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

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DISCUSSION

1. ANALYSIS OF RAT CrAT EXPRESSION IN YEAST

In the present work, we have cloned, for the first time, the cDNA encoding rat CrAT (Accession number AJ620886). Prior to this study, an mRNA sequence of rat CrAT had been reported, based on the *in silico* localization of several open reading frames (ORFs) (accession number XM_242301) observed in the genomic sequence of rat CrAT (accession number NW_047651). The genomic organization of the postulated rat CrAT was similar to CrATs from various organisms (van der Leij, 2000). However, it was also suggested to contain an extra exon (exon 7bis) not found in any other organism, solely on the basis of the observation of an ORF of 81 nt within intron 7. Our findings after sequencing the rat CrAT cDNA did not reveal the proposed exon 7bis. To establish whether this exon is present in mature CrAT mRNA, a shorter cDNA fragment comprising the putative exon 7bis was amplified by PCR. Since no such sequence was found, it was concluded that rat CrAT does not contain an exon 7bis. Nor was such an exon observed in any published CrAT cDNA sequence or in more than 200 published EST sequences from rat CrAT, and no such amino acid sequence can be aligned with protein sequences of CPT I, CPT II or COT from various organisms. Alternatively, if such an exon were to exist, expression of the processed mRNA containing exon 7bis in rat testis would be undetectable, even using PCR amplification.

We also postulated the presence of an N-terminal 29-amino acid sequence that would act as a mitochondrial targeting signal (MTS) in rat CrAT. In human mitochondrial CrAT, this MTS is clipped off during translocation through the inner mitochondrial membrane (Corti, 1994b) and the cleavage probably occurs between residues 29 and 30, where the sequence more closely resembles the well-conserved consensus cleavage site RXY[↓]S/A (RFK[↓]A in human CrAT) (Gavel, 1990). Since this sequence is also conserved in rat CrAT, we hypothesized that the mature form of rat mitochondrial CrAT (CrAT mit) would be 597 amino acids long.

The use of a mutant strain of *S. cerevisiae* (FY23 Δ cat2) devoid of CrAT, COT and CPT activities allowed us to express wt and mutant rat CrAT and COT cDNAs and study their kinetic characteristics. Comparison of the biochemical properties of the

yeast-expressed CrAT with those of the native protein (Miyazawa, 1983a; Colucci, 1988) revealed similar affinity for L-carnitine and acetyl-CoA, and exactly the same acyl-CoA specificity. This is the first time that mammalian CrAT has been expressed in yeast, and it has been demonstrated that *S. cerevisiae* is a valid model to study the structure-function relationship of CrAT. The same applies to rat COT expressed in yeast, as its kinetic characteristics and acyl-CoA specificity are very similar to those of the rat COT purified from liver (Miyazawa, 1983b).

2. ANALYSIS OF THE FOUR AMINO ACIDS IDENTIFIED BY BIOINFORMATIC METHODS IN CrAT

We attempted to identify the amino acids that are responsible for the different acyl-CoA selectivity of the members of the carnitine acyltransferase family. Before the publication of the 3-D structures of CrAT and COT, we attempted to address this issue using a bioinformatic approach, based on analysis of the complete alignment of the carnitine/choline acyltransferase family. In a protein family alignment, the positions considered as tree determinants or subfamily conserved residues are usually accepted as implicated in key catalytic activities, being responsible for the different enzymatic activities or substrate specificities of the different subfamilies in the alignment. A clear example of this type of analysis is the previously published analysis of the interaction of several members of the acyltransferase family with the inhibitor malonyl-CoA (Morillas, 2003).

The bioinformatic analysis revealed four amino acids that were present in all of the enzymes that catalyze short-chain acyl-CoAs (CrAT and ChAT) and absent in those enzymes that use medium- and long-chain acyl-CoAs as substrates (COT and CPTs). In rat CrAT, these amino acids are Ser¹¹⁹, Phe¹³⁸, Gly²⁴⁹ and Ala⁵¹⁵. Although we considered it highly likely that these amino acids were involved in determining acyl-CoA specificity, mutagenesis studies on these residues indicated that this is not the case. Neither of the mutants tested was able to enhance activity with the medium-chain acyl-CoA substrate decanoyl-CoA.

