MTP (P40939)[i]

Mitochondrial trifunctional protein

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Asterisks before titles (*) indicate junctures at which decisions were made or the kinetic implication of a certain set of data disregarded.

[i] *Note on search: the EC number also gives you references to crotonase activity. The protein number, however (P40939) gives you nice protein-specific literature pertaining to the MTP, which can be used on Uniprot.*

Contents

BACKGROUND

Uchida *et al.* (1) purified and characterised the mitochondrial trifunctional protein (MTP) from rat liver – a membrane-associated protein containing 2-enoyl-CoA hydratase (ECH), 3-hydroxyacyl-CoA dehydrogenase (HACD), and 3-ketothiolase (KACT) activities – and confirmed that it had unique activity, separate from other known mitochondrial proteins. In the same year, Carpenter *et al*. (2) isolated the human MTP from liver. Before this, "it had been thought that multifunctional proteins are only present in peroxisomes and prokaryotic organisms and that the mitochondrial β-oxidation system in higher animals consists of structurally independent and monofunctional protein entities."

MTP is principally active towards medium and long-chain substrates (3), lacking activity towards C4-enoyl-CoA (1,3,4).

STRUCTURE

MTP is an oligopeptide consisting of equimolar amounts of large (79 kDa) and small polypeptides (51 and 49 kDa). The basic composition of such an oligomer is $\alpha_2\beta_2$, but higher oligomers ($\alpha_4\beta_4$, $\alpha_6\beta_6$) are also seen *in vitro* (5). The question remains whether these higher oligomers are physiologically relevant or *in vitro* artefacts, with arguments to both sides having been made (6,7). However, for modelling purposes, this is not so important.

The larger, α-subunit, which contains the ECH and HACD activities (enoyl-CoA to ketoacyl-CoA) is encoded by the HADHA (human ADHA) gene and the smaller β-subunit is encoded by the HADHB gene (human ADHB) which is responsible for the KACT activity (Eaton *et al.* (8) Fig. 1).

"It is also of particular interest that the genes for the α- and β-subunits are adjacent to each other, but head-to-head, on chromosome 2p23, and are probably transcribed from the same bidirectional promoter region; hence expression and induction of the two subunits may be co-ordinated." (9).

The subunits localise at the mitochondrial inner membrane, along with VLCAD and Complex I of the ETC, perhaps indicating a metabolon of long-chain-specific mFAO enzymes (Eaton *et al.* (8)).

SUBCELLULAR LOCALISATION

Binding of this MTP to the membrane was found to be tighter than that of VLCAD since the trifunctional protein could not be solubilized without a detergent (1). Fould *et al.* (6) found – by cosedimentation and surface plasmon resonance analyses – that MTP interacted strongly with cardiolipin and phosphatidylcholine, suggesting a direct reaction of MTP with the inner mitochondrial membrane.

Within a $\alpha_2\beta_2$ polymer, the two β-subunits make a tightly bound homodimer at the centre, while the two α-subunits are bound to each side of the β_2 dimer, creating an arc; on its concave side, this arc binds to the mitochondrial inner membrane (Fig. 4., (5)).

Liang and colleagues (7) also report conserved patches of positively charged residues on the concave surface of the oligomers, and imagine that these could – in conjunction with physical association between cardiolipins and the MTP protein – be useful in binding to the (probably) curved cristae that protrude into the mitochondrion from the inner mitochondrial membrane.

Kispal *et al.* (10) discuss the binding protein responsible for anchoring 3-hydroxyacyl-CoA dehydrogenase (at that point they did not properly discern between MTP and M/SCHAD yet, though this likely refers to MTP, which is membrane associated): they mention that previous authors had not seen intermediate accumulation in mFAO, but that ruptured mitochondria did show the accumulation of intermediates: this suggests strong channelling. What is more, Kispal and colleagues (10) found the binding protein to bind specifically to the hydroxyacyl-CoA dehydrogenase and not to other dehydrogenases, like fumarase, malic dehydrogenase, etc; also, binding to the protein doubled HCAD activity – all of this suggests a highly specific mechanism of membrane-localisation.

* Unexplored kinetic implication: effect of membrane binding

The work of Kispal *et al.* (10) suggests that HACD activity measured in isolated HACD enzymes is a two-fold underestimation of *reverse* HACD activity. Since HACD is the rate-limiting step (see later), this would mean at least a two-fold increase in MTP activity. If MTP activity proves important for the model, please refer back to this point.

FUNCTION

Enoyl-CoA hydratase activity (ECH)

There are several enzymes catalysing the enoyl-CoA hydratase reaction in higher animals:

1. CROT (5);
2. enoyl-CoA hydratase/3-hydroxyacyl- CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (trifunctional protein) (6) are present in mitochondria;
3. enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein (bifunctional protein) (7);
4. medium-chain D-hydroxyacyl-CoA dehydratase is present in peroxisomes (8);
    a. there is also a microsomal isozyme catalysing enoyl-CoA hydration (9), which might be a cognate of the peroxisomal dehydratase;
5. The presence of long-chain enoyl-CoA hydratase (10) was also described, but it has the same properties as MTP (pH optimum around 8.5, highest activity towards C8-enoyl-CoA, no

activity towards C4-enoyl-CoA, membrane associated) and it was described before MTP was known. So, it might be MTP activity. **However,** Jackson *et al.* (11) later report a matrix-associated long-chain enoyl-CoA hydratase enzyme which is distinct from MTP and CROT.

* Unexplored matrix associated long-chain ECH enzyme

Though the reports of long-chain ECH activity in pig heart from Schulz (10) might be ascribed to MTP activity. Jackson *et al.* (11) later report a matrix-associated long-chain enoyl-CoA hydratase enzyme which is distinct from MTP and CROT. They meticulously exclude both MTP (by both subfractionating human liver mitochondria into matrix and membrane fractions, and by assaying cells from severely MTP-deficient patients) and CROT activity (by immunoprecipitation of CROT). The presence of such an enzyme would have implications for the observed kinetics of CROT: in studies where CROT was not explicitly isolated and identified (as in Jiang *et al.* (12)), then the presence of the long-chain ECH might be confounding the data.

Physiological inhibitors of crotonase might also inhibit MTP

Crotonase

Waterson & Hill (18) tested the following compounds for potential inhibition of activation of crotonase: CoA, acetyl-CoA, butyryl-CoA, pantetheine, ATP, ADP, AMP, GTP, UTP, CTP, adenine, adenosine, acetoacetate, ethyl acetoacetate, and crotonate, and found no inhibition.

* Unexplored kinetic implication: Inhibition by acetoacetyl-CoA

Inhibition of crotonase by active-site binding of the enolate tautomer of acetoacetyl-CoA was reported by Waterson & Hill (18) with a K_i = 1.6 µM. Since the enolate tautomer is the active inhibitor and the keto-tautomer isn't (Fig 1. Keto-Enol), the apparent K_i value of acetoacetyl-CoA would be higher (lower affinity).

Waterson & Hill (18) find K_i = 30 µM at pH = 7.5 in bovine liver

Fong & Schulz (19) find K_i = 14 µM at pH = 8.0 in pig heart

Stern ((20), Table I) observed that only 4.9% of acetoacetyl-CoA is in the enolate form at pH = 7.55 while 12.2% is at pH = 8.13. This may explain the differences between the two measurements.

Acetoacetyl-CoA can, hypothetically, enter the active site of MTP's ECH activity: "[T]he N-terminal part of the polyfunctional enzymes shows similarity to mECH and mECI, whereas the C-terminal part aligns to mitochondrial monofunctional 3-hydroxyacyl-CoA dehydrogenase." (21). Therefore, there may be grounds to suspect that MTP is also inhibited by acetoacetyl-CoA competitively binding the active site where enoyl-CoAs would otherwise bind. This remains to be explored, however.

He *et al.* (22): "The matrix concentration of acetoacetyl-CoA may never be high enough to sufficiently inhibit crotonase so that the hydration of enoyl-CoA would become rate limiting in β-oxidation. This is especially true if the hydration of long-chain enoyl-CoAs is catalyzed by long chain enoyl-CoA hydratase and not by crotonase which exhibits little activity toward long-chain substrates."

* Unexplored kinetic effect: slight inhibition by hydroxyacyl-CoA

Jin *et al.* (23) observed the accumulation of long-chain hydroxyacyl-CoA intermediates in isolated mitochondria incubated with fatty acid substrates. Long-chain hydroxyacyl-CoAs also have a very strong competitive K_i with respect to crotonase (0.35 µM for hydroxyhexadecanoyl-CoA).

We can be relatively certain that this strong inhibition is not recapitulated entirely in MTP, as can be seen in Fig 3 by He *et al.* (22). This seems to be a product inhibition of crotonase and not an allosteric effect on ECH activity. MTP is inhibited by 30% of its maximal activity at 7 µM, which is also substantial.

I disregard this for now.

3-Hydroxyacyl-CoA Dehydrogenase (HACD)

3-hydroxyacyl-CoA dehydrogenation is the conversion of 3-hydroxyacyl-CoA to 3-ketoacyl-CoA. Several enzymes catalyse this reaction:

1. 3-hydroxyacyl-CoA dehydrogenase (HADH – what Van Eunen *et al.* (24) refer to as M/SCHAD, or medium/short-chain acyl-CoA dehydrogenase) (25)
2. A short-chain (shorter than C4) 3-hydroxyacyl-CoA dehydrogenase (SCHAD), also known as 3-hydroxy-2-methylacyl-CoA dehydrogenase or 17β-hydroxysteroid dehydrogenase type 10 (HSD10), which acts in leucine metabolism and also the metabolism of steroids and cholic acids (26,27).
3. Mitochondrial trifunctional protein (MTP; (1))
4. Peroxisomal bifunctional protein (PBF; (28))
5. A mitochondrial long-chain 3-hydroxyacyl-CoA dehydrogenase (mLCHAD; (29)) which was reported in bovine and ovine liver, but not in human liver so far.
6. He *et al.* (30) reported the presence of a homotetrameric human short-chain L-3-hydroxyacyl-CoA dehydrogenase with an identical N-termal segment to the bovine mLCHAD (29). This enzyme is identical to an endoplasmic reticulum amyloid β-peptide-binding protein (ERAB) and is not seen in the liver. Whether it is identical or related to HSD10 (26) of mLCHAD (29) is not known.

3-keto-CoA thiolase (KACT)

To our knowledge, only two mitochondrial enzymes catalyse the conversion of 3-ketoacyl-CoAs to acetyl-CoA and shortened acyl-CoAs:

1. Medium-chain 3-ketoacyl thiolase (MCKAT; (31))
2. Mitochondrial trifunctional protein (MTP, containing LCