MASS SPECTROMETRY

Mass spectrometry was performed on a Micromass (Manchester, UK) Quattro triple quadrupole mass spectrometer fitted with an electrospray ionisation source. Solvent A (acetonitrile/water/formic acid: 30/70/0.05(v/v/v)) was used as carrier solvent and was supplied to the ionisation source by an LKB/Pharmacia (Sweden) pump. For direct injection of the acetyl carnitine standard, the flow rate was 20 μ l/minute and 5 μ l of the standard was injected through a Rheodyne injection valve. The molecular ion ($[M+H]^+$) of acetylcarnitine was observed using a capillary voltage of 3.5 kV, a source temperature of 80°C and a cone voltage setting of 20 V. To obtain the fragment pattern of acetylcarnitine, the molecular ion was dissociated in the fragmentation cell by collision-induced dissociation at an argon pressure of 2.8×10^{-3} mbar, applying a collision energy of 20 eV. The resultant fragments were scanned in the second analyser.

Quantification of acetylcarnitine in the incubation samples was accomplished by HPLC/MS/MS. A Phenomenex (Torrance, USA) Luna C8 150X2.00 mm (3 μ) column was used for separation, with solvent A as mobile phase at a flow rate of 100 μ l/minute delivered by the above mentioned pump. Ten microlitres of sample were injected by a Waters 747 (UK) autosampler. The eluent from the column was directed into the electrospray ionisation source of the mass spectrometer. The capillary voltage, cone voltage, argon pressure and collision energy used were as mentioned above. Detection was by multiple reaction monitoring, using the molecular ion of acetylcarnitine as precursor ion and the fragment at $m/z = 85$ as product ion. Chromatographic peaks representing acetylcarnitine were integrated and the concentration in the incubation samples was calculated from a dilution range of known concentrations of standard acetylcarnitine, prepared in the same buffer as used for the enzyme assay and diluted to a final concentration of 90/10 (v/v): acetonitrile/15 mMTris.HCl. The calculations were performed automatically by the Quantify program of the MassLynx program and expressed as ng/ml.

3.2.4 RESULTS

The electrospray mass spectrum of acetylcarnitine is depicted in Figure 1A. The molecular ion ($[M+H]^+$) at $m/z = 204$ is observed as the base peak in the spectrum. Fragmentation of the molecular ion resulted in the fragmentation spectrum shown in Figure 1B. Using the same conditions, the molecular ion of carnitine was detected at $m/z = 164$ and fragmentation produced an ion spectrum in which the most abundant fragment ion was observed at $m/z = 43$ (not shown).

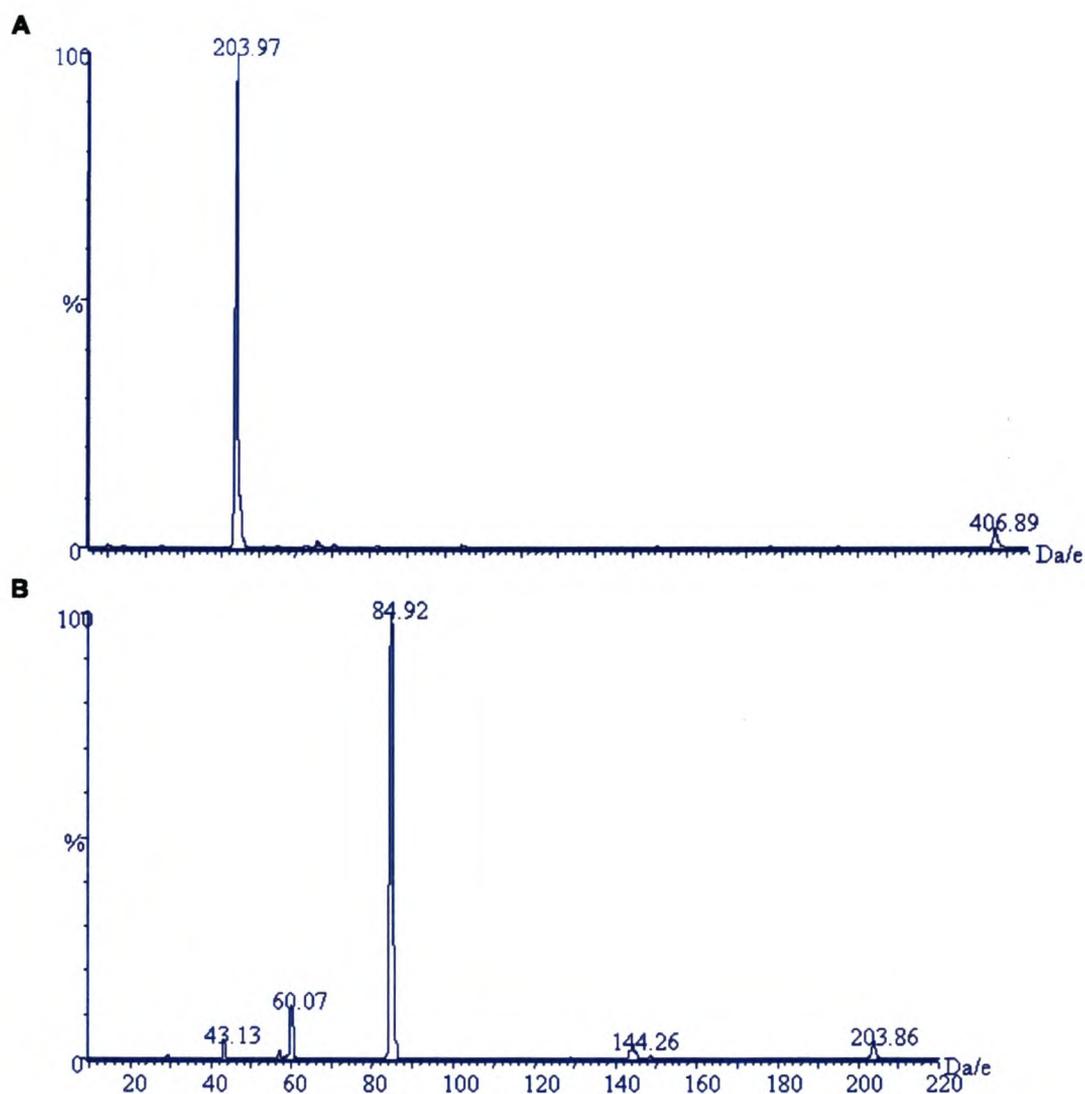


Fig. 1. Electrospray mass spectrum of the parent ion from acetylcarnitine (A) at $m/z = 204$. The fragmentation resulted in the fragmentation ion at $m/z = 84$ (B), which was used for detection.

For the quantification of acetylcarnitine, the molecular ion at $m/z = 204$ and the fragment ion at $m/z = 85$ were used as precursor- and product ions in the multiple reaction monitoring mode of detection. Carnitine was detected by using the precursor- and product ions at $m/z = 164$ and 43 respectively, in the multiple reaction monitoring mode. The components of the assay mixture were separated by HPLC before detection, and Figure 2 indicates that there is no cross-over contamination of the acetylcarnitine due to the presence of carnitine in the reaction mixture.

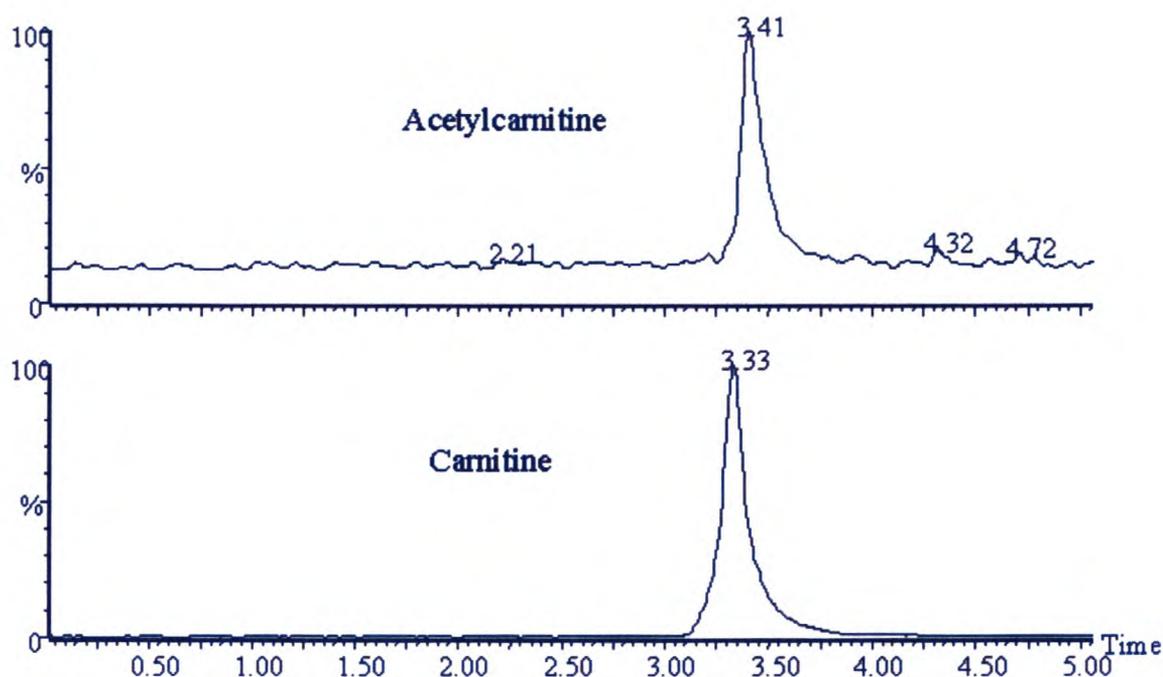


Fig.2. HPLC chromatogram of the separated components in the reaction mixture. The figure clearly indicates that there is no crossing over.

The concentration of acetylcarnitine in each incubation sample was calculated from a calibration curve established by using known concentrations of acetylcarnitine analysed under the same conditions as those used for the assay samples. The standard curve, with a coefficient of determination of 0.9990, is depicted in Figure 3.

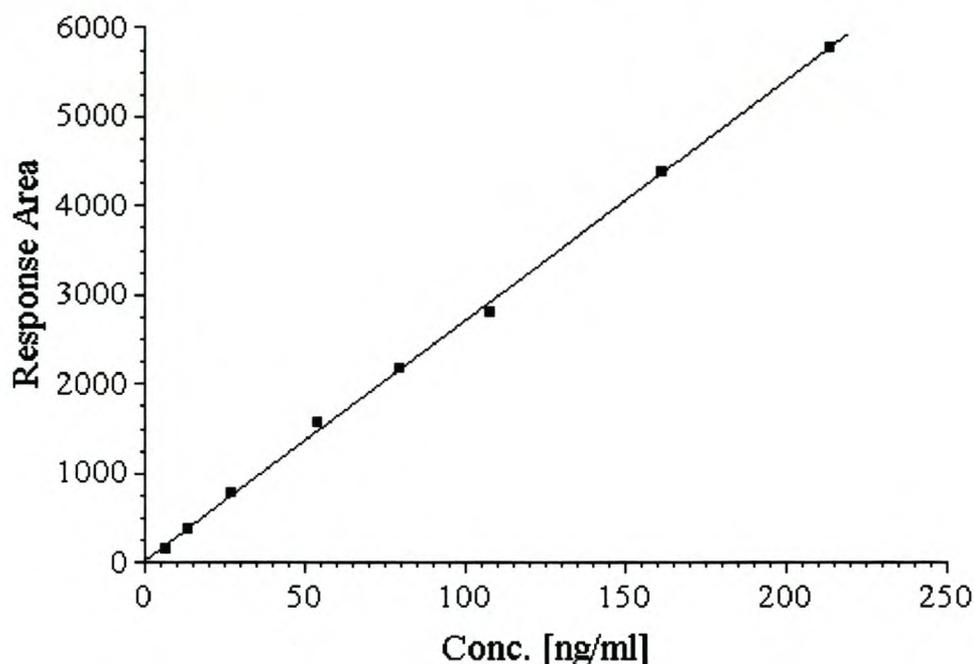


Fig.3. The calibration graph of acetylcarnitine-hydrochloride. The standard curve was used to determinate the concentration of acetylcarnitine formed during the enzyme assay.

Figure 4 represents the results obtained when CAT activity was measured in a wild-type yeast strain grown on ethanol. A linear increase in the conversion of acetyl-CoA to acetylcarnitine is clearly observed. The absence of acetylcarnitine at time zero is indicated clearly. The zero value was not included in the determination of activity, however, as some acetylcarnitine may have been transferred from the yeast crude extract. The specific CAT activity measured in these conditions was 7 pmol of acetylcarnitine formed per min and ng of protein. The result was highly reproducible in six independently conducted experiments (Fig. 4).

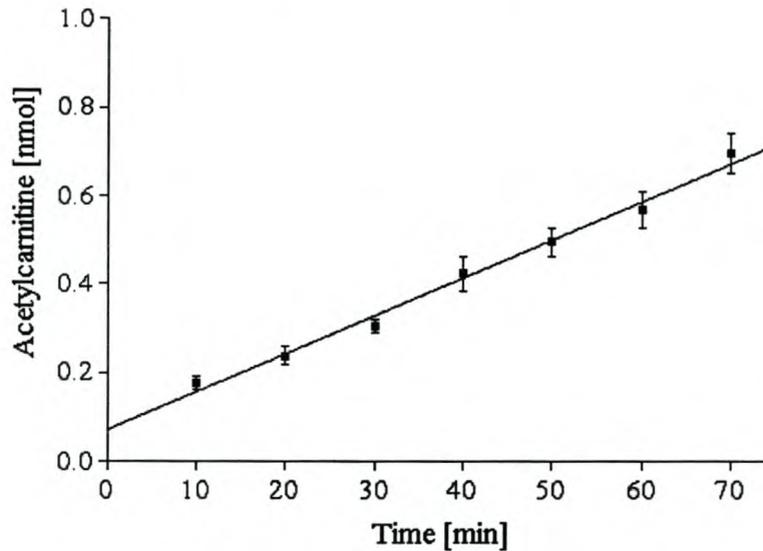


Fig. 4. Time-dependent formation of acetylcarnitine due to the presence of CAT enzymes in the yeast crude extract. The reaction was performed as described under materials and methods. The graph presents the average of six repetitions.

In a second set of experiments, the effect of various protein concentrations on the formation of acetylcarnitine was measured. Here too, a near linear increase in product formation for increasing protein concentration over a fixed period of time was observed. The reaction was stopped either after 20 min (Fig. 5A) or after 40 min (Fig. 5B).

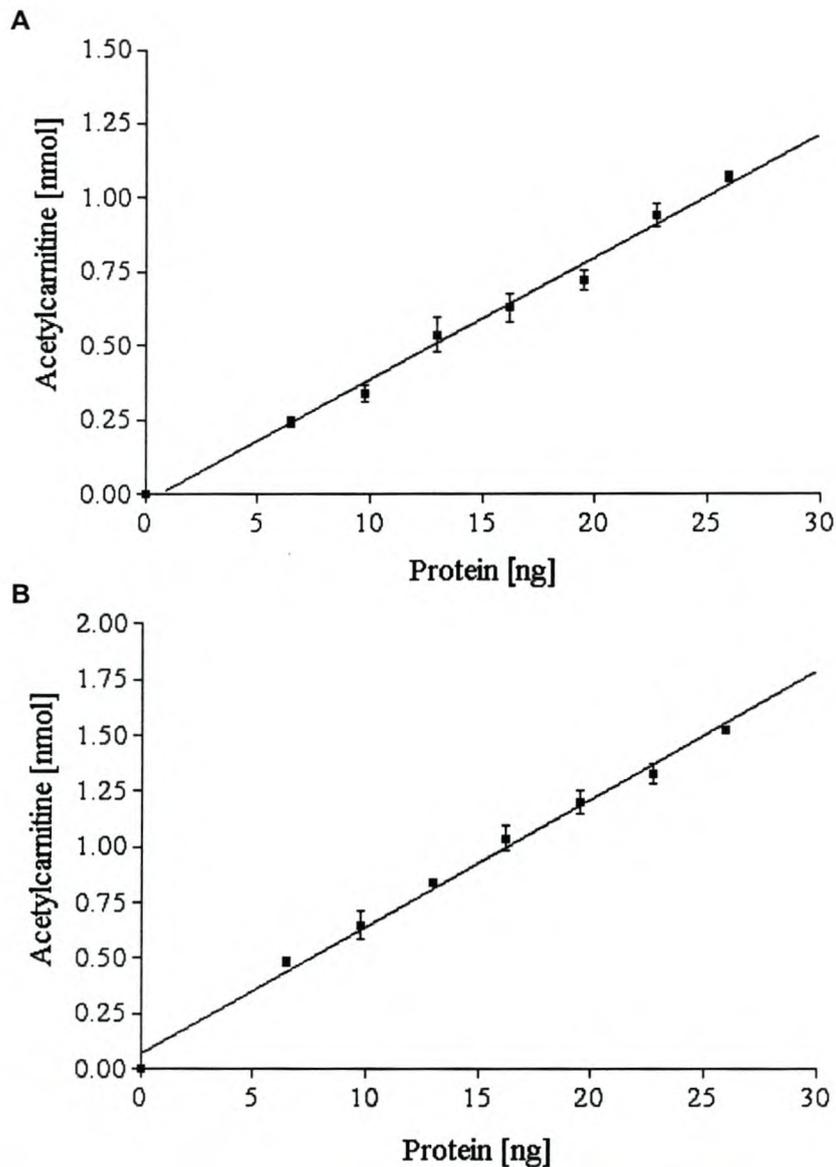


Fig. 5. Protein-dependent formation of acetylcarnitine due to the presence of CAT enzymes in the yeast crude extract. The reaction was performed with different protein concentrations and was stopped either after 20 min (A) or after 40 min (B). The graph presents the average of six repetitions

As a control, the mutant strain with the three deleted *CAT* genes showed no activity, even when grown on other non-fermentable carbon sources, including oleic acid and acetate (data not shown). These data confirm that *S. cerevisiae* has only three *CAT*-encoding genes. In a second control, a reaction mixture containing distilled water instead of carnitine also showed no activity.