After the publication of the CrAT crystal, the locations of these amino acids were mapped onto the 3-D structure of CrAT (Fig. 1). We observed that, with the exception of the Ser¹¹⁹, the amino acids were positioned far away (at least 15 Å) from the active site and the catalytic histidine (His³⁴³). This observation would explain why none of these amino acids is involved in substrate acyl-CoA specificity in the CrAT enzyme. The partial loss of activity with acetyl-CoA in three of the four mutants (S119N, F138R and G249A), along with the almost complete abolition of activity of the quadruple mutant, indicate that these amino acids may be important in the maintenance of the 3-D structure.

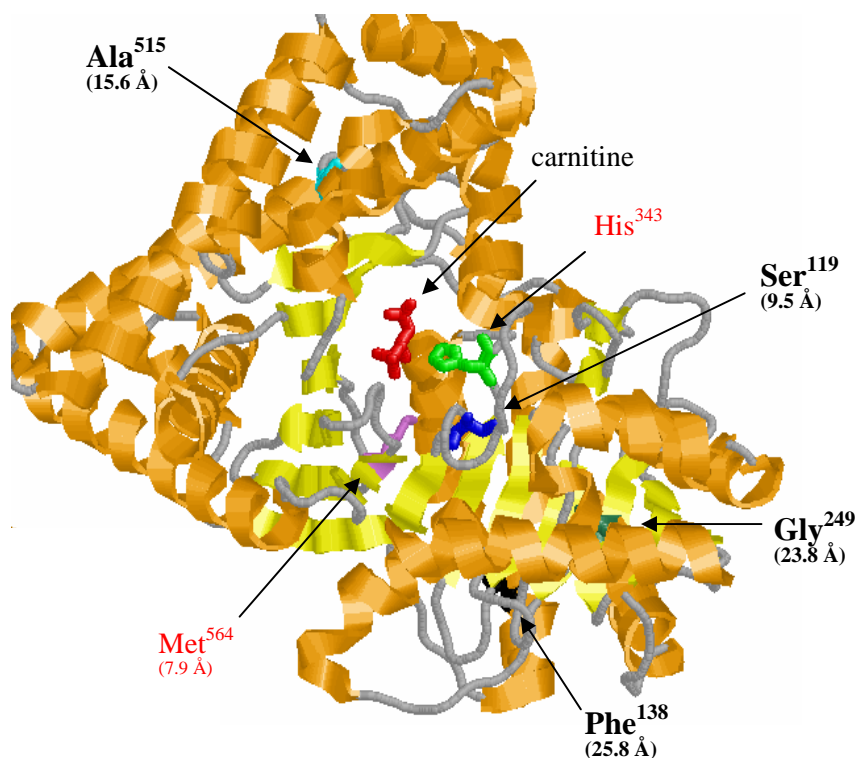


Fig. 1. Location of the putative amino acids involved in acyl-CoA specificity in the 3-D structure of mouse CrAT. The figure shows a *ribbon plot* representation of the mouse 3-D structure. β -sheets are shown in orange, and α -helices in yellow. A stick representation of the molecule of carnitine (red) is included. The position of CrAT catalytic His³⁴³ (green), Ser¹¹⁹ (blue), Phe¹³⁸ (black), Gly²⁴⁹ (greenblue), Ala⁵¹⁵ (cyan), and the Met residue involved in acyl-CoA specificity (purple) are also indicated. The distance between the side chain of each amino acid residue and that of the catalytic His³⁴³ is shown in parentheses.

3. ROLE OF CrAT His³⁴³ AND Glu³⁴⁷ IN CATALYSIS

The carnitine acyltransferases seem to share a common mechanism for transesterification. It is generally accepted that a highly conserved histidine is essential for catalysis in all members of the carnitine/choline acyltransferase family. In rat CrAT the residue implicated in this process is His³⁴³. Mutant H343A was expressed in *S. cerevisiae* and the activity of the mutant was abolished, supporting its catalytic role.

It has also been postulated that an aspartate may be involved in catalysis, leading to the suggestion of a catalytic triad-type mechanism, similar to the serine proteases (Brown, 1994). In the human CrAT structure, a functional conserved glutamate, Glu³⁴⁷, interacts with the catalytic histidine (Wu, 2003). Mutation of Glu³⁴⁷ to Ala in CrAT (mutant E347A) abolished enzyme activity, as seen in CPT II and L-CPT I (Brown, 1994; Morillas, 2004). Therefore, Glu³⁴⁷, together with His³⁴³, could be directly involved in catalysis. It is most likely that Glu³⁴⁷ potentiates the catalytic activity of His³⁴³ and also stabilizes the positive charge that develops on the histidine ring after deprotonation of the hydroxyl group of carnitine (Wu, 2003). Deprotonation of carnitine facilitates the nucleophilic attack of the carbonyl carbon of the thioester bond of acetyl-CoA, resulting in the formation of a tetrahedral intermediate. In the active site Ser⁵⁵⁴, the second serine residue of the strictly conserved STS motif, helps to stabilize the negatively charged oxyanion intermediate. Thus, our experimental results support the hypothesis that CrAT, as well as other carnitine acyltransferases appear to utilize a His-Glu/Asp catalytic-dyad to carry out catalysis, while the transition state is stabilized by a Ser residue.

Interestingly, the identity of some of the key catalytic residues in CrAT are the same as those identified in the bacterial carbohydrate acetylating enzymes such as *N*-acetyl-glucosamine-1-phosphate uridyltransferase (GlmU) (EC 2.3.1.157). These enzymes are not related to CrAT in structure or sequence. In GlmU, a histidine residue has been proposed to deprotonate the amine on the carbohydrate to activate it for nucleophilic attack on acetyl-CoA. This His is activated by a conserved glutamate residue and the tetrahedral transition state is stabilized by a serine residue (Sulzenbacher, 2001). This suggests that a wide range of acyltransferase enzymes

appear to utilize a common catalytic mechanism, which has been conserved during evolution.

4. CrAT Met⁵⁶⁴ AND COT Gly⁵⁵³ DETERMINE THE ACYL-CoA SPECIFICITY OF BOTH ENZYMES

Substrate specificity in the carnitine acyltransferase family is now well defined, based on studies of various groups: CrAT catalyzes short-chain acyl-CoAs (Miyazawa, 1983a), whereas COT catalyzes medium-chain acyl-CoAs containing between 6 and 12 carbons (Farrell, 1984) and CPTs catalyze long-chain fatty acyl-CoAs (Miyazawa, 1983b). We hypothesized that a structural feature of the enzyme might be responsible for substrate specificity, depending on the length of the acyl-CoAs. We addressed this question with CrAT and COT.

The putative amino acid residue responsible for governing the access of the acyl-CoA was identified by close examination of the catalytic site in CrAT crystals. The report of the crystal structure of mouse CrAT (Jogl, 2003) suggested Met⁵⁶⁴ as a candidate, because its side chain is voluminous and projects into the putative cavity where the acyl-CoA may enter. Our bioinformatic approach failed to identify Met⁵⁶⁴; in our protein alignment we compared all the known sequences of the carnitine/choline acyltransferase family from several organisms (including mammals, fungi and some invertebrates), and a Met residue at this position is only conserved in mammalian CrATs. In yeast CrATs, a Trp or Tyr is found at the same position, and in choline acetyltransferases the equivalent amino acid is a cysteine (see Appendix). Although a Met residue is not conserved in all short-chain acyl-CoA enzymes, it is conservatively substituted for a voluminous residue that could play the same role. When we performed a second alignment that only considered the mammalian representatives (14 in total), the presence of either a Gly or a Met residue in the same position was found to be a common feature in all long-chain and short-chain acyl-CoA enzymes, respectively. It was clear that not only COT but also CPT I and CPT II contain a glycine at this position. This suggested that the smaller glycine was probably the residue that allows free access of the long-chain fatty acyl-CoAs, whereas the larger side chain of Met⁵⁶⁴

prevented access of long-chain acyl-CoAs. The CrAT mutant M564G produced positive results that support these predictions.

The increases in activity and catalytic efficiency towards medium-chain acyl-CoAs in the CrAT mutant M564G were not accompanied by changes in K_m for acyl-CoA. Maximal changes were observed in V_{max} and catalytic efficiency, suggesting that these increases in catalytic activity are attributable to the altered accessibility of the catalytic site. In the wt CrAT enzyme, the Michaelis constants for substrates ranging from acetyl- to octanoyl-CoA are very similar. The same behaviour had been observed in purified CrAT from mouse (Farrell, 1984): K_m 's for acyl-coA for the purified enzyme were invariant over the range C₂ to C₈ ($18 \pm 2 \mu\text{M}$). This poses the question as to whether the various acyl-CoAs in the wt model are located in the hydrophobic pocket or in the catalytic channel. In the latter case, acyl-CoAs would displace carnitine from its site. This appears to occur, as the carnitine K_m increases with increasing chain length of acyl-CoA. Carnitine is presumably displaced and catalysis is prevented, as indicated by the low V_{max} of the wt CrAT in the presence of long-chain acyl-CoAs. If the hydrophobic pocket is open, as shown in the CrAT mutant M564G, the long hydrocarbon chain can occupy it. Consequently, the carnitine K_m decreases with increasing carbon-chain length for acyl-CoAs (except for myristoyl-CoA) and catalytic efficiencies for medium- and long-chain acyl-CoA increase and catalysis proceeds. This is supported by a previous enzymatic study in pigeon CrAT (Chase, 1967). Chase observed that the binding of medium-chain acyl-CoAs to CrAT lowered its affinity for L-carnitine, that is, the K_m of the enzyme for L-carnitine increases with increasing medium-chain acyl-CoA concentration, whereas when short-chain acyl-CoAs were used, the K_m for carnitine was independent of the concentration of the acyl-CoA.

We have also demonstrated that, in the reverse case, mutation of the orthologous glycine (Gly⁵⁵³) to methionine in rat COT converts this enzyme into a pseudo-CrAT. Results on the activity of the COT mutant G553M with different acyl-CoAs used as substrate are very similar to those for wt CrAT. The same applies to CrAT mutant M564G and wt COT: the pattern of activities in relation to acyl-CoAs of different lengths is very similar. In both cases the enzymes containing glycine rather than methionine, CrAT mutant M564G and wt COT, present high enzyme activities with

medium and long fatty acyl-CoAs, while the activity toward acetyl-CoA is low. These findings are consistent with those obtained by Jogl (2005), who reported that the mouse COT G553M mutant can no longer utilize octanoyl-CoA as a substrate, although the activity with the short-chain acetyl-CoA substrate is maintained.

In addition, our 3-D models confirm the important role of CrAT Met⁵⁶⁴ and COT Gly⁵⁵³ in determining the acyl-CoA specificity of both enzymes. Models for the enzymes containing Met (wt CrAT and COT G553M) are structurally equivalent and reveal a shallow cavity that it is only accessible to short-chain acyl-CoAs. In contrast, the model for CrAT mutant M564G is very similar to the one for wt COT. In both models, the small size of the side chain of the glycine, Gly⁵⁶⁴ in CrAT and Gly⁵⁵³ in COT, reveals a deep cavity accessible to longer acyl groups, and these models correlate with the preference of both enzymes for medium-chain acyl-CoAs. Moreover, the position of the fatty acyl part of myristoyl-CoA in the CrAT M564G model is very similar to that modelled for the palmitoyl part of palmitoyl-CoA in the hydrophobic pocket of CPT I (Morillas, 2004). This is consistent with the crystal structures of the M564G mutant, alone and in complex with carnitine (Hsiao, 2004). The 3-D structures also reveal a deep binding pocket that can accommodate a larger acyl group.

An alternative explanation to the model of methionine/glycine acting as a gate to permit the location of longer acyl-CoAs could be based on increased flexibility of the mutant form, since glycine does not constrain the backbone psi/phi angles. To differentiate between these two models, an intermediate situation was tested in which the Met⁵⁶⁴ residue in CrAT was substituted by alanine. The presence of a methyl group in the side chain of alanine would mimic the presence of the methionine in terms of backbone flexibility (reducing the degrees of freedom of the phi/psi angles of the backbone) but, in contrast, it would also reflect the small size of the glycine residue. The similarity of the results obtained to those obtained in the Gly mutant strongly supports the gate model.

5. RE-ENGINEERING CrAT AND COT INTO A PSEUDO CPT

The 3-D model for the positioning of long-chain acyl-CoAs in the CrAT M564G mutant (Fig. 24, Results), allowed us to identify a second amino acid residue, Asp³⁵⁶, which could be involved in determining acyl-CoA specificity in CrAT. The replacement of Asp³⁵⁶ with alanine, along with the mutation of Met⁵⁶⁴ to Gly, allows CrAT to catalyze long-chain acyl-CoAs such as palmitoyl- and stearoyl-CoA. The CrAT double mutant D356A/M564G behaves as a CPT in terms of acyl-CoA specificity. The model for the positioning of long-chain acyl-CoAs in CrAT mutant D356A/M564G confirms our experimental results, since it reveals a deeper cavity that allows the positioning of palmitoyl- and stearoyl-CoA. This cavity is almost completely occupied when stearoyl-CoA is introduced. Even allowing full rotation of acyl chain bonds, it was impossible to obtain an active-enzyme compatible model for longer substrates. Longer substrates could perhaps be accommodated in the hydrophobic pocket if the side chain of Thr⁵⁶⁰ were to be removed. However, the fact that this residue is part of the external surface of the protein introduces new variables (e.g., contacts of the end of the acyl chain with surrounding water) that make it difficult to develop a model using current *in silico* methods.

