

**Alternative Exon Usage in the Single CPT1 Gene of *Drosophila* Generates Functional Diversity in the Kinetic Properties of the Enzyme**

*Differential expression of alternatively spliced variants in drosophila tissues*

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2 The abbreviations used are: RT, reverse transcription; EST, expressed sequence tag; contig, group of overlapping clones.

## ABSTRACT

The *Drosophila melanogaster* genome contains only one CPT1 gene (Jackson, V. N., Cameron, J. M., Zammit, V. A., and Price, N. T. (1999) *Biochem. J.* 341, 483–489). We have now extended our original observation to all insect genomes that have been sequenced, suggesting that a single CPT1 gene is a universal feature of insect genomes. We hypothesized that insects may be able to generate kinetically distinct variants by alternative splicing of their single CPT1 gene. Analysis of the insect genomes revealed that (a) the single CPT1 gene in each and every insect genome contains two alternative exons and (ii) in all cases, the putative alternative splicing site occurs within a small region corresponding to 21 amino acid residues that are known to be essential for the binding of substrates and of malonyl-CoA in mammalian CPT1A. We performed PCR analyses of mRNA from different *Drosophila* tissues; both of the anticipated splice variants of CPT1 mRNA were found to be expressed in all of the tissues tested (both in larvae and adults), with the expression level for one of the splice variants being significantly different between flight muscle and the fat body of adult *Drosophila*. Heterologous expression of the full-length cDNAs corresponding to the two putative variants of *Drosophila* CPT1 in the yeast *Pichia pastoris* revealed two important differences between the properties of the two variants: (i) their affinity ( $K_{0.5}$ ) for one of the substrates, palmitoyl-CoA, differed by 5-fold, and (ii) the sensitivity to inhibition by malonyl-CoA at fixed, higher palmitoyl-CoA concentrations was 2-fold different and associated with different kinetics of inhibition. These data indicate that alternative splicing that specifically affects a structurally crucial region of the protein is an important mechanism through which functional diversity of CPT1 kinetics is generated from the single gene that occurs in insects.

## INTRODUCTION

Lipid metabolism in insects has largely been investigated from the perspective of energy supply to flight muscle, particularly in migratory flight. Because their lipid metabolism is less complex than in vertebrates, insects are considered a good model system in which to study the fundamental aspects of fat metabolism. The increasing availability of insect gene sequence data and completely sequenced genomes allows comparative analyses with mammalian data and suggests that many of the fundamental molecular mechanisms involved in fatty acid metabolism and their regulation are conserved. Although in most insects there are major differences in the glycerolipids used to transport lipid between tissues (diacylglycerol in insects *versus* triacylglycerol in vertebrates), the transfer of the long chain acyl moiety across the mitochondrial inner membrane is likely to be substantially similar to that in vertebrates and to involve the carnitine shuttle (1). We have previously shown that the fruit fly *Drosophila melanogaster* has only a single CPT1 (carnitine palmitoyltransferase 1) gene (2). When we expressed the full-length cDNA (Berkeley *Drosophila* Genome Project), we found that it encodes a functional enzyme in terms of both its catalytic activity and malonyl-CoA inhibition. It has been suggested that insect genomes, which contain relatively few genes for a metazoan (~14,000), increase their gene expression complexity through alternative splicing (3). Because the expression of CPT1 enzymes with different kinetic characteristics is central to the regulation and coordination of whole body fatty acid metabolism in mammalian species (4), we hypothesized that *D. melanogaster* and other insects may overcome the restrictions that might be imposed by the existence of only a single gene, through the generation of alternative transcripts so as to generate kinetically distinct CPT1 variants. Therefore, in the present study we have tested our hypothesis by (i) searching for alternative CPT1 exons in the insect genomes that have been sequenced to date; (ii) quantifying the level of expression of the two variant mRNA species predicted to exist in *Drosophila* tissues, using RT-PCR2 and real time PCR; and (iii) heterologously expressing in the yeast *Pichia pastoris* both of the two cDNAs that can be generated by alternative splicing of *Drosophila* CPT1 followed by characterization of the kinetics of the two variant proteins. We find that each of the single CPT1 genes in the insect genomes currently available contains alternative exons. In addition, both mRNA variants are expressed in all of the *Drosophila* tissues tested (larval and adult stages), with one variant being preferentially expressed in flight muscle compared with the fat body. The two proteins resulting from expression in *P. pastoris* of each cDNA variant display different kinetic characteristics, particularly with reference to their affinity for acyl-CoA substrate and the mechanism of inhibition by malonyl-CoA.

## EXPERIMENTAL PROCEDURES

*Alternative Exon Search in the CPT1 Gene of Insects*—The CPT1 gene sequences of *D. melanogaster*, *Anopheles gambiae*, and *Aedes aegypti* were retrieved from the Ensembl website. The CPT1 genomic sequence from *Apis mellifera* was identified by searching the honey bee genome assembly 4.0 for sequences similar to the partial Am CPT1 EST (PubMed reference BE844575). CPT1 genomic sequences were similarly identified for the mosquito by utilizing the VectorBase BLAST facility (5). Additional BLAST analysis was performed via Flybase (6). The ClustalW algorithm was used for multiple sequence alignments.

*Preparation of Total RNA from D. melanogaster Tissues and cDNA Synthesis*—Total RNA was extracted from *Drosophila* tissues that were pooled from several animals. The tissues were dissected, placed directly into QIAzol, and homogenized using QIAshredder columns (Qiagen). Total RNA was then prepared using the RNeasy lipid tissue mini kit (Qiagen), including an on-column DNase digestion (RNase-Free DNase Set; Qiagen), according to the manufacturer's protocol. RNA quantity and purity was determined using a Nanodrop spectrophotometer. A quantity (150 ng) of total RNA was then reverse-transcribed for 50 min at 37 °C in a 20- $\mu$ l reaction mixture containing 1x first strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>), 500 ng of oligo(dT) (18-mer) primer, 0.5 mM dNTPs,

10 mM dithiothreitol, 40 units of RNaseOUT (Invitrogen), and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen), followed by inactivation of the reaction by incubation for 15 min at 70 °C and storage at -20 °C. Control reactions were performed in which the aliquot containing reverse transcriptase was replaced with distilled water.