3.2.5 DISCUSSION

The yeast *S. cerevisiae* is an important model organism used to study molecular processes in eukaryotic cells. Due to the ease of genetic manipulation, this organism has contributed greatly to advancing the understanding of the molecular mechanisms involved in metabolic processes. In order to have a better understanding of the role

of each of the CAT enzymes in the metabolism of yeast, it is important to study the activity of these enzymes collectively and individually in a wide range of physiological conditions. An assay was developed, based on the time-dependent formation of acetylcarnitine from free carnitine and acetyl-CoA. Acetyl carnitine was measured by HPLC in combination with tandem mass spectrometry.

Due to the presence of non-volatile salts in the incubation mixture, it was necessary to separate the acetylcarnitine from the more ionic compounds prior to detection in the mass spectrometer. Contrary to previously reported methods using MS/MS for the detection of acetylcarnitine, there is no extraction step of the assay samples used in this method. The HPLC step is sufficient for the removal of excess salts present in the assay samples. Simultaneous elution of the analyte and high concentrations of strong ionic compounds could suppress the ionisation of the analyte and negatively influence the lower limit of quantification. The inclusion of the separation step, accomplished by online HPLC on a C8 column, successfully sequestered acetylcarnitine for optimum sensitivity of detection. To optimise ionisation and detection, a low concentration of buffer was used and the samples were diluted by a factor of ten in acetonitrile, prior to separation. The low buffer concentration did not affect the buffering capacity of the enzyme assay mixture and the enzyme activity remained unaffected.

A high concentration of carnitine, used as precursor in the enzyme reactions, was also present in the reaction mixture. As indicated in Figure 2, carnitine and acetylcarnitine were not completely resolved by the HPLC system employed. The presence of carnitine, however, did not influence the detection and quantification of acetylcarnitine, due to the fact that different combinations of precursor and fragment ions were used in the detection of carnitine and acetylcarnitine. This demonstrates the inherent specificity of the multiple reaction-monitoring mode of detection and also provides the possibility of simultaneous quantification of both carnitine and acetylcarnitine in a single step.

3.2.6 ACKNOWLEDGEMENTS

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3.3 UNPUBLISHED RESULTS

The interactions between the carnitine shuttle system and other metabolic pathways that utilise acetyl-CoA for energy production or biomass building are not understood very well. Furthermore, the specific roles of the three carnitine acetyltransferases are only partially established. Besides the activity levels of the three CATs, we looked at possible interactions and relations to other metabolic pathways. The following section provides a summary of the experiments that were initiated to reveal some of these interactions. These results will form part of future publications.

3.3.1 INTRODUCTION

The β -oxidation of fatty acids in mammalian cells takes place in both mitochondria and peroxisomes. Medium-chain and long-chain fatty acids are catabolised primarily in mitochondria whereas very long-chain fatty acids and certain branched-chain fatty acids are handled primarily by peroxisomes (Wanders *et al.*, 1995; Leenders *et al.*, 1996; Schulz, 1991). The importance of peroxisomal β -oxidation is emphasised by the existence of inherited diseases in man that are caused by an impairment in peroxisomal β -oxidation (Wanders *et al.*, 1995).

It is generally accepted that mammalian fatty acid β -oxidation in peroxisomes is incomplete and only involves chain shortening of fatty acids to produce acetyl-CoA and/or propionyl-CoA plus medium-chain acyl-CoAs. These are then transported as carnitine esters to the mitochondria, where they are further oxidised to CO₂ and H₂O (Bieber, 1988; Reddy and Mannaerts, 1994).

In contrast to the process in mammals, the degradation of fatty acids in yeast takes place exclusively in peroxisomes (Kanau *et al.*, 1995). The acetyl-CoA produced has to be transported to the mitochondria for complete oxidation to CO₂ and H₂O. Two pathways for further utilisation of peroxisomal acetyl-CoA have been identified (Van Roermund *et al.*, 1995). In the first, acetyl-CoA enters the peroxisomal glyoxylate cycle to produce succinate, which is subsequently transported to the mitochondria, probably via a putative dicarboxylate carrier, Acr1p (Palmieri *et al.*, 1997). The second pathway involves the intraperoxisomal conversion of acetyl-CoA into acetylcarnitine, which is catalysed by carnitine acetyltransferase (CAT), and the transport of acetylcarnitine to the mitochondria, where mitochondrial CAT catalyses the reverse reaction to form carnitine and acetyl-CoA to enter the tricarboxylic acid cycle (TCA) for energy production. This process is referred to as the carnitine shuttle. In addition, this shuttle also plays a role when yeast cells are grown on other non-fermentable carbon sources, such as acetate and ethanol. The metabolism of these compounds results in the production of acetyl-CoA in the cytoplasm, and this product needs to be transported to the mitochondria for energy production (Schmalix and Bandlow, 1993; Stemple *et al.*, 1998).

The existence of two pathways for the utilisation of peroxisomal acetyl-CoA was suggested on the basis of results showing that disruption of either the *CIT2* gene, encoding the peroxisomal glyoxylate cycle enzyme citrate synthase (Cit2p), or the *CAT2* gene, encoding the peroxisomal and mitochondrial carnitine acetyltransferase, did not affect the growth of yeast on oleate, whereas a mutant with both genes disrupted ($\Delta cit2/\Delta cat2$) failed to grow on this carbon source, due to an inability to oxidise this fatty acid (Van Roermund *et al.*, 1995).

Besides Cat2p, two additional CATs have been identified in *S. cerevisiae*. Cat2p is responsible for >95% of the total CAT activity in oleate-grown yeast cells (Kispal *et al.*, 1993). A second gene, *YAT1*, codes for a CAT that presumably is associated with the outer surface of the mitochondria and contributes an estimated 5% of total CAT activity in acetate- and ethanol-grown cells (Schmalix and Bandlow, 1993). A third gene, *YAT2*, codes for a CAT that has been suggested to be cytosolic and that shows a high contribution to CAT activity in ethanol-grown cells (Swiegers *et al.*, 2001). The sequence homologies among the three CAT-encoding genes are extensive. Swiegers *et al.* (2001) showed that, in a strain with a disrupted peroxisomal citrate synthase, all three carnitine acetyltransferases are essential for growth on non-fermentable carbon sources and are unable to cross-complement each other.

This rather surprising set of data raises the question about the specific function of each of the three CAT enzymes in *S. cerevisiae*, since our current understanding of the carnitine shuttle does not require the existence of three independent carnitine acetyltransferases to ensure survival on all non-fermentable carbon sources.

The role of carnitine in the metabolism of *S. cerevisiae* has not been investigated beyond the shuttling of acetyl residues. However, data suggest that carnitine does have broader functions. To further investigate possible roles, we have investigated the intracellular localisation of the enzymes, confirming existing results about the localisation of Cat2p and Yat1p by employing C-terminal GFP tagging, while additionally localising Yat2p. In order to study the enzymes in detail and to investigate functions beyond the shuttling of acetyl-CoA, we generated strains carrying a single, double and triple deletion of the three CAT genes to assess phenotypical differences. Here we show that, in all conditions tested, there are no phenotypical differences between a wild type strain and strains carrying a single, double and triple deletion of CAT genes, and that all these strains show similar growth patterns in fermentable and non-fermentable carbon sources.

In addition, we show that overexpression of any of the three CAT genes does not result in cross-complementation of the growth defect on non-fermentable carbon sources in strains where one of the other two CAT genes has been disrupted in a $\Delta cit2$ background.

We also show that the constitutive expression of the *Schizosaccharomyces pombe* malate permease (*MAE1*) gene can compensate for the growth defect of a double mutant strain on non-fermentable media supplemented with L-malic acid.

3.3.2 MATERIALS AND METHODS

3.3.2.1 YEAST STRAINS AND CULTURE CONDITIONS

All yeast strains used in this study are derived from strain FY23 and are listed in **Table 3.1**. Yeasts were grown either on rich YPD (1% yeast extract, 2% peptone, 2% glucose) or on minimal YND media, containing 0.67% (w/v) yeast nitrogen base (YNB) without amino acids (DIFCO) and 2% (w/v) glucose supplemented with amino acids according to the specific requirements of the respective strains. To study the phenotypical effect of overexpression of each carnitine acetyltransferase, two different media with non-fermentable carbon sources were used. Both media contained 0.67% (w/v) YNB without amino acids (DIFCO), 2% of agar and either 2% (v/v) ethanol (YNE) or 2% (w/v) glycerol (YNG) as sole carbon source, supplemented with 10 mg/L of L-carnitine (+C). For the localisation studies, yeast was grown in test tubes containing 5 ml of either YNE [0.67% (w/v) YNB without amino acids (DIFCO) and 2% (v/v) ethanol] or YNO [0.67% (w/v) YNB without amino acids (DIFCO) and 0.1% oleic acid] media. To investigate the effect of the *Schizosaccharomyces pombe* malate permease (*MAE1*) gene, strains were spotted on YNEM media, containing 0.67% (w/v) YNB without amino acids (DIFCO), 0.01% Bromocresol green indicator, 2% (v/v) ethanol, 1% (w/v) L-malic acid and 2% agar. For the enzymatic assay, yeast strains were grown as a preculture in a YND medium to an optical density (OD₆₀₀) of 3.0, corresponding to ca. 1.2×10^8 cells/ml. From this preculture, different media were inoculated to an initial OD₆₀₀ of 0.15. These include (i) YNE, (ii) YNG, (iii) YNA [0.67% (w/v) YNB without amino acids and 2% (w/v) acetate] and (iv) YNO.

3.3.2.2 DNA MANIPULATION

All plasmids used in this study are listed in **Table 3.2** and the primers are listed in **Table 3.3**. Standard DNA techniques were carried out as described by Sambrook *et al.* (1989). Standard procedures for the isolation and manipulation of DNA were used throughout the study (Ausubel *et al.*, 1994). Restriction enzymes, T4 DNA-ligase and Expand Hi-Fidelity polymerase used in the enzymatic manipulation of DNA were obtained from Roche Diagnostics (Randburg, South Africa) and used according to the specifications of the supplier. *Escherichia coli* DH5 α (GIBCO-BRL/Life Technologies) was used as host for the construction and propagation of all plasmids. Sequencing of all plasmids was carried out on an ABI PRISMTM automated sequencer.

Table 3.1 Description of strains used in this study

Yeast strains	Relevant genotype	Sources and references
FY23	<i>MATa leu2 trp1 ura3</i>	Winston <i>et al.</i> 1995
FY23 Δ yat1	<i>MATa trp1 ura3 Δyat1::LEU2</i>	Swiegers <i>et al.</i> 2001
FY23 Δ yat2	<i>MATa trp1 ura3 Δyat2::LEU2</i>	Swiegers <i>et al.</i> 2001
FY23 Δ cat2	<i>MATa trp1 ura3 Δcat2::LEU2</i>	Swiegers <i>et al.</i> 2001
FY23 Δ yat2 Δ yat1	<i>MATa ura3 Δyat2::LEU2 Δyat1::TRP1</i>	This study
FY23 Δ yat1 Δ cat2	<i>MATa trp1 Δyat1::LEU2 Δcat2::URA3</i>	This study
FY23 Δ yat2 Δ cat2	<i>MATa trp1 Δyat2::LEU2 Δcat2::URA3</i>	This study
FY23 Δ yat2 Δ cat2 Δ yat1	<i>MATa yat2::LEU2 cat2::URA3 yat1::TRP1</i>	This study
FY23 Δ cit2 Δ yat1	<i>MATa ura3 Δcit2::TRP1 Δyat1::LEU2</i>	Swiegers <i>et al.</i> 2001
FY23 Δ cit2 Δ yat2	<i>MATa ura3 Δcit2::TRP1 Δyat2::LEU2</i>	Swiegers <i>et al.</i> 2001
FY23 Δ cit2 Δ cat2	<i>MATa ura3 Δcit2::TRP1 Δcat2::LEU2</i>	Swiegers <i>et al.</i> 2001

Table 3.2 Description of plasmids used in this study

Plasmids	Relevant genotype	Sources and references
YCplac33	<i>CEN4 URA3</i>	Gietz and Sugino 1988
YEplac112	<i>2μ TRP1</i>	Gietz and Sugino 1988
YEplac181	<i>2μ LEU2</i>	Gietz and Sugino 1988
YDp-W	<i>TRP1</i>	Berben <i>et al.</i> 1991
YDp-U	<i>URA3</i>	Berben <i>et al.</i> 1991
PGEM-T-easy-YAT1		Swiegers <i>et al.</i> 2001
PGEM-T-easy-YAT2		Swiegers <i>et al.</i> 2001
PGEM-T-easy-CAT2		Swiegers <i>et al.</i> 2001
p Δ cat2	<i>Δcat2::URA3</i>	This study
p Δ yat1	<i>Δyat1::LEU2</i>	Swiegers <i>et al.</i> 2001
p Δ yat1	<i>Δyat1::TRP1</i>	This study
YEplac112-T-GFP2-B-BGL	<i>2μ TRP1</i>	Received from M. Bester Westermann and Neubert 2000
pYES2-mtBFP	<i>2μ URA3</i>	
YCplac33-YAT1-GFP	<i>CEN4 URA3 YAT1</i>	This study
YCplac33-YAT2-GFP	<i>CEN4 URA3 YAT2</i>	This study
YCplac33-CAT2-GFP	<i>CEN4 URA3 CAT2</i>	This study
PGEM-T-easy-Cat2p		This study
YEplac 112-mtBFP	<i>2μ TRP1</i>	This study
YEplac181-BFP-P	<i>2μ LEU2</i>	This study
YCplac33-PGKpt	<i>CEN4 URA3 PGK_P PGK_T</i>	Received from M. Bester
YCplac33-PGKp-YAT1-PGKt	<i>CEN4 URA3 YAT1</i>	This study
YCplac33-PGKp-YAT2-PGKt	<i>CEN4 URA3 YAT2</i>	This study
YCplac33-PGKp-CAT2-PGKt	<i>CEN4 URA3 CAT2</i>	This study
pHV3	<i>2μ LEU2 PGK_P MAE1 PGK_T</i>	Volschenk <i>et al.</i> , 1997
YCplac33-PGKp-MAE1-PGKt	<i>CEN4 URA3 MAE1</i>	This study

Table 3.3 Oligonucleotides used in this study

Primer	Sequence
CAT2F1	5'-GACACTGTTGCGCCAAATTTTCG-3'
CAT2R1	5'-ATAAGCAAGGCACAATATCC-3'
YAT1F1	5'-ATCAGCATCAGCATCAGC-3'
YAT1R1	5'-AGAGGTAATCCAAACGACG-3'
YAT1-GFP-F	*5'-GATCGAATTCGTGGAAATCATCGCGCGCAAGCCA-3'
YAT1-GFP-R	*5'-GATCGGTACCACCGGACACGCTCACGTCGAAGTA-3'
YAT2-GFP-R	*5'-GATCGGTACCTTGATCTAAGGTCGCCACCTTTCT-3'
YAT2-GFP-F	*5'-GATCGAATTCGAGGCAGCCCGTGTTGCGTCACAA-3'
CAT2-GFP-F	*5'-GATCGAATTCCTTTCTTGAAATTCTGTCAAATCT-3'
CAT2-GFP-R	*5'-GATCGGTACCTAACTTTGCTTTTCGTTTATTCTC-3'
Cat2p	5'-GATCCTGCAGTCGCGAGAGTGCTTTCTTTTAG-3'
BFP-P(R)	*5'-GATCAAGCTTTTATAACTTTGCTTTGTATAGTTCATCCATGCCAT-3'
BFP-P(F)	*5'-GATCTCGCGAATGAGTAAAGGAGAAGAACTTTTCAC-3'
mtBFP-F	*5'-GATCTCGCGAATGGCCTCCACTCGT-3'
mtBFP-R	*5'-GATCAAGCTTTTATTTGTATAGTTCATCCATGCCATGT-3'
CAT2ov-F	*5'-GATCGAATTCATGAGGATCTGTCAATTCGA-3'
CAT2ov-R	*5'-GATCCTCGAGTCATAACTTTGCTTTTCG-3'
YAT1ov-F	*5'-GATCGAATTCATGCCAACTTAAAGAGACT-3'
YAT1ov-R	*5'-GATCCTCGAGTCAACCGGACACGCTCA-3'
YAT2ov-F	*5'-GATCGAATTCATGTCAAGCGGCAGTA-3'
YAT2ov-R	*5'-GATCGTCGACTTATTGATCTAAGGTCGCC-3'
5'-mae1	*5'-GATCGAATTCATGGGTGAACTCAAGGAAATC-3'
3'-mae1	*5'-GATCAGATCTTTAAACGCTTTCATGTTCACT-3'

*Underlined sequences indicate introduced restriction sites

3.3.2.3 CLONING AND DISRUPTION OF GENES

3.3.2.3.1 CONSTRUCTION OF THE DOUBLE DISRUPTIONS OF CAT GENES

To create the double mutants FY23 Δ *yat1* Δ *cat2* and FY23 Δ *yat2* Δ *cat2*, the *CAT2* gene was disrupted in either the FY23 Δ *yat1* or FY23 Δ *yat2* strain (Swiegers *et al.*, 2001). For this purpose, a 595 bp *Bam*HI/*Bgl*II fragment internal to the *CAT2* gene of the plasmid PGEM-T-easy-*CAT2* was replaced by a 1.1 kb *URA3* gene from plasmid YDp-U. The resulting construct, p Δ *cat2*::*URA3*, was used to isolate a 2.9 kb disruption cassette containing the *URA3* gene plus *CAT2* flanking regions, and this was transformed into FY23 Δ *yat1* and FY23 Δ *yat2*. Transformants were isolated on selective media and the disruption was verified by polymerase chain reaction (PCR) using the primers CAT2F1 and CATR1 (Table 3.3). To create the FY23 Δ *yat1* Δ *yat2* double mutant, a Δ *yat1* disruption construct was created by using the plasmid p Δ *yat1*::*LEU2* and removing the *LEU2* gene as a 1.6 kb *Bam*HI fragment and replacing it with a 0.8 kb *TRP1* fragment from plasmid YDp-W. The resulting disruption construct, p Δ *yat1*::*TRP1*, was used to isolate a 1.9 kb fragment containing the *TRP1* gene and *YAT1* flanking regions. This was transformed into strain FY23 Δ *yat2* and the disruption was verified using primers YAT1F1 and YAT1R1.

3.3.2.3.2 CONSTRUCTION OF THE CAT-GFP AND BFP PLASMIDS

The 759 bp GFP open reading frame contains a *Kpn*I site nine nucleotides after the start codon. The *YAT2* gene was amplified from the PGEM-T-easy-*YAT2* plasmid using the primers YAT2-GFP-F and YAT2-GFP-R. In the reverse primer, the stop codon was replaced with a *Kpn*I restriction site (Table 3.3). The resulting 3.1 kb fragment was inserted as a *Eco*RI/*Kpn*I fragment into the YEplac112-T-GFP2-B-BGL plasmid. From this construct, a 4.0 kb *Xba*I/*Nar*I fragment was subcloned into the YCplac 33 vector. The *YAT1* and *CAT2* genes and their respective promoter regions were amplified from the PGEM-T-easy-*YAT1* or PGEM-T-easy-*CAT2* plasmid, using either the YAT1-GFP-F and YAT1-GFP-R or the CAT2-GFP-F and CAT2-GFP-R primers. The resulting *Eco*RI/*Kpn*I fragments of 3.1 kb were subsequently ligated in the YCplac33-*YAT2*-GFP plasmid digested with *Eco*RI and *Kpn*I containing the GFP open reading frame.

For co-localisation studies, the blue fluorescent protein (BFP)-encoding BFP gene, with either a mitochondrial or a peroxisomal signal sequence, was used and expressed under the promoter of *CAT2*. The promoter was amplified from the YCplac33-*CAT2*-GFP plasmid by using the primers CAT2-GFP-F and Cat2p. The resulting 1.2 kb fragment was blunt-ligated into the pGEM-Teasy vector and digested with *Eco*RI and *Pst*I, purified and subcloned into YEplac181 and YEplac121. To fuse the mitochondrial-targeted blue fluorescent protein to the promoter, the BFP and the mitochondrial pre-sequence were amplified by PCR, using the mtBFP-F and

mtBFP-R primers and the pYES2-mtBFP plasmid as template. The resulting 960 base pair fragment was cloned into the plasmids as a *NruI/HindIII* digest. To construct the peroxisomal-targeted BFP, a primer was designed containing the peroxisomal targeting signal type 1 (PTS-1) at the C-terminus of the open reading frame (**Table 3.3**). Using the primers BFP-P(R) and BFP-P(F), a 630 base pair fragment was obtained by PCR, using the pYES2-mtBFP plasmid as template, and cloned as a *NruI/HindIII* fragment into the YEplac181 and YEplac121 vectors containing the *CAT2* promoter.

3.3.2.3.3 CONSTRUCTION OF THE OVEREXPRESSION PLASMIDS

The *CAT2* gene was amplified from the YCplac33-*CAT2-GFP* plasmid template by PCR, using primers CAT2ov-F and CAT2ov-R (**Table 3.3**). The resulting 2.0 kb fragment was blunt-ligated into the pGEM-Teasy vector. The *YAT1* gene was amplified from the YCplac33-*YAT1-GFP* plasmid template by PCR, using the YAT1ov-F and YAT1ov-R primers. A 2.1 kb fragment was ligated into the pGEM-Teasy vector. The remaining carnitine acetyltransferase gene, *YAT2*, was amplified from the YCplac33-*YAT2-GFP* plasmid template by PCR using the YAT2ov-F and YAT2ov-R primers. A 2.7 kb fragment was ligated into the pGEM-Teasy vector. The pGEM-Teasy-*CAT2* and pGEM-Teasy-*YAT1* plasmids were digested with *EcoRI* and *XhoI*, resulting in fragments of 2037 and 2104 base pairs respectively. The pGEM-Teasy-*YAT2* plasmid was digested with *EcoRI* and *SaI* and resulted in a fragment of 2796 base pairs. These fragments were ligated into the *EcoRI/XhoI* site of the YCplac33-*PGKpt* plasmid. The resulting three plasmids, YCplac33-*PGKpt-CAT2*, YCplac33-*PGKpt-YAT1* and YCplac33-*PGKpt-YAT2*, contained either the *CAT2*, *YAT1* or *YAT2* gene under control of the phosphoglycerate kinase (*PGK*) promoter. These constructs were used in overexpression experiments.

3.3.2.3.4 CONSTRUCTION OF THE *MAE1* EXPRESSION PLASMID

The *Schizosaccharomyces pombe* malate permease gene, *MAE1*, was amplified from the pHV1 (Volschenk *et al.*, 1997) plasmid template by PCR, using primers 5'-*mae1* and 3'-*mae1* (**Table 3.3**). The resulting 1.3 kb fragment was blunt-ligated into the pGEM-Teasy vector and the pGEM-Teasy-*MAE1* plasmid was subsequently digested with *EcoRI* and *BglII*. The resulting fragment of 1323 base pairs, corresponding to the open reading frame of the *MAE1* gene, was then ligated into the *EcoRI/BglII* site of the YCplac33-*PGKpt* plasmid.

3.3.2.4 FLUORESCENT MICROSCOPY

In order to determine the intracellular location of the three proteins Yat1p, Yat2p and Cat2p *in vivo*, each was C-terminally tagged with GFP. Centromeric shuttle vectors carrying in-frame fusions of *GFP* to the 3' end of *YAT1*, *YAT2* and *CAT2* under transcriptional control of their respective native promoters were introduced into the haploid yeast FY23. For direct fluorescent visualisation of the yeast peroxisomes and mitochondria, both organelles were targeted with a BFPp controlled by the promoter of *CAT2* on a multi-copy plasmid. A Nikon E400 microscope with UV source and appropriate filter sets was used to visualise fluorescence. Images were taken with a Nikon COOLPIX 990 digital camera. Scion Image for Windows was used to capture video images, and Microsoft Photo Editor 3.0 was used for editing the images.

3.3.3 RESULTS

3.3.3.1 EFFECT OF CAT GENE DELETION ON GROWTH SPEED

To monitor if single *CAT* gene deletions or the combination in various double and in the triple disrupted strains have an effect on growth, growth curves were obtained in fermentable (YND) and non-fermentable (YNE) media supplemented with L-carnitine. As shown in **Figure 3.6 A, B and C**, no significant difference in growth characteristic could be observed. Mean generation time in the exponential growth phase and the final biomass production were similar. From **Figure 3.6 B and C** it also is clear, that in strains with a functional citrate synthase, the inability to shuttle acetyl-CoA via the carnitine system does not affect the growth speed, since the mean generation time of the strains in YNE media with and without supplementation of L-carnitine showed no significant differences. However, there are small differences in the exponential growth phase, which might be linked to the use of different auxotrophic markers. Oleate as sole carbon source could not be tested, due to the high background absorbance of oleic acid and Tween 80, which makes the readings unreliable.

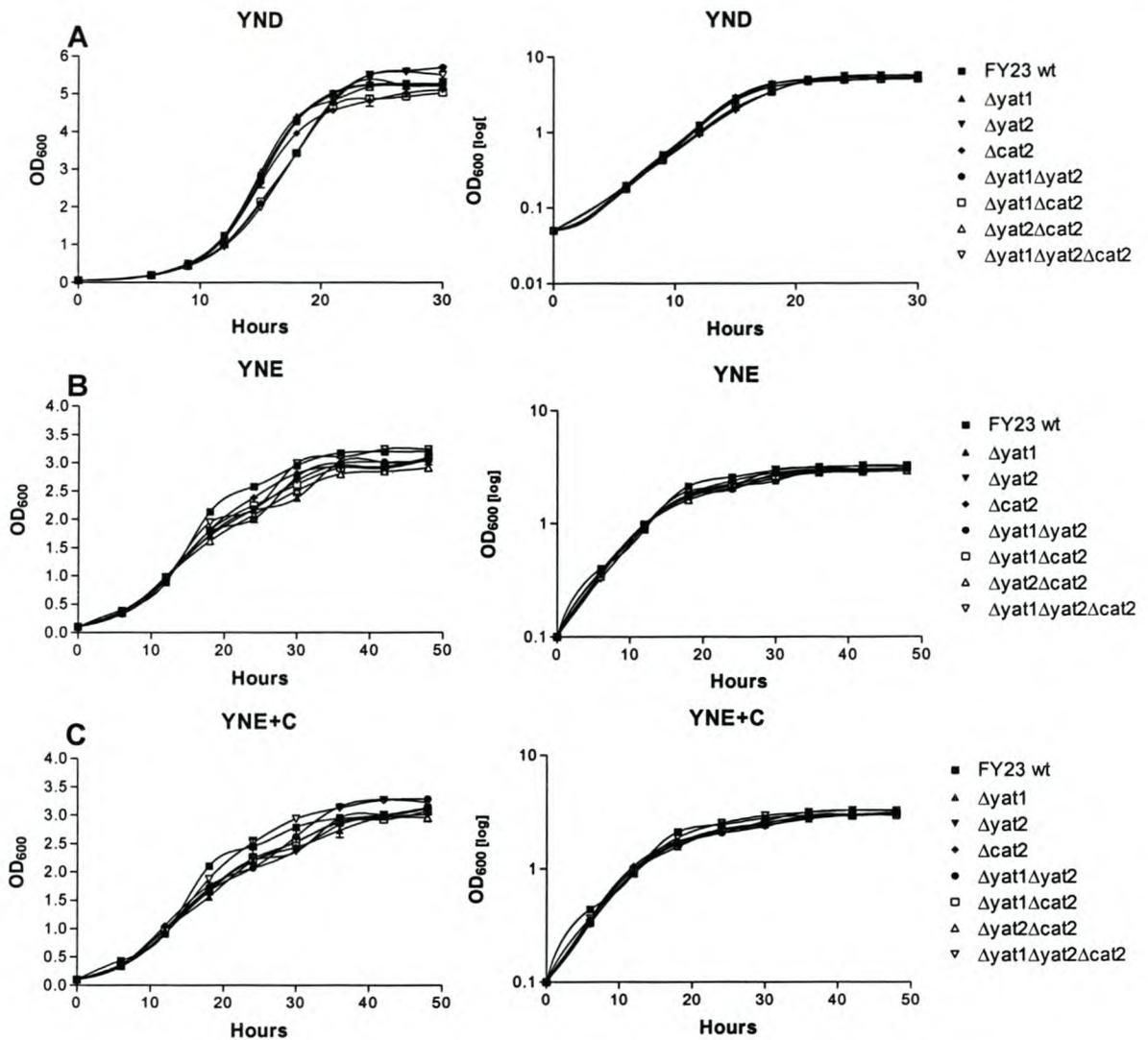


Figure 3.6 Growth curves of single, double and triple CAT gene deletions. Graphs show a linear scale on the left and a semi-log scale on the right side. (A) YND media, (B) YNE media and (C) YNE media supplemented with L-carnitine. The graph presents the average of three repetitions.

3.3.3.2 OVEREXPRESSION OF CAT GENES DOES NOT RESULT IN CROSS-COMPLEMENTATION

To assess if the overexpression of one of the three CAT genes under the constitutive *PGK* promoter could overcome the growth defect on non-fermentable carbon sources in the $\Delta cit2$ background, the double mutants (FY23 $\Delta cit2\Delta yat1$; FY23 $\Delta cit2\Delta yat2$ and FY23 $\Delta cit2\Delta cat2$) were transformed with the YCplac33-PKGpt plasmid carrying either the *YAT1*, *YAT2* or *CAT2* gene. Transformants were streaked out on YNE or YNG agar plates, supplemented with L-carnitine. The plates were incubated at 30°C for 14 days. In the $\Delta cit2$ background, the overexpression of the deleted CAT gene could rescue growth (**Figure 3.7 A-C**), indicating the functionality of the genes on the plasmid. However, in none of the cases could a cross-complementation be observed, indicating a specific and unique function for each of the three CAT genes.

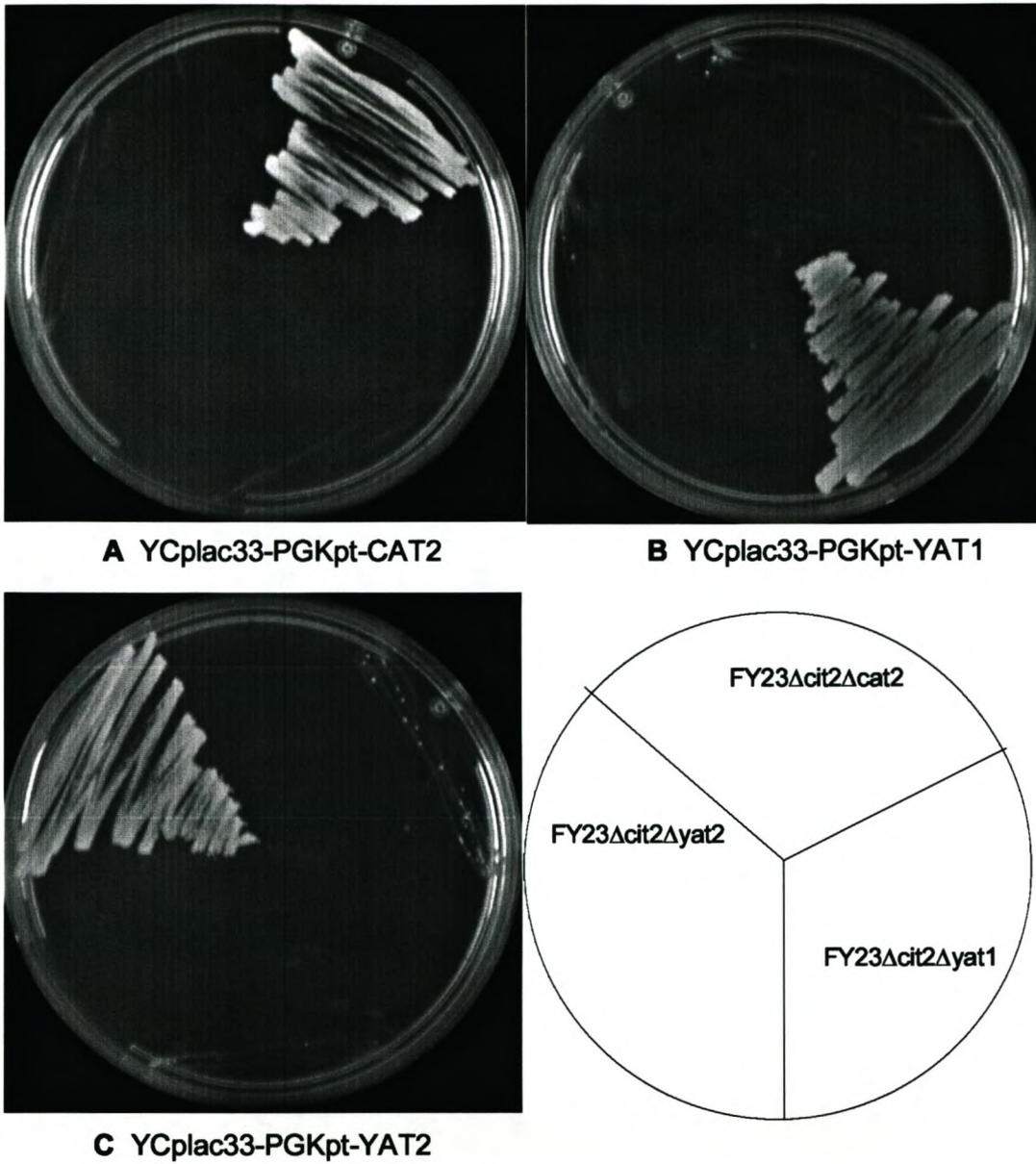


Figure 3.7 Overexpression of the CAT genes under the PGK promoter. Strain layout as indicated. (A) overexpression of *CAT2*, (B) overexpression of *YAT1* and (C) overexpression of *YAT2*.

3.3.3.3 EXPRESSION OF THE *SCHIZOSACCHAROMYCES POMBE* MALATE PERMEASE GENE (*MAE1*) COMPENSATES FOR THE GROWTH DEFECT ON NON-FERMENTABLE CARBON SOURCES

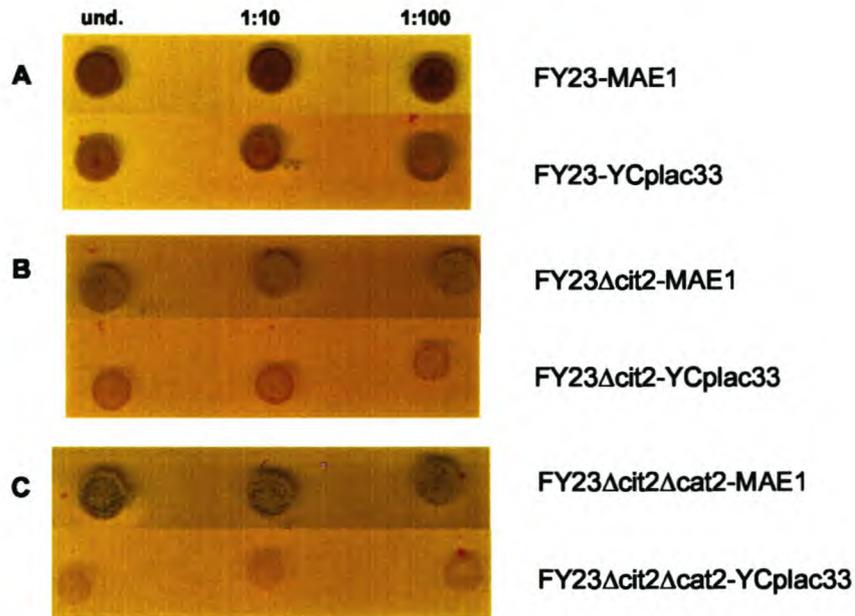


Figure 3.8 Expression of the *Schizosaccharomyces pombe* malate permease gene (*MAE1*) in (A) the wild type strain, (B) in the $\Delta cit2$ background and (C) in the $\Delta cit2\Delta cat2$ double mutant. The growth medium used was YNEM, which leads to a green colouration of colonies if acids are consumed.

Peroxisomally produced acetyl-CoA can be utilised in two ways, either through shuttling via the carnitine system, or by forming C_4 compounds, in particular malate and succinate. In order to assess a possible function for the three CATs in the secondary metabolism of these C_4 compounds, we generated a strain that has an efficient uptake of C_4 dicarboxylic acid by expressing the *Schizosaccharomyces pombe* malate permease gene (*MAE1*). To investigate if the expression of *MAE1* in media supplemented with malate as additional carbon source could overcome the carnitine dependency in the $\Delta cit2$ background, or if it would reveal specific roles for the three CATs, the strains were transformed with YCplac33-PKGpt plasmid carrying the *MAE1* gene or the YCplac33-PKGpt plasmid as control. Strains were grown in YND media to an optical density of OD_{600} of 1 and then spotted in a range of dilutions on YNEM media with and without carnitine. The media used contained Bromocresol green indicator. A drop in pH will lead to a green colouration of the colonies. The plates were incubated at 30°C for 14 days. As shown in **Figure 3.8 (A-C)**, the expression of the *Schizosaccharomyces pombe* malate permease (*MAE1*) gene complemented the carnitine dependency of a $\Delta cit2$ deletion strain. All the spotted colonies carrying the *MAE1* gene grew to a similar degree. However, a slight difference in colour was observed between the wild type containing the *MAE1* gene and the $\Delta cit2$ mutants, suggesting that malate utilisation proceeds more efficiently in

the presence of a functional *CIT2* gene. **Figure 3.8 A** shows that the expression of the *MAE1* gene improves growth speed in the wild type. In the $\Delta cit2$ mutant and the $\Delta cit2\Delta cat$ double mutants, the presence of the *MAE1* gene did not lead to phenotypical differences when compared to the strains transformed with the plasmid carrying no insert. The strains grew to a similar extent (**Figure 3.8 B and C**), and no phenotypical difference could be observed between the three double mutants (data not shown). In addition, strains carrying the *MAE1* gene also utilised succinate as sole carbon source (data not shown). The presence or absence of carnitine did not change the growth behaviour of the strains.

3.3.3.4 LOCALISATION STUDIES OF THE THREE CAT-ENCODING GENES

3.3.3.5

In order to determine the exact intracellular localisation of each CAT protein (Yat1p, Yat2p and Cat2p) *in vivo*, each was C-terminally tagged with GFP. Wild type strain FY23 was transformed with the centromeric plasmid YCplac33, carrying in-frame fusions of *GFP* to the 3' end of *YAT1*, *YAT2* and *CAT2* under transcriptional control of their respective native promoters. To differentiate between cellular organelles, e.g. mitochondria and peroxisomes, strains were co-transformed with shuttle vectors YEplac112 and YEplac181, carrying the BFP with either a mitochondrial or a peroxisomal signal sequence under the transcriptional control of the *CAT2* promoter.

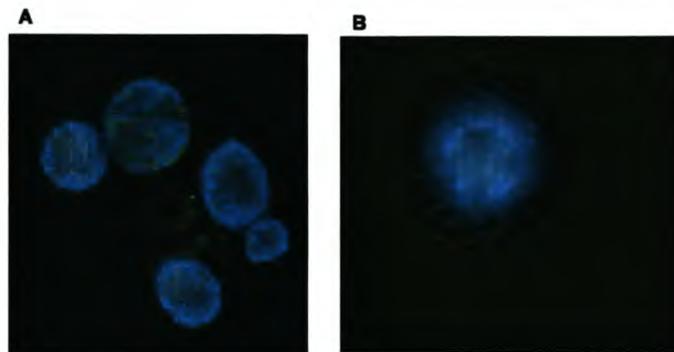


Figure 3.9 Expression of *BFP* in a FY23 wild type strain either with (A) a mitochondrial signal sequence in ethanol media, or (B) with the peroxisomal signal sequence in oleic acid media.

Figure 3.9 shows the blue fluorescent protein that was used as a control to distinguish between the mitochondria and the peroxisome. In all co-transformed strains, similar fluorescent signals could be observed for the targeted BFP. The following figures (3.10-3.11) therefore show only the green fluorescent protein.

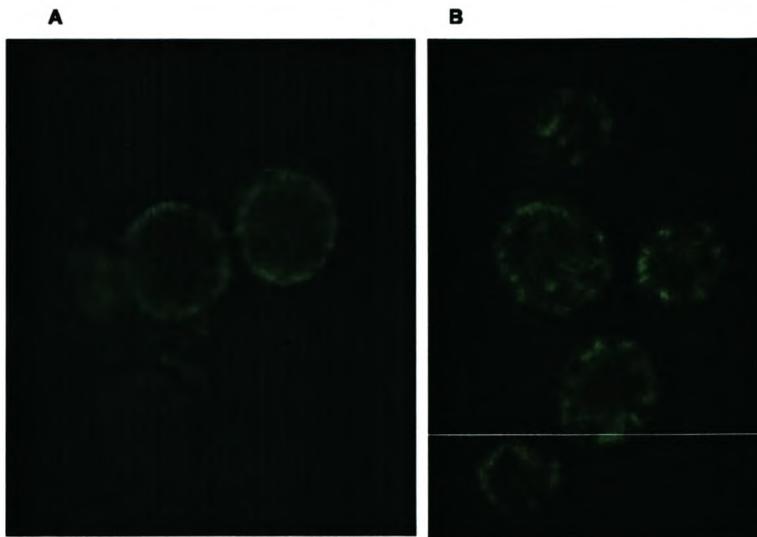


Figure 3.10 *YAT1-GFP* in **A** ethanol media and **B** oleic acid media

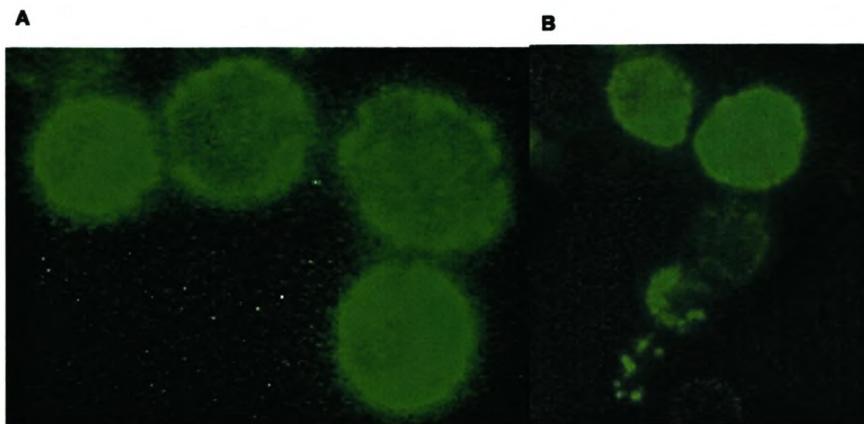


Figure 3.11 *CAT2-GFP* in **A** ethanol media and **B** oleic acid media

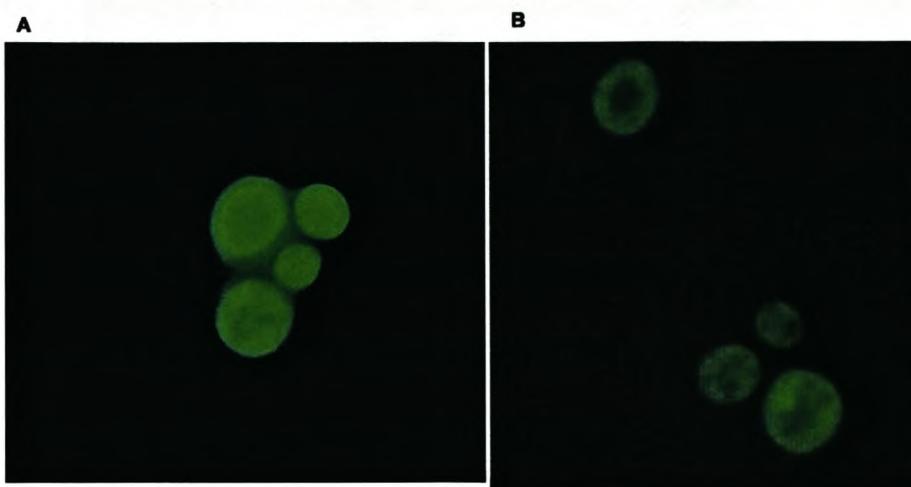


Figure 3.12 *YAT2-GFP* in **A** ethanol media and **B** oleic acid media

From **Figure 3.10**, it is clearly visible that *Yat1p* is linked with the mitochondria, which confirms existing data. However, the fluorescent signal is much weaker than for *Cat2p*. *Cat2p* is associated with the mitochondria and the peroxisome, since, in both

cases, there is a strong signal in the mitochondria and the peroxisome in ethanol and oleic acid (**Figure 3.11**). Yat2p, on the other hand, appears to be distributed throughout the cell, suggesting a cytoplasmic localisation (**Figure 3.12**).

3.3.3.5 CAT ACTIVITY IN VARIOUS DOUBLE AND TRIPLE CAT DELETION STRAINS

The new method for CAT determination was used to analyse CAT activity in a FY23 wild type strain, as well as in the three double mutant strains. Culture conditions were identical as described in **3.2.3.3**. In a first set of experiments, total CAT activity of a FY23 wild type strain was analysed in four different non-fermentable carbon sources. FY23 mutant strains, in which only one functional CAT was present, were analysed in the same conditions. Samples were taken after 10, 20, 40, 60 and 80 min.

As indicated in **Figure 3.13**, the wild type strain showed similar activity patterns in all the media tested. Interestingly, the activity was slightly higher in glycerol and ethanol compared with oleic acid and acetate as sole carbon source.