Another approximation to broaden the specificity of CrAT to longer acyl-CoAs was the replacement of a cluster of three residues in rat CrAT (⁵⁶³VMS⁵⁶⁵) with the orthologous amino acids in CPTs (three glycines). The resulting CrAT triple mutant GGG produced positive results, since it showed a higher activity with palmitoyl-CoA than the single mutant M564G. Unfortunately, we were not able to continue the characterization of this triple mutant. Our first mitochondrial protein extracts showed a high carnitine acyltransferase activity, but then we could not reproduce the same levels of activity in the following protein extracts, in which activity was reduced by 90%. CrAT triple mutant GGG was expressed in yeast at similar levels to the wt, thereby ruling out the possibility that the marked reduction of activity was caused by low protein expression. In addition, when Hsiao (2004) attempted the overexpression of this triple mutant in *E. coli*, they found that the mutant was unstable in solution and they were unable to characterize it.

Our model for the positioning of octanoylcarnitine in the fatty acid binding site of rat COT allowed us to identify two amino acids, Ile⁵⁵¹ and Asp³⁴³, that seem to interfere with the correct positioning of acyl-groups longer than eight carbons and might control the access of long-chain substrates in COT. Both amino acids were replaced individually with Ala in COT but neither mutant displayed enhanced activity with long-chain substrates. In a different study, structure-based mutations in mouse COT also failed in the attempt to expand the enzyme activity toward long-chain acyl-CoAs (Jogl, 2005). The amino acids Met³³⁵ and Cys³²³, which in the COT crystal interact with each other and the C8 atom of the octanoyl group, were mutated individually to their counterparts in CPT, but neither mutation allowed the enzyme to catalyze longer fatty acids as substrates.

6. MODEL FOR ACYL-CoA CHAIN-LENGTH DISCRIMINATION IN THE CARNITINE ACYLTRANSFERASE FAMILY

Taking into account all the previous results, we propose a model for the acyl-CoA chain-length discrimination in CrAT based on the presence of two amino acids, Met⁵⁶⁴ and Asp³⁵⁶, which act as checkpoints at different stages of the entry of acyl-CoAs to the fatty acid binding site. The bulkier side chain of Met⁵⁶⁴ forms the floor of the shallow fatty acid binding pocket of CrAT and impedes the entry of medium- and long-chain acyl-CoAs; this explains CrAT selectivity for short-chain acyl-CoAs. The replacement of this Met with the smaller Gly (M564G) opens the binding site and reveals a deeper hydrophobic pocket that can accommodate acyl-CoAs of up to fourteen carbons. The hydrophobic chain of longer acyl-CoAs cannot fit in the pocket in the presence of the side chain of Asp³⁵⁶ and, therefore, cannot be catalyzed by the enzyme. The substitution of this amino acid with a smaller, uncharged residue (D356A), along with the M564G mutation, completely opens the binding site and allows entry of long-chain acyl-CoAs, such as palmitoyl- and stearoyl-CoA.

It should be noted that replacement of Met⁵⁶⁴ with Gly is based on both structural considerations and sequence alignments (Met is replaced by Gly in COTs and CPTs), and this substitution could be considered as an evolutionary development that was

selected because it enabled the carnitine acyltransferase family to catalyze longer acyl-CoAs. On the other hand, our replacement of the Asp³⁵⁶ with Ala was only based on structural considerations. Alignment of the proteins of the family shows high variability in the region close to the Asp³⁵⁶; the orthologous residues corresponding to CrAT Asp³⁵⁶ are His/Tyr in COT, Asn in CPT II, and Glu in CPT I. In these enzymes, whose natural conformation accepts long chain substrates, the cavity has adopted a slightly different shape to that of acetyltransferases. Small displacements of beta strands E1 and E13, and alpha helix H12 lead to a wider cavity in which long substrates can adopt less restrictive conformations. This situation can be extrapolated from CPT I models (Morillas, 2004) and was observed in mouse COT structures (Jogl, 2005). Thus, the presence of bulky or charged residues in this position for long-chain enzymes is not as critical in terms of substrate fitting as it is for mutated CrAT, which has a narrower, hidden cavity. Although the mutation of Asp³⁵⁶ to Ala, along with the mutation of Met⁵⁶⁴ to Gly, creates an artificial enzyme that behaves as a CPT in terms of acyl-CoA specificity, it is clear that the replacement of this Asp³⁵⁶ with Ala is not the strategy followed by nature to handle long-chain substrates in CPTs, in contrast to the replacement of Met⁵⁶⁴ with Gly. Selectivity for long-chain substrates may be determined by more complex factors than specific amino acid variations between the different members of the family, possibly by interactions among several different residues at the same time as variations in secondary structural elements. The most striking difference in the CPT I enzymes is the presence of a 13-amino acid insertion between beta strands E13 and E14 that may form a more flexible pocket that can accommodate long-chain acyl groups. Interestingly, this insertion is located just before the Met⁵⁶⁴ residue.

7. EFFECT OF C75-CoA ON CrAT ACTIVITY

Our experimental results show that C75-CoA is a potent inhibitor of CrAT mutants M564G and D356A/M564G. In contrast, C75-CoA exhibits no inhibitory effect on wt CrAT. Thus, only when the hydrophobic pocket of CrAT is opened and accessible to longer acyl-CoAs, as happens in both CrAT mutants, is the protein sensitive to C75-CoA inhibition. Taking into account these results and the fact that the chemical structure of C75-CoA resembles that of a medium-chain acyl-CoA (Fig. 2A

and B), it can be suggested that the inhibitor might occupy the same pocket in CrAT mutants as the acyl-CoAs substrates, and that the mechanism of inhibition might be based on mutual exclusion, as has been shown for CPT I (Bentebibel, 2006).

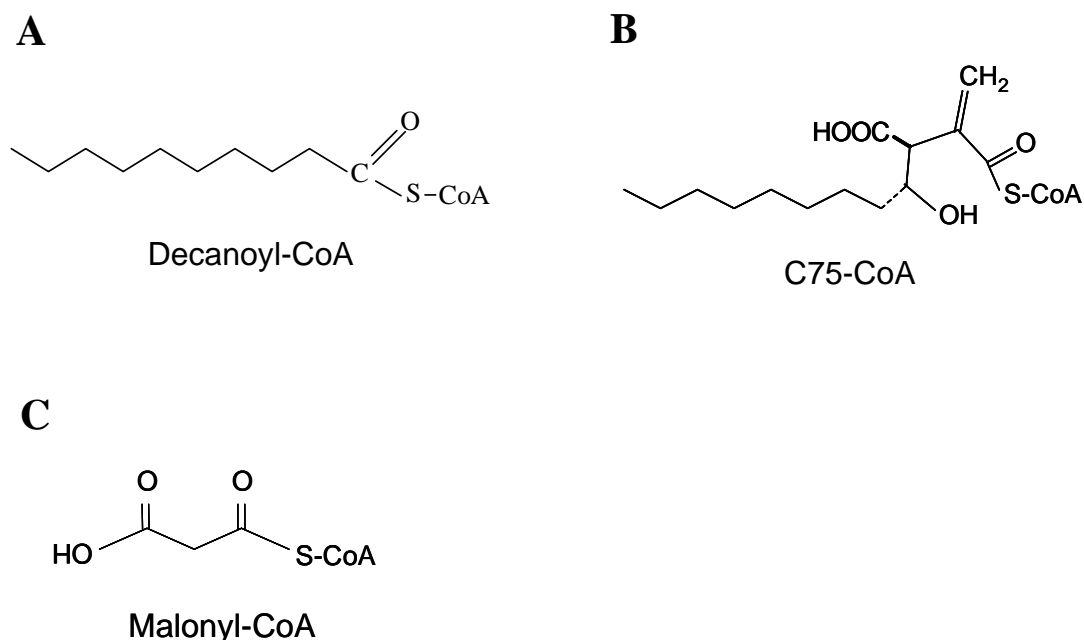


Fig. 2. Chemical structure of decanoyl-CoA, malonyl-CoA, and C75-CoA.

The IC_{50} and K_i values show that the CrAT double mutant is more sensitive to C75-CoA than the single mutant. This could be due to the fact that the binding of the hydrocarbon chain of the C75-CoA molecule is favoured in the deeper and more hydrophobic cavity of the CrAT double mutant. However, neither CrAT mutant is as sensitive as CPT I to C75-CoA, since the IC_{50} and K_i values for C75-CoA acting on CPT I are 50- and 19-fold lower than that observed for the CrAT double mutant. These results indicate that there are other factors, apart from the presence of a hydrophobic pocket for the binding of the aliphatic chain of the inhibitor, that contribute to the inhibitory potency of C75-CoA toward its targets. The chemical structure of the C75 “head”, which contains a carboxylic group, resembles that of the molecule of malonyl-CoA, the physiological inhibitor of CPT I (Fig. 2C). It can be hypothesized that the C75-CoA “head” could mimic the interaction of malonyl-CoA with CPT I, and that would explain why C75-CoA is such a potent inhibitor of CPT I.

It is well known that malonyl-CoA is not an inhibitor of CrAT activity. Moreover, we demonstrated that malonyl-CoA does not inhibit either the CrAT mutant M564G or the mutant D356A/M564G. Therefore, the lack of an interaction between either of the CrAT mutants and malonyl-CoA would explain their limited sensitivity to the inhibitor.

8. MODEL FOR CARNITINE-CHOLINE DISCRIMINATION IN CrAT

Our results indicate two factors that contribute to the discrimination between choline and carnitine in CrAT, each to a similar extent. Firstly, the electrostatic interaction of Thr⁴⁶⁵ and Arg⁵¹⁸ with the carboxylate group of carnitine; when this interaction is eliminated in the CrAT triple mutant, its catalytic efficiency towards carnitine decreases dramatically and there is a 5-fold increase in catalytic efficiency toward choline. The second factor is the side-chain volume of the residues around the carnitine in the active site of CrAT. The replacement of Ala¹⁰⁶ in CrAT with methionine in the CrAT quadruple mutant reduces the volume of the carnitine binding site, and this almost completely blocks carnitine acetyltransferase activity and leads to a 9-fold increase in the catalytic efficiency towards choline, nearly doubling the effect of the abolition of the electrostatic interactions.

The fact that our improvement of the catalytic efficiency of CrAT towards choline (9-fold) was not as successful as that achieved by Cronin (1997) with ChAT towards carnitine (1620-fold) is not due to a failure in the acquisition of choline activity of CrAT mutants, since their catalytic activity with choline is only 3 times lower than that of mutant ChAT with carnitine (172 vs 469 M⁻¹s⁻¹). This discrepancy is attributable to the fact that wt CrAT is very active toward choline ($K_{cat}/K_m = 18.2 \text{ M}^{-1}\text{s}^{-1}$) while wt ChAT has a very low carnitine acetyltransferase activity ($K_{cat}/K_m = 0.289 \text{ M}^{-1}\text{s}^{-1}$). The relatively high choline acetyltransferase activity of wt CrAT could be due to the fact that choline can enter the carnitine binding pocket without any steric hindrance.

9. CrAT OVEREXPRESSION AND ESTER FORMATION IN YEAST

GC/MS analysis of the fermentation products obtained with the different *CAT2*-overexpression strains indicated that the expression levels of *CAT2* have only a modest effect on ester production. For some esters, including the important flavour components ethyl acetate and 3-methyl-butyl acetate (isoamyl acetate), overexpression of mitochondrial Cat2p caused a significant reduction compared with control cells. At the same time, overexpression of mitochondrial Cat2p produced a 50% increase in the levels of 2-methyl propanol. α -Keto butyrate is a precursor of both propanol and ethyl butanoate. If more propanol is made then it means less of the α -keto butyrate would be channelled into ethyl butanoate. Indeed, the results show that significantly less ethyl butanoate is formed when mitochondrial Cat2p is overexpressed. Compared to mitochondrial Cat2p, overexpression of cytosolic Cat2p resulted in minor decreases in the formation of some esters. The reason for this smaller contribution of cytosolic Cat2p to ester synthesis is unclear. We expected this cytosolic isoform to have a greater effect than the mitochondrial isoform because the yeast alcohol acetyltransferase I, Atf1p, responsible for the major part of volatile acetate ester production in yeast, is localized in lipid particles in the cytosol (Verstrepen, 2004).

It should be noted that the fermentation media was supplemented with L-carnitine because *S. cerevisiae* is unable to synthesize carnitine (van Roermund, 1999). Although CrAT catalyses a reversible reaction, the addition of L-carnitine would favour the forward reaction by a mass action effect, and would therefore lead to the formation of acylcarnitine and free CoA (Fig. 3). This would cause a decrease in the acetyl-CoA/CoA ratio and, consequently, a decrease in ester formation, consistent with observations from our analyses of fermentation products. Under these fermentation conditions, *CAT2* overexpression produces a similar, although more modest, effect to that observed in an *ATF1* deletion strain (Verstrepen, 2003b). These results are consistent with the finding that ethyl acetate, formed primarily in the cytosol, was more reduced in the *CAT2* cyt-overexpression strain than in the strain overexpressing *CAT2* mit. On the other hand, 3-methyl butyl acetate (isoamyl acetate), which is dependent on

mitochondrial metabolism, was more reduced in the *CAT2* mit than in the *CAT2* cyt-overexpressing strain.

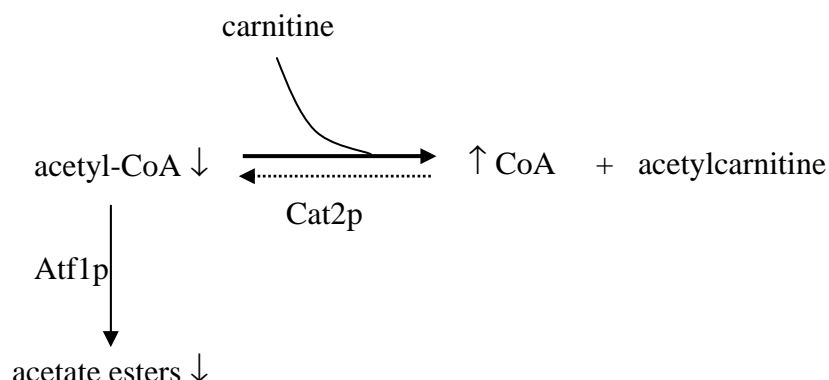


Fig. 3. **Effect of the overexpressing *CAT2* under conditions of increased carnitine availability.** The forward carnitine acetyltransferase reaction is indicated with an arrow and the reverse reaction is indicated with a dashed arrow.

The observation that overexpression of *CAT2* resulted in only a very limited effect on the formation of esters during fermentation could be due to the following factors. First of all, we used a laboratory strain (FY23) for the fermentation trials, and this strain produces between 5- and 10-fold lower concentrations of esters than a wine strain. The low levels of some esters, such as ethyl dodecanoate or 3-methyl propyl acetate, made their quantification difficult. It is quite possible that more extreme changes in the composition of volatile esters would be seen if *CAT2* were overexpressed in a commercial wine yeast such as VIN13. Therefore, modulation of volatile ester composition by modification of carnitine acetyltransferase metabolism is probably still a viable option. Secondly, in order to see a major effect of *CAT2* overexpression, this gene should have been overexpressed together with *ATF1* and/or pantothenate kinase (*panK*) to increase the levels of esters and CoA, respectively. It has been demonstrated that overexpression of *ATF1* in yeast leads to a 2- to 10-fold increase in the levels of some acetate esters (Lilly, 2000). Moreover, it has been reported that overexpression of *panK* along with simultaneous supplementation of pantothenic acid in *E. coli* leads to an increase in intracellular CoA and acetyl-CoA levels (Vadali, 2004b). In these conditions, *CAT2* could play an important role because of its involvement in modulation of the acyl-CoA/CoA ratio.

10. FUTURE: FURTHER INVESTIGATION

The amino acid substitutions in rat CrAT performed in this study reveal several residues that are involved in acyl-CoA and choline substrate recognition and provide insights into the molecular requirements for their correct positioning for efficient catalysis. The carnitine acyltransferase family has received much attention, as they are considered promising targets for the development of drugs against type II diabetes and other human diseases (Anderson, 1998; Wagman, 2001). The CrAT mutant D356A/M5464G catalyses acyl-CoAs over a wide range of chain length, from acetyl- to palmitoyl-CoA, thereby mimicking the natural proteins CrAT, COT and CPT. Long-chain acyl-CoAs are candidate mediators of insulin resistance. Therefore, this double mutant may be useful for studies of the influence of fatty acids on insulin resistance. If this CrAT double mutant were to be overexpressed, these harmful long-chain acyl-CoAs would be transformed into acylcarnitine derivatives and then excreted in the urine, thus detoxifying selective acyl residues and releasing free CoA. Overexpression of this mutant CrAT offers two advantages over the overexpression of CPT I. Firstly, the CrAT mutant reacts with a broader series of fatty acid acyl-CoAs than CPT I and would facilitate the excretion of a wider range of harmful acyl residues as acylcarnitines. Secondly, the activity of the CrAT mutant D356A/M5464G is not inhibited by malonyl-CoA: this is unlike wt CPT I but similar to wt CrAT. The results presented in this study not only help us to understand structure-function relationships within the acyltransferase family but may also facilitate studies on obesity, NIDDM, and patients with defective β -oxidation. Moreover, our results open the possibility of biotechnological applications of the enzymes of the carnitine acyltransferase family in the wine industry.