#### *RT-PCR Amplification of Splice Variants from D. melanogaster*

*Tissues*—PCRs were carried out in a total volume of 50 µl containing 7.5 ng of cDNA, 0.2 µM of each primer, and 25 µl of BioMix Red (Bioline). The reactions were incubated for 1 min at 94 °C, followed by 40 cycles consisting of 30 s at 94 °C, 30 s at 64 °C, and 20 s at 72 °C. A final extension of 10 min at 72 °C was performed, and the reaction was subsequently cooled to 4 °C. The primers used were CPT6A-3 (CACACATGTGGGAGAACCTG) and CPT7-1 (TGCAGGGCCATTTGTATGTA) to amplify exon 6A-containing sequences and CPT6B-4 (TGGGACACCTGTGGGAGTAT) and CPT7-1 (TGCAGGGCCATTTGTATGTA) for exon 6B-containing sequences. Control reactions were performed with template from reverse transcription reactions from which the reverse transcriptase was omitted.

*Real Time PCR Analysis of CPT1 Expression*—Quantitative PCR amplification was performed in a 20-µl reaction mixture consisting of 0.2–1.0 µl of cDNA, 0.5 µM of each primer, and 10 µl of PowerSYBR Green PCR Master Mix (Applied Biosystems) using an ABI PRISM 7000 system. Specific primer pairs were used to amplify total CPT1 (CPT1-4-1, GCAAGTGCAAATTGAGGAAA; CPT1-5-5, AAGTGCTCCTCACCTTCCAC), exon 6A-containing CPT1 (CPT1-5-2, CCGCTGGTTTGACAAGTG; CPT1-6A-4, TCATCGACGATCAGGTTCTC), and exon 6b-containing CPT1 (CPT1-6B-1, AATGGTCGCGTTGGCTTC; CPT1-6B-2, TCCCAAACCGGTGCATC). The reactions were incubated for 2 min at 50 °C, followed by 10 min at 95 °C. This was followed by 40 cycles consisting of 15 s at 95 °C and 1 min at 60 °C. All of the samples were subjected to a melting curve analysis consisting of 15 s at 95 °C, 20 s at 60 °C, and 15 s at 95 °C. The relative mRNA level in each sample was calculated by constructing a standard curve and then by normalization relative to the expression of the ribosomal rp49 gene (primers used: rp49-1, CCGCCACCAGTCGGATC; rp49-2, TTGGGCTTGCGCCATT). The relative expression level of the mRNA in the adult fat body was arbitrarily set to 1.0 by dividing the expression values of all samples by that of the adult fat body. The specificity of the primers was verified by examining the melting curves of each sample. Additional controls included either substituting the cDNA with distilled water or using cDNA from reactions performed without reverse transcriptase. Statistical analysis was performed using a two-tailed Student's *t* test (SPSS software).

*Heterologous Expression and Kinetic Characterization of Drosophila CPT1 Splice Variants*—The Berkeley *Drosophila* Genome Project EST clone GH21765 was obtained from Research Genetics (Carlsbad, CA). The BsgI-HindIII fragment of our previously described *D. melanogaster* cDNA in pGAPZ (which contains exon 6A) was replaced with the corresponding fragment from EST clone GH21765 (which contains exon 6B). Following verification of the sequence, the cDNA was expressed in the yeast *P. pastoris* (which lacks endogenous CPT activity) as described previously (2). CPT1 activity was measured using cell-free yeast extracts as described previously. Kinetic characterization was performed as in Ref. 7. In assays designed for the determination of the IC<sub>50</sub> formalonyl-CoA, the palmitoyl-CoA and carnitine concentrations used were 135 and 500 µM, respectively. To obtain the K<sub>0.5</sub> (concentration of substrate that gives 50% of maximal activity) for palmitoyl-CoA, the concentration of carnitine was fixed at 500 µM. To obtain K<sub>0.5</sub> for carnitine, palmitoyl-CoA was fixed at 135 µM (in the presence of 0.1% dialyzed bovine serum albumin). Curve fitting was carried out using Sigma-Plot software with nonlinear regression analysis. Where possible (see below), determination of K<sub>i</sub> values for malonyl-CoA was performed using primary and Dixon plots as described previously (8, 9). Statistical analysis was performed using two-tailed Student's *t* tests (Sigma-Plot software).

## RESULTS

### ***Evidence for Splice Variation in the Transcription of Insect CPT1***

We have previously shown, using Southern blotting, that *Drosophila* has only one CPT1 gene (2). The subsequent availability of the sequence of the complete *D. melanogaster* genome (10) confirmed this finding. The published annotated sequences for the genomes of the mosquitoes *A. gambiae* and *A. aegypti*, the honey bee *A. mellifera*, the wasp *Nasonia vitripennis*, and the beetle *Tribolium castaneum* show that these genera too have only one CPT1 gene (11). In view of the importance of the existence of CPT1 isoforms with different kinetic characteristics in mammals, we hypothesized that insects may be able to generate CPT1 kinetic diversity through alternative splicing of exons within their single gene.

Initial searching for potential alternative exons was performed on the *Drosophila* gene in which we identified a putative exon that was an alternative to the known exon 6. It is located in intron 5 and starts 7063 bp downstream of the first exon. The putative alternative exon was therefore designated 6A, and the known exon 6 was renamed exon 6B. Exon 6B is 66 bp long, whereas exon 6A is 60 bp long. Consequently, replacement of exon 6B with 6A would retain the same reading frame (Fig. 1A). By using either exon 6A or 6B in the *Drosophila* CPT1 sequence, two splice variants of this gene could be generated (Fig. 1B). The *Drosophila* CPT1 gene spans 10.2 kb, including proximal 5' regulatory sequences, 3.65 kb of which contain eight wholly or partially protein-coding exons. As shown in Fig. 1C, only four of the exonic boundaries are conserved between insect and mammalian CPT1 genes, among which there is complete conservation of the boundaries of the 18 protein-coding exons (12–14). In particular, the boundaries of exons flanking the alternatively spliced exons are not conserved between insect and human CPT1A (Fig. 1C). This difference may have arisen because the position of these boundaries would facilitate the alternative splicing of the insect sequences (to obtain functionally different isoforms) but would be redundant in mammals because of the existence of separate CPT1 genes.

### ***Potential for Alternative Splicing of the CPT1 Gene in Other Insects***

We further analyzed the sequences of the CPT1 gene of all insects for which sufficient sequence information is available (see "Experimental Procedures"). We find that all contain putative alternative exons that can be used mutually exclusively in alternative splicing of the CPT1 gene. In particular, analysis of the genomes of four other insect genomes identified very similar arrangements within their respective CPT1 genes (Fig. 2).

### ***EST Evidence for the Existence of Splice Variants in Vivo***

Evidence that alternative splicing actually occurs *in vivo* was initially obtained by exploiting the availability of a large number of *Drosophila* EST sequences, which enabled us to search for the occurrence of CPT1 splice variants post-transcription. The majority of the CPT1 cDNAs identified were from full-length clones, and thus sequence coverage was largely restricted to the 5' ends of cDNAs. However, a single EST sequence (accession number AI402528) revealed a potential splice variant that differed from our previously characterized cDNA (2). This EST was derived from clone GH21765 obtained from a pooled male and female adult head library. The sequence had a region corresponding to 66 bp, present within the cDNA sequence we published previously (2). This region replaced 60 bp of the original clone and had a distinct sequence while retaining the same reading frame. As predicted from the gene sequence analysis, the EST data base indicated that the two variant CPT1 molecules would result from inclusion of *either* exon 6A *or* 6B (Fig. 1A) in mature transcripts, with no evidence of both exons being included simultaneously. Because the exon lengths are both multiples of 3, alternative transcripts would exist containing either exon without the introduction of a frame shift. However, it is also possible that alternative transcripts could exist containing both exons without the introduction of a frame shift. Indeed analysis of the 158 reported ESTs for *Drosophila* CPT1 indicates the existence of four ESTs that encode transcripts bearing both exons and two that contain neither exon. As discussed below, it is highly unlikely that that these (nonalternative) transcripts represent

functional proteins, because the region of the CPT1 protein affected is crucial to the folding around the catalytic site, and the functional protein is unlikely to tolerate its deletion or duplication.

There is also evidence that alternative splicing of the *CPT1* gene gives rise to alternative transcripts in other insect orders outside the fly family (Diptera). BLAST examination of the EST data base for the wasp *N. vitripennis* (Hymenoptera) revealed three clones (GE394463, GE355704, and GE460931) encoding cDNA sequences homologous to exon 6A of *Drosophila* and one sequence (GE450688) corresponding to exon 6B. Both of the ESTs map to the same region of Scaffold 12 of the wasp genomic sequence and are likely to encode alternative exons.

### **Detection and Quantification of CPT1 Splice Variant mRNA Species in Tissues of *Drosophila***

To test directly whether the CPT1 splice variant mRNA are expressed in tissues of *D. melanogaster*, we prepared total RNA from various *Drosophila* tissues of the larval and adult stages. RT-PCR analysis demonstrated that both variants containing either exon 6A or exon 6B are expressed in all of the tissues examined (Fig. 3A). A parallel experiment in which the reverse transcriptase was omitted during synthesis demonstrates that the PCR products were specific and that no genomic contamination was present (Fig. 3A, lower panel). There was also a very minor band that migrated to a position immediately above the amplification product for the 6A-specific sequence. This band was ~60 bp larger than the major product and is likely to represent a transcript that has incorporated both exons into the same molecule. However, this minor transcript may not be translated and is likely to represent an aberrant splicing intermediate.

Having established that both DmCPT1 variants are expressed in *Drosophila* tissues, we analyzed the relative quantities of transcripts containing either exon 6A or 6B. We chose to analyze the relative mRNA contents of the two variants in adult flight muscle and fat body specifically because of the known differences in the metabolism of fatty acids in these two insect tissues; fatty acids (derived from circulating diacylglycerol mobilized from the fat body) are major fuels for ATP formation in flight muscle (15, 16). This analysis clearly demonstrated that although the relative amount of exon 6B-containing transcripts does not differ between these tissues, exon 6A-containing mRNA is present to a significantly greater extent in flight muscle than in the fat body (Fig. 3B). We also analyzed the total CPT1 mRNA content (*i.e.* containing either exon 6A or 6B) of these tissues, and it was found to be similar in flight muscle and fat body (Fig. 3B).

### **Kinetic Properties of the Two Variant Forms of the DmCPT I Protein Expressed in *Pichia***

**Affinities for Substrates**—To determine whether the two DmCPT1 splice variants have different kinetic properties, as might be predicted from the identity of the region of the protein affected by alternative splicing, constructs corresponding to the two variants were individually expressed in the yeast *P. pastoris* (which lacks endogenous CPT activity), and their kinetic characteristics were determined in mitochondria-enriched cell-free extracts prepared from spheroplasts. As can be seen from Table 1, the major difference in their kinetic parameters was in the  $K_{0.5}$  for palmitoyl-CoA ( $K_{0.5} = 104 \pm 8$  and  $520 \pm 53 \mu\text{M}$  for DmCPT1-6A and DmCPT1-6B, respectively). The  $K_{0.5}$  for carnitine was 1.5-fold higher for the DmCPT1-6B variant ( $p=0.016$ ; Table 1) than for the exon 6A variant.

**Inhibition by Malonyl-CoA**—Both variants had low IC<sub>50</sub> values for malonyl-CoA (concentration of malonyl-CoA required to inhibit CPT activity by 50%) relative to human and rat CPT1A, confirming the high sensitivity of the CPT1-6B variant to malonyl-CoA inhibition we reported originally (2). However, whereas at low palmitoyl-CoA concentrations (35  $\mu\text{M}$ ; see “Experimental Procedures”) the IC<sub>50</sub> values for malonyl-CoA were very similar for the two DmCPT1 variants (Table 1), they diverged considerably at higher palmitoyl-CoA concentrations (Table 1). Thus, at 70  $\mu\text{M}$  palmitoyl-CoA, DmCPT1-6A showed a 2.3-fold higher IC<sub>50</sub> value for malonyl-CoA than that for DmCPT1-6B (2.32  $\pm$  0.32 versus 1.01  $\pm$  0.16  $\mu\text{M}$  for CPT I-6A and -6B, respectively;  $p = 0.027$ ). We sought to determine the  $K_i$  values for malonyl-CoA for each of the two variants through the use of secondary Dixon plots constructed from primary plots obtained using increasing concentrations of malonyl-CoA at multiple palmitoyl-CoA

concentrations. We found that this was possible only for DmCPT1–6A ( $K_i = 0.64$  and  $0.70 \mu\text{M}$  malonyl-CoA,  $n=2$ ) as only this variant showed pure competitive inhibition of CPT1 activity by malonyl-CoA with respect to palmitoyl-CoA (data not shown). By contrast, it was not possible to use the same graphical methods to calculate a  $K_i$  value for DmCPT1–6B because this displayed mixed competitive inhibition (not shown). This difference in the kinetic characteristics of the two variants agrees with the marked divergence observed between the respective IC50 values at higher palmitoyl-CoA concentrations.

## DISCUSSION

In the present study we have extended our observation, obtained originally for *Drosophila* (2), that insect genomes contain only one CPT1 gene in contrast to mammalian species in which three genes have been identified (14). In view of the importance of the distinctive kinetic characteristics of mammalian CPT1 isoforms to their function in the various tissues in which they occur (17), we hypothesized that insects may generate functional (kinetic) diversity from their single gene through alternative splicing. Therefore, we searched for potential alternative exons within the *Drosophila* gene and identified a putative exon (located in intron 5) that was an alternative to the known exon 6, starting 7063 bp downstream of the first exon. Importantly, the lengths of the two alternative exons differ by 6 base pairs; this enables in-frame alternative usage. Subsequent analysis of the genomes of five other insects belonging to different orders: *A. gambiae*, *A. aegypti* (mosquitoes), *N. vitripennis* (wasp), *T. castaneum* (beetle), and *A. mellifera* (honey bee), identified very similar arrangements. Thus, all insect CPT1 genes analyzed contain potential alternative exons, even though the genes are organized slightly differently in the different insects.

In the three Diptera genomes analyzed, the alternative exons occur within the region of CPT1 that codes for residues that contribute significantly to the catalytic domain of the enzyme, and they can generate splice variants upon translation. In the honey bee gene, the relevant sequences are located in two separate contigs of the incomplete *Apis* genome and could potentially originate from different genes. However, given the high similarity with the *Drosophila* exons 6A and 6B (Fig. 2), it is highly likely that these sequences represent alternative exons for the *A. mellifera* CPT1 gene.

The existence of homologous exons also within a hymenopteran suggests that this mechanism is widely conserved within the Insecta and that alternative splicing is used generally by insects to generate CPT1 variants from their single gene. EST data base sequences for both *Drosophila* and other insects have confirmed that both variants are expressed *in vivo*.

The present experimental evidence that mRNAs corresponding to 6A and 6B in separate transcripts are present in all *Drosophila* tissues tested (from both larval and adult stages) confirmed the above predictions. The detection of a minor amount of transcript corresponding to mRNA potentially containing both 6A and 6B sequences suggests that this may be an aberrant splicing intermediate that arises as a very minor mRNA species without necessarily being translated. It is very unlikely that if this mRNA were to be translated, the resulting protein would be functional because the same region of the protein encoded by both the 6A and 6B exons is highly critical for enzyme function, and protein folding is unlikely to tolerate the effective duplication of this region in such a critical region of the protein structure.

The alternative exon sequences (of all insect CPT1 genes analyzed) encode a small region of the primary sequence that includes the absolutely conserved catalytic His residue found in all of the members of the choline and carnitine acyltransferase families (His471 in *Drosophila* CPT1; Fig. 2). This residue has been shown to be important for catalytic function in mammalian CPT1A and 1B, carnitine octanoyltransferase, and CPT2. In addition, the two alternative DmCPT1 sequences (corresponding to exons 6A or 6B, respectively) each encode residues known to be important for binding of long chain acyl-CoA substrate or the inhibitor malonyl-CoA (18–22). Thus, alternative splicing specifically alters a small region of the DmCPT I amino acid sequence intimately involved in substrate and inhibitor binding. This suggests that if both variants are expressed *in vivo*, they are likely to have different kinetic characteristics.

The kinetic data we obtained on the carnitine acyltransferase activity resulting when each *Drosophila* variant was separately and heterologously expressed in *Pichia* showed that this was indeed the case. Two major differences were observed, both of which related to acyl-CoA binding (carnitine kinetics were unaffected). The  $K_m$  of the DmCPT1- 6A variant for palmitoyl- CoA was 5-fold lower than that for the 6B variant. This suggests that the DmCPT1–6A variant enzyme would be expected to be more active in the presence of the low levels of unbound long chain acyl-CoA esters that occur physiologically (23). This difference between the two variants is likely to be further pronounced *in vivo* because of the lower sensitivity of the 6A variant to malonyl-CoA when the concentration of palmitoyl-CoA is raised (70  $\mu$ M) toward the  $K_m$  value of this variant (104 +/- 8  $\mu$ M in the presence of albumin, see “Results”). This difference in sensitivity to malonyl-CoA inhibition at higher palmitoyl-CoA concentrations was due to the acyl-CoA substrate being able to overcome competitively the malonyl-CoA inhibition in the case of the 6A variant but not for the 6B variant (which showed noncompetitive inhibition, thus preventing the determination of an absolute value for  $K_i$ ). The overall *in vivo* effect of these differences between the kinetic characteristics of the two proteins is anticipated to be that the CPT1–6A variant is more intrinsically active at physiological concentrations of long chain acyl-CoA and malonyl-CoA, for any comparable level of protein expression of the two variants.

Therefore, the differences in the kinetic properties of the two variants may be physiologically relevant with respect to the different patterns of fatty acid metabolism in different insect tissues. It was significant to note that, whereas the level of total DmCPT1 mRNA transcripts (6A 6B) was very similar between adult fat body and flight muscle, the 6A transcript was much more highly expressed in flight muscle than in the fat body. This preponderance of variant 6A in flight muscle may be related to the much higher capacity for fatty acid oxidation in this tissue required to provide the necessary rate of ATP formation during flight (15, 16). This observation suggests that the unequal transcription of the two variants may be an important mechanism through which differential regulation of fatty acid oxidation in the two tissues is exerted. Therefore, in addition to showing that both variants are expressed in all of the *Drosophila* tissues tested, our data also show that potentially physiologically relevant differences in the alternative transcription of the 6A and 6B exons occur in tissues with different requirements for fatty acid metabolism.

Apart from the above differences, we found that both variants of *Drosophila* CPT1 are highly sensitive to malonyl-CoA inhibition in absolute terms (Fig. 4) (compare Ref. 8). The IC50 values for both variants were comparable with that of the more sensitive of the two mammalian isoforms, CPT1B, when this too was expressed in *Pichia* (8). By contrast, the  $K_{0.5}$  values for palmitoyl-CoA for both variants were higher (~2- and 10-fold higher, respectively) than those for rat CPT1A and CPT1B isoforms when they were expressed and assayed under the same conditions (7). Both variants of *Drosophila* CPT1 are highly sensitive to malonyl-CoA inhibition at 35  $\mu$ M palmitoyl-CoA, which is considerably lower than the  $K_{0.5}$  values of either variant (Fig. 4) (2). At this concentration, the values for both variants were comparable with that of the more sensitive of the two mammalian isoforms, CPT1B, when this too was expressed in *Pichia* (2). However, a direct comparison of the IC50 values between the insect and the mammalian isoforms may not be possible because of the different range of  $K_{0.5}$  values for palmitoyl- CoA in insects and mammals, and the possibility that “free” intracellular long chain acyl-CoA concentrations may be different. By contrast, the two *Drosophila* variants had very similar affinities for L-carnitine, which were intermediate between those of rat CPT1A ( $K_{0.5}$  = 153  $\mu$ M carnitine) and CPT1B ( $K_{0.5}$  = 779  $\mu$ M carnitine) expressed and assayed under the same conditions (7).

Therefore, it would appear that the effects of changing the primary sequence of the CPT1 protein in the region affected by alternative splicing affects the conformation surrounding the acyl-CoA-binding pocket but not the carnitine-binding pocket, which is predicted to be accessed from the opposite aspect of the molecule in mammalian CPT1 molecular models (20). The relationship between malonyl-CoA and palmitoyl-CoA binding is the primary difference between the two DmCPT1 proteins resulting from alternative exon usage in *Drosophila*.



Although predicted by our previous observations (2), the high sensitivity of the enzyme to malonyl-CoA is surprising in view of the observation that the residues involved have since been suggested to play a major role in malonyl-CoA inhibition of mammalian CPT1 isoforms. Thus, the equivalent of His277 has been suggested to be important for malonyl-CoA sensitivity of CPT1 (18), and this is supported from its substitution in the malonyl-CoA-insensitive CPT2 (18). However, in all of the insect CPT1 genes sequenced, His277 is not conserved. Therefore, this raises the prospect that His277 in rat CPT1A is not directly involved in determining the malonyl-CoA sensitivity of the enzyme. In this respect, it is important to note that the effects of the H277A mutation (but not those of H483A) in rat CPT1A were only observed for the protein that was expressed in *Saccharomyces cerevisiae* during the first 1 h of induction of CPT1A expression; protein synthesized 20 h after the induction of gene expression did not show the loss of malonyl-CoA sensitivity associated with the H277A mutation (22).

Other residues within the region affected (*e.g.* equivalent to Ala478 and His483 in the rat enzyme) have been implicated in malonyl-CoA binding based on their conservation in mammalian CPT1A and CPT1B and carnitine octanoyltransferase (which is also a malonyl-CoA-sensitive enzyme) but not in CPT2 or carnitine acetyltransferase, which are malonyl-CoA insensitive (18). A role for these residues in substrate and inhibitor binding has been verified experimentally. Thus, for example, the H483A mutant of rat CPT1A shows a 50% decrease in the affinity for malonyl-CoA (18) and the A478D mutation affected the affinity for palmitoyl-CoA (18). Interestingly, Ala478 is not conserved in the most recently described mammalian isoform, CPT I-C, which has a very low catalytic constant when carnitine and long chain acyl-CoA are used as substrates (14, 24). In all of the insect *CPT1* genes studied, the residues equivalent to His483 and Ala478 in rat CPT1A are both encoded within the alternatively spliced exons (6A/6B) and, importantly, are conserved in both variants generated from each of the insect *CPT1* genes analyzed (Fig. 2) except for the CPT1-6A variants of *A. mellifera* and *N. vitripennis*. Elucidation of the significance of the nonconservation of this residue in these two variants is beyond the scope of the present study.

Structure modeling techniques have been used to generate *in silico* three-dimensional structures of the acyl-CoA binding region and the malonyl-CoA-binding sites of rat and human CPT1A (19–21, 25). The crystal structures for the soluble members of the carnitine acyltransferase family: carnitine acetyltransferase and carnitine octanoyltransferase and the loosely membrane-bound CPT2 (26–34), have been determined, and the structure of carnitine acetyltransferase has been used as a template for remote homology design analysis to refine a previous model for human CPT1A (21). More recently, structural homology modeling of both the N-terminal (using *Xenopus laevis* transcription factor IIIa as structural homologue) and C-terminal segments has been performed (20). This modeling revealed that one of the two malonyl-CoA-binding sites of mammalian CPT1A is predicted to be very close to the interface between the interacting N- and C-terminal segments (17) of the human protein (20). In these latest models the residues mentioned above (Ala478 and His483) are predicted to be very close to the malonyl-CoA molecule in the computed models; Ala478 was proposed as being involved in the formation of both malonyl-CoA-binding sites (O and A) of human CPT1A (20). Similarly (25) suggested that Ala478 in rat CPT1A is located within/near the catalytic site and may participate in the binding of malonyl-CoA at one (with the lower affinity) of the two inhibitor-binding sites. In the present context, it is interesting that the conservation of these residues is accompanied by apparently minor differences in primary sequence between the variants generated by alternative splicing from the single insect CPT1 gene but that the resultant transcripts can generate proteins with the significant differences in kinetic parameters of the translated proteins that were observed in the present study. It will be instructive to discover whether modeling of the *Drosophila* (and other insect) variant sequences can provide additional information about the interaction between LC-acyl-CoA and malonyl-CoA binding to CPT1.

Four further residues previously identified as absolutely conserved in all mammalian CPT1 sequences but substituted in malonyl-CoA-insensitive carnitine acyltransferases and in choline acyltransferase members of the family of proteins (22) namely, Thr314, Asn464, Met593, and Cys608 of rat CPT1A, are also all conserved in the CPT1 genes of all four insects studied.

Finally, based on the existence of cDNAs with alternative 5' ends, we previously postulated that DmCPT1 has two promoters (2). Alignment of these alternative ends with the complete gene sequence that has since been published has now confirmed this. As in the case of the mammalian *CPT1A* (35, 36) and *CPT1B* promoters (37–40), those for DmCPT1 are TATAless. Both promoter regions of the *D. melanogaster* CPT1 contain a number of sequence motifs with a high degree of similarity to known transcription factor-binding sites. Judging by the high content of both exons (1A and 1B) of the ESTs that cover the 5' end of *Drosophila* CPT1, it would appear that both promoters are active *in vivo*. However, exon 1B-containing sequences are almost completely absent from embryo-derived ESTs, whereas most of those containing exon 1B are derived from adult head libraries, suggesting that promoter 2 is not extensively used during embryonic development but is active in the adult.

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## Figure Legends:

**FIGURE 1. Alignment of the nucleotide and amino acid sequences of exons and of splice sites within the *Drosophila* CPT 1 gene that give rise to CPT1 variants through alternative splicing and exonic structures of mammalian and insect CPT1 genes.** A, the sequences of the alternative exons in *Drosophila*. B, schematic diagram depicting the proposed splice sites in transcripts of types 6A and 6B; the numbers of the residues affected (Fig. 2) are indicated. C, comparison of the exonic structures of mammalian and insect CPT1 genes. The exon boundaries are shown for protein coding exons for human liver-type CPT1 (*CPT1A*; top), *D. melanogaster* CPT1 (middle), and *A. gambiae* CPT1 (bottom). The only protein coding regions of exon 12 are shown for *A. gambiae* CPT1. The relative exon sizes are shown only approximately to scale. Noncoding regions of exons are shaded in gray. The alternatively spliced exons are shown in black; conserved boundaries are indicated.

**FIGURE 2. Alignment of the affected predicted protein sequences in insect CPT1 splice variants with the sequences of the corresponding regions of the rat and human CPT1 (isoforms 1A, 1B, and 1C).** The sequences of the alternative exons in the CPT1 gene of *Drosophila* (*Dm*), *A. gambiae* (*Ag*), *A. aegypti* (*Aa*), *N. vitripennis* (*Nv*), and *A. mellifera* (*Am*) are aligned with the sequences of the three CPT1 isoforms (1A, 1B, and 1C) from rat and human (*Hs*). The position of the conserved catalytic His residue (His473 in rat and human) is shown by an asterisk. The conservation of other residues previously suggested by structure modeling to be close to both malonyl-CoA and carnitine in the computed models (Asp477 and Ala478;  $\phi$  and  $\delta$ , respectively) or catalytic activity (His483, #) are also indicated. The exon boundaries in the insect and mammalian sequences are indicated by arrows (above and below, respectively). CPT1 sequences for *Drosophila* were taken from Flybase. *A. gambiae* exon translations correspond to the positions on chromosome 3: 8B 49788552–49788614; 8A 49789247–49789312 and are numbered from annotated protein sequence AGAP010164-PA. *A. aegypti* exon translations correspond to positions on supercontig 157 and are numbered from the predicted protein AAEL005458-PA. *A. mellifera* sequences are derived from two partial gene sequences from genome contigs: Exon A, AmELGUn\_WGA3411\_4; and Exon B, AmELGUn\_WGA5157\_4. *N. vitripennis* sequences are derived from translations of ESTs aligning to Scaffold 12 of the genomic sequence: Exon A GE394463.1 and Exon B GE450688.1 both corresponding to gene LOC100121693 and the predicted protein XP\_001605305. The numbers are amino acids from the start of the protein. These could not be assigned to the *Apis* sequence because the *Apis* genome has not been fully assembled and annotated.

**FIGURE 3. Analysis of the expression of CPT1 splice variant mRNA in tissues of *D. melanogaster*.** A, splice variant mRNA expression in different tissues from *D. melanogaster* was analyzed by RT-PCR using specific primers for either exon 6A or 6B (forward primers) and for exon 7 (reverse primer). A specific band of 277 bp (6A isoform) or 274 bp (6B isoform) is evident in each tissue analyzed. The weak upper band obtained with the primer pair amplifying the exon 6A-containing sequence likely represents a partially spliced transcript including both exons 6A and 6B with a predicted size of 337 bp. B, quantitative PCR analysis of the mRNA of splice variants in adult fat and muscle tissue. A forward primer specific for exon 5 was used in combination with reverse primers specific for either exon 6A (left panel) or 6B (center panel). Total CPT1mRNA was measured with primers specific for exons 4 and 5 (right panel). mRNA contents were normalized to that of the expression of rp49, a ribosomal protein. All of the values are the means (+/- S.D.) expressed relative to the level of transcript found in fat tissue, which is set to 1.0. The asterisk signifies that the difference in variant 6AmRNA expression in flight muscle and fat body is statistically significant ( $p < 0.001$ ; two-tailed  $t$  test).

**FIGURE 4. Malonyl-CoA inhibition and substrate dependence of the activity of DmCPT1–6A and DmCPT1–6B variants expressed in the yeast *P. pastoris*.** The effect of increasing concentrations of

malonyl-CoA on CPT activity (A) of the DmCPT1-6A and DmCPT1-6B variants, measured at 35  $\mu$ M palmitoyl-CoA. In B and C, the velocity *versus* substrate concentrations curves for carnitine and palmitoyl-CoA, respectively, are shown. The values (means  $\pm$  S.E.) for three to five separate, independent yeast preparations are expressed as percentages of the maximal activity computed for the individual preparations. Where *error bars* are not apparent, they lie within the symbols. The data points for the 6A variant are indicated by *filled symbols*; those for 6B are given by *open symbols*.

Figure 1

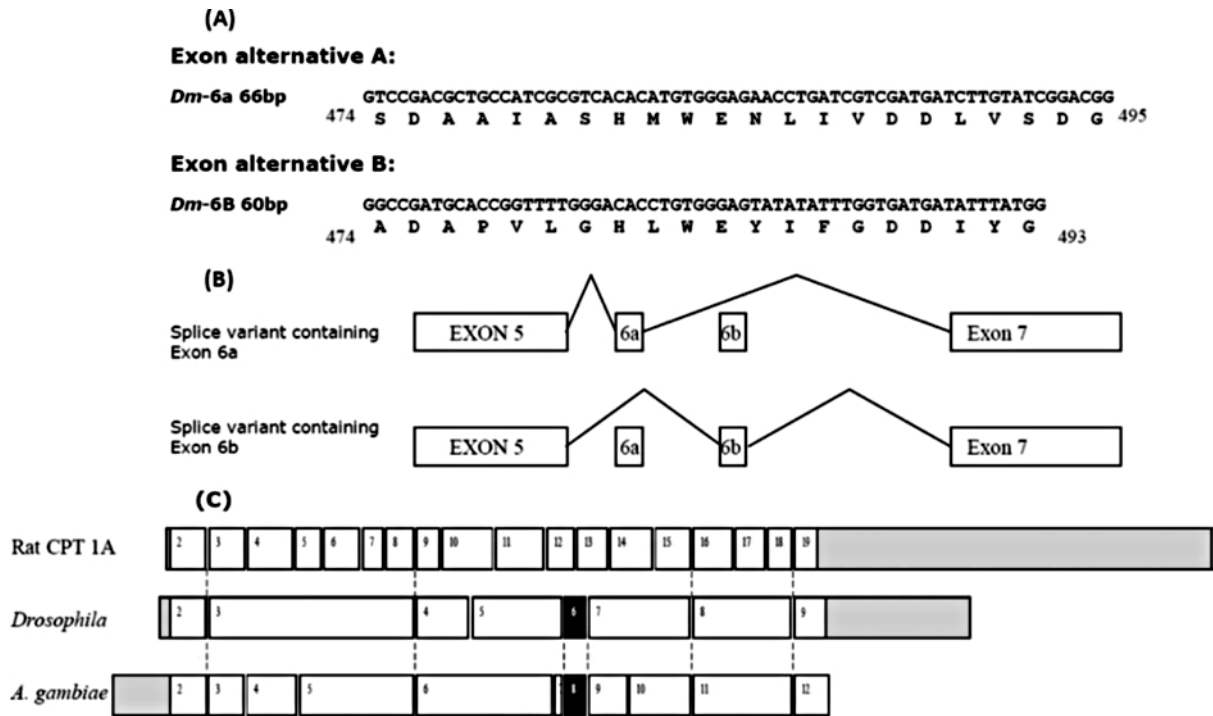


Figure 2

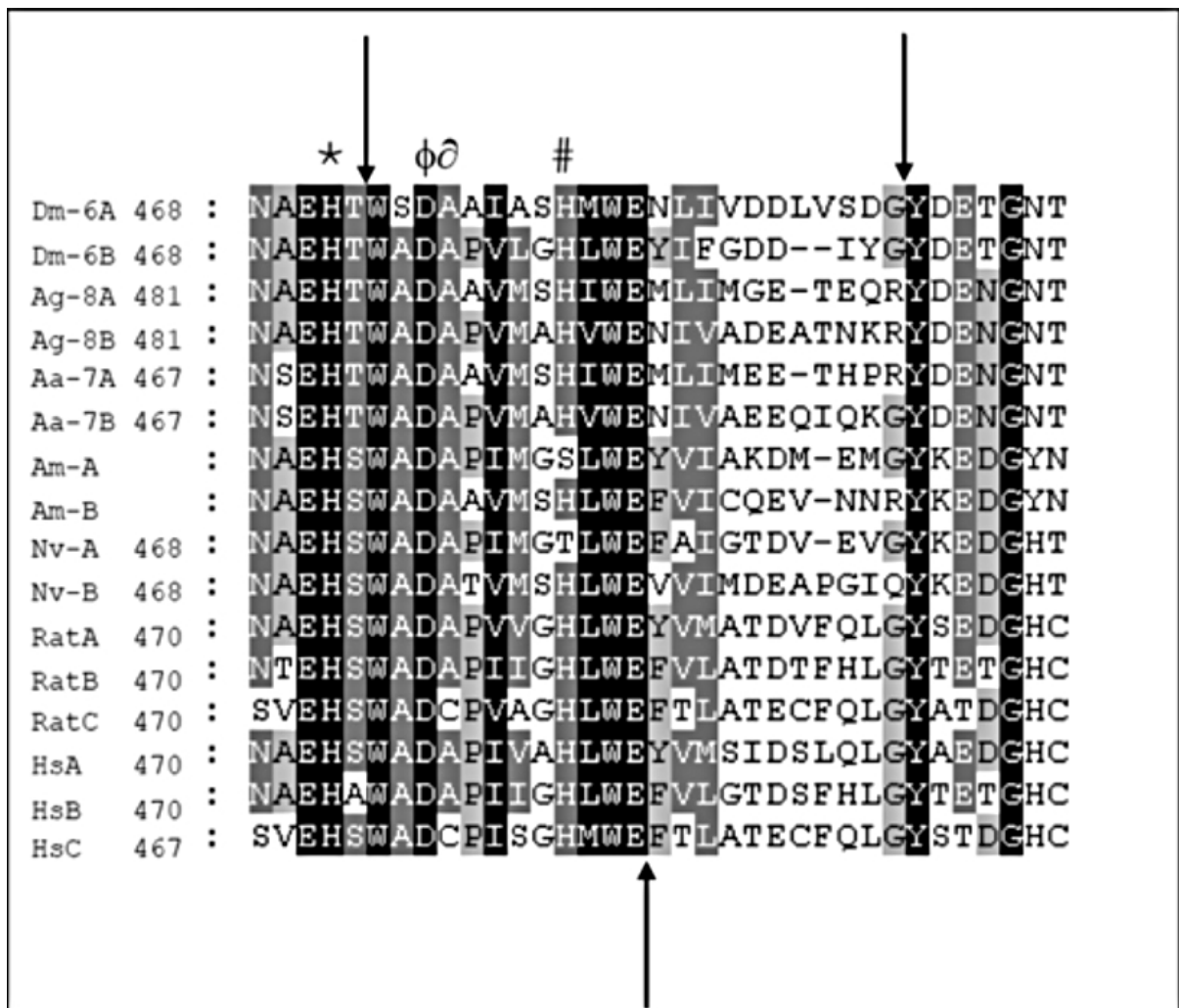


Figure 3

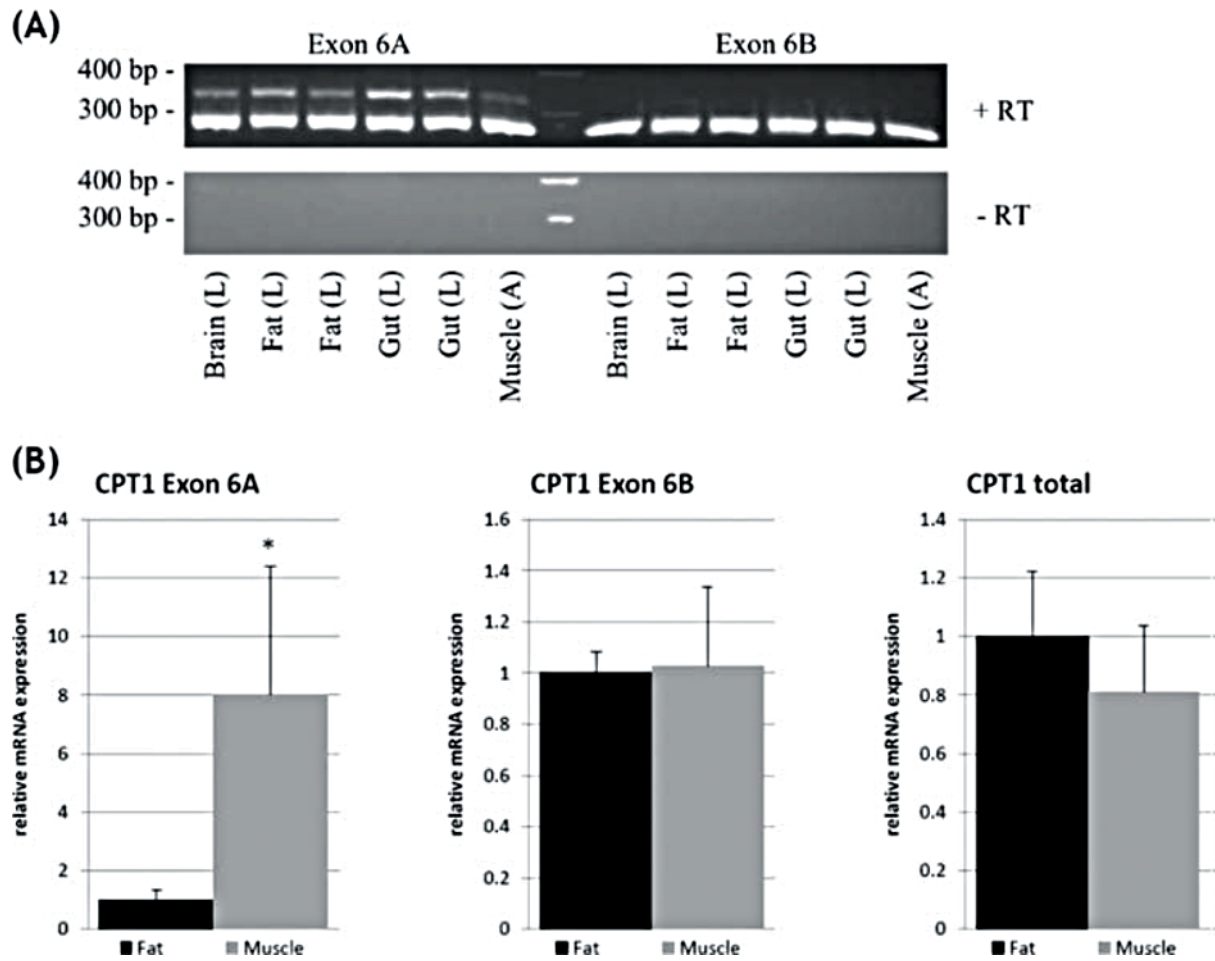
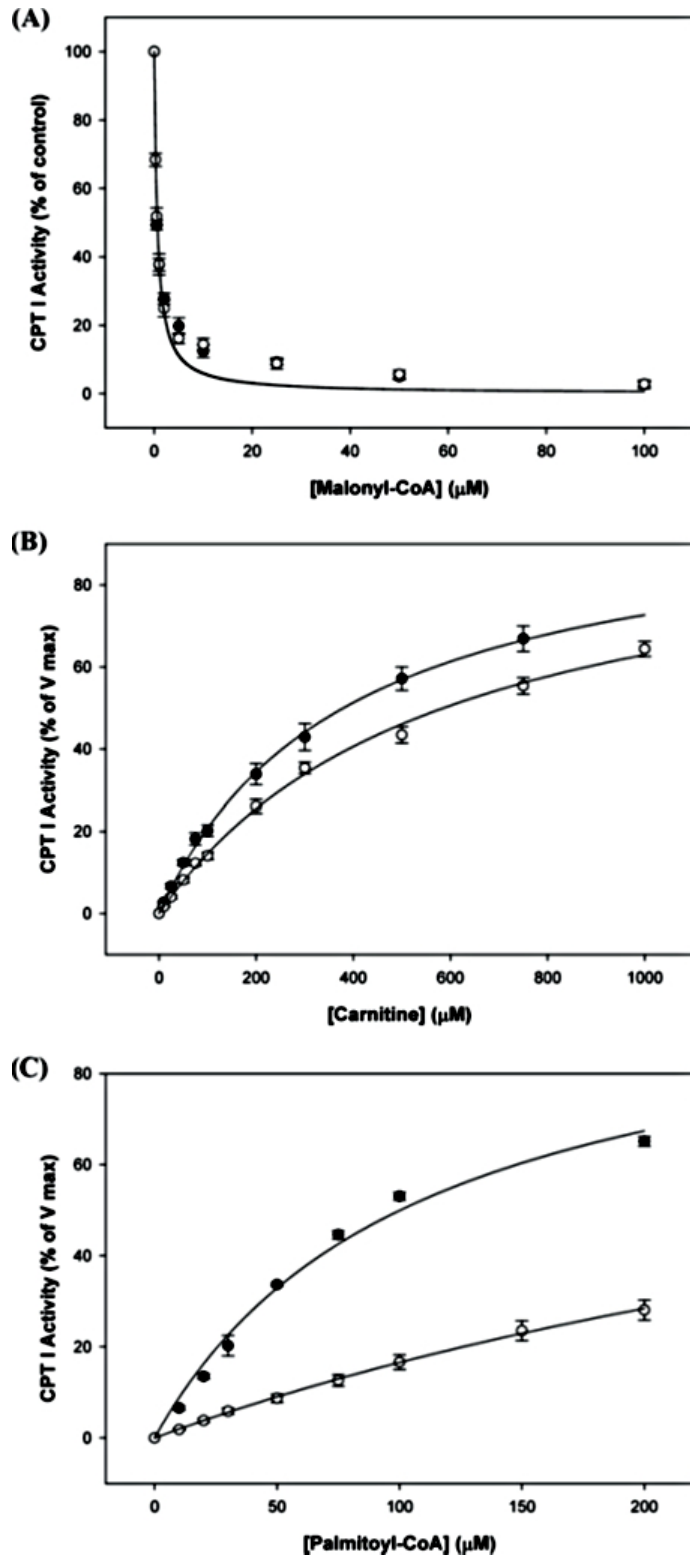




Figure 4



## Table 1

### Comparison of kinetic parameters of DmCPT1–6A and Dm CPT1–6B expressed in *P. pastoris*

DmCPT1–6A and DmCPT1–6B were expressed in *P. pastoris* as described under “Experimental Procedures,” and enzyme activity was measured in cell-free mitochondria enriched extracts. For all of the parameters, the values are the means +/- S.D. for the number of separate preparations indicated.

	$K_{0.5}$		$IC_{50}$	
	Carnitine	Palmitoyl-CoA	Malonyl-CoA with 35 mM palmitoyl-CoA	Malonyl-CoA with 70 mM palmitoyl-CoA
	$\mu M$		$\mu M$	
DmCPT1–6A	406 +/- 30 ( $n = 4$ )	104 +/- 8 ( $n = 4$ ) <sup>a</sup>	0.74 +/- 0.1 ( $n = 3$ )	2.32 +/- 0.32 ( $n = 4$ ) <sup>a</sup>
DmCPT1–6B	591 +/- 46 ( $n = 4$ )	520 +/- 53 ( $n = 4$ )	0.76 +/- 0.1 ( $n = 3$ )	1.01 +/- 0.16 ( $n = 4$ )

<sup>a</sup> These values are statistically significantly different ( $p > 0.01$ ) between the DmCPT1–6A and the DmCPT1–6B variants for the parameter indicated.