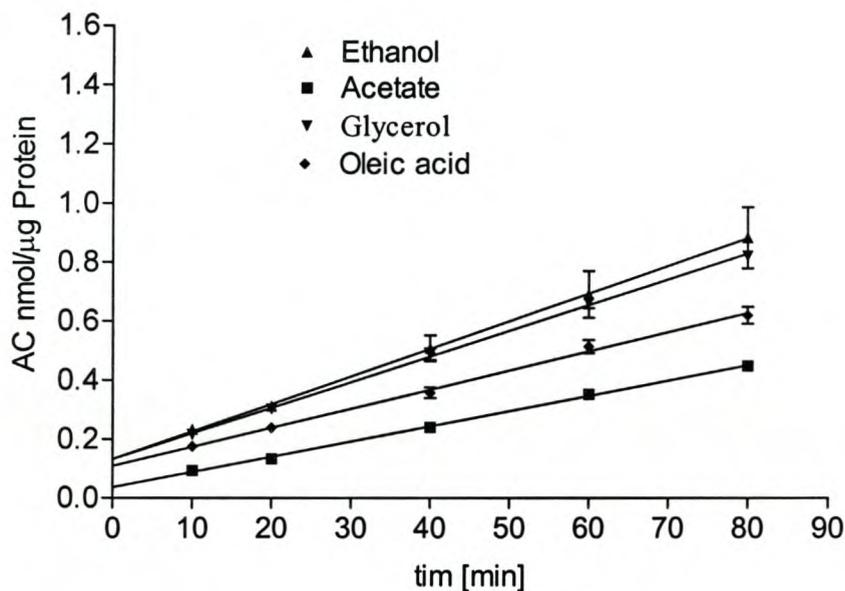


Figure 3.13 Total CAT activity in the FY23 wild type strain. The graph presents the average of three independent repetitions, error bars included.

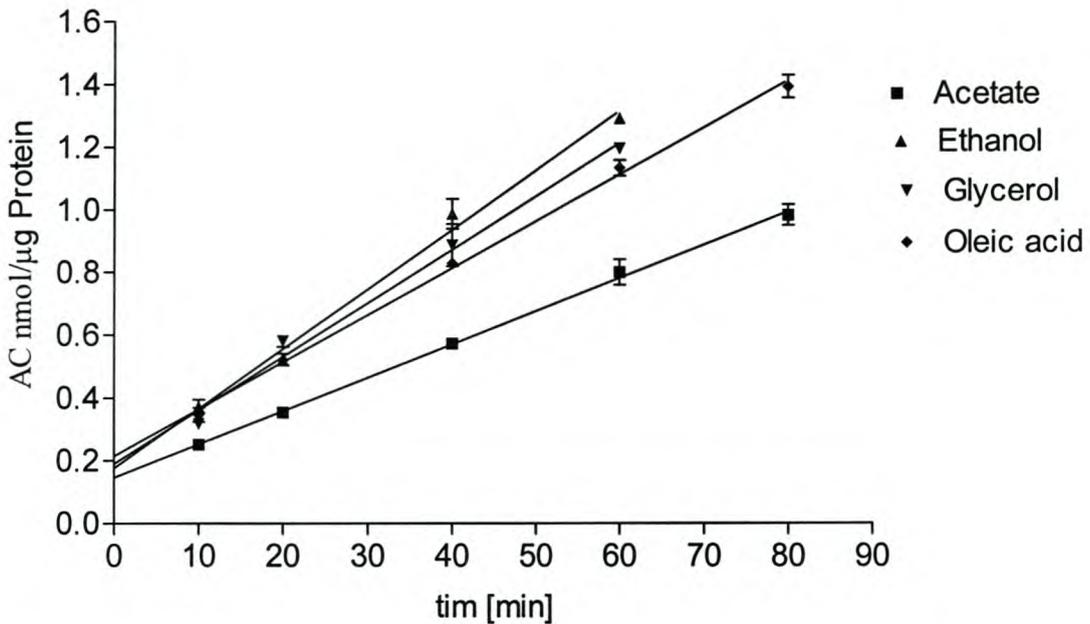


Figure 3.14 Total CAT activity in the FY23 Δ yat1 Δ yat2 strain. The graph presents the average of three independent repetitions, error bars included.

Figure 3.14 presents the CAT activity for the FY23 Δ yat1 Δ yat2 double mutant. Once again, the level of activity was similar in the four media, except in acetate, where activity was 30% lower.

Interestingly, the FY23 Δ yat1 Δ yat2 mutant showed significantly higher CAT activity than the FY23 wild type. Levels of activity in the double mutant were up to 40% higher than in the wild type in similar physiological conditions. No CAT activity could be detected under the same conditions in the other two double mutants, FY23 Δ yat1 Δ cat2 and FY23 Δ yat2 Δ cat2 respectively, and for the triple mutant FY23 Δ yat1 Δ yat2 Δ cat2 (data not shown).

The data suggest that Yat1p and Yat2p could have a higher affinity for the reverse reaction, or could regulate the amount of acetylcarnitine produced by Cat2p. To assess whether Yat1p and Yat2p are the CATs responsible for the reverse reaction rather than the forward reaction, the same assay will be performed using acetylcarnitine and free CoA as substrates. Due to time limitations, this data has not yet been obtained.

3.3.3.6 DISCUSSION AND CONCLUSION

The aim of this study was to investigate the specific roles of the three CAT genes that are encoded by the yeast genome. From the deletion phenotypes, it is clear that, in strains with a functional peroxisomal citrate synthase, the shuttle system has no effect on growth speed. All strains grew similarly. The overexpression of any of the three CAT genes could not cross-complement the deletion of another CAT gene, indicating that each of the genes fulfills a unique and very specific function in the shuttling and regulation of acetyl-CoA.

The provision of C₄ compounds through the expression of the *Schizosaccharomyces pombe* malate permease gene (*MAE1*) was able to compensate for the growth defect of the strains on non-fermentable carbon sources, but no phenotypical differences between the different CAT mutants could be observed, indicating that the effect is a compensation for the peroxisomal citrate synthase and not of the CAT genes.

The localisation studies confirmed the existing data. We showed that Cat2p is a peroxisomal and mitochondrial carnitine acetyltransferase and that Yat1p is associated with the mitochondria. In addition, we tentatively localised Yat2p in the cytosol.

The first set of data obtained with the novel method to analyse the formation of acetylcarnitine in a time-dependent reaction from acetyl-CoA and carnitine as precursors suggests that Yat1p and Yat2p may be responsible for the reverse reaction, e.g. the conversion of acetylcarnitine and free CoA to acetyl-CoA and carnitine. This hypothesis is based on the finding that the FY23 Δ *yat1* Δ *yat2* strain showed a significantly higher total CAT activity when compared to that of the wild type strain with three functional CATs. Furthermore, the low or non-measurable activity of the mutant strains FY23 Δ *yat1* Δ *cat2* and FY23 Δ *yat2* Δ *cat2*, when monitoring the forward reaction also supports this assumption. However, up to date, no proof for this assumption has been obtained, due to technical problems with the HPLC-electrospray mass spectrometer.

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Chapter 4

GENERAL DISCUSSION AND CONCLUSION

4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 CONCLUDING REMARKS AND PERSPECTIVES

In the past few decades, *S. cerevisiae* and other yeast species have been used as model organisms for the study of many processes that take place in the eukaryotic cell. The carnitine system has been shown to play a significant role in mammalian systems, in particular in the regulation of the acetyl-CoA / free CoA ratio, the β -oxidation of long-chain fatty acids and the translocation of acetyl units into mitochondria (reviewed in Bremer, 1983). In *S. cerevisiae*, the function of carnitine seems to be limited to the transfer of short-chain activated acetyl residues, either from the cytosol or the peroxisomes to the mitochondrion. It was the aim of this study to highlight the importance of the carnitine system in both the mammalian system, especially in humans, and in the eukaryote *S. cerevisiae*.

The literature review focusses on various aspects of carnitine transport and transfer in mammals and, to a lesser extent, in *S. cerevisiae*. We discuss the discovery and the biosynthesis of carnitine as an important co-factor for enzymatic activities (Bremer, 1983; Carter *et al.*, 1995). After uptake, the acylation state of the mobile carnitine pool is linked to that of the limited and compartmentalised free CoA-SH pools by the action of the family of carnitine acyltransferases and the mitochondrial membrane transporter. Therefore, the genes and sequences of the carriers and the acyltransferases were discussed along with mutations that affect activity. After summarising the enzymatic background, recent molecular studies on the carnitine acyltransferases were described to provide a clear picture of the role and function of these freely reversible enzymes. The enzymatic regulation and chemical mechanism were also discussed in relation to the different inhibitors under study for their potential to control diseases linked to lipid metabolism (reviewed in Ramsay *et al.*, 2001).

The experimental section of this thesis, on the other hand, investigates the roles of three carnitine acetyltransferase-encoding genes, named *YAT1*, *YAT2* and *CAT2*, in *S. cerevisiae* (Kispal *et al.*, 1993; Schmalix and Bandlow, 1993; Swiegers *et al.*, 2001). The development of an accurate and precise method to analyse the formation of acetylcarnitine by HPLC electrospray mass spectrometry was a necessary first step in this study. Besides high sequence similarity, our phenotypical data from a single, double and triple deletion of *CAT* genes on growth speed and overexpression of the *CAT* genes did not reveal other specific functions besides the shuttling of acetyl-CoA.

Our data clearly show that each enzyme fulfils a unique function in the transfer of activated acetyl groups between cellular compartments. The study generated interesting data, but leaves the question of specific functions for each carnitine acetyltransferase in *S. cerevisiae* largely unresolved.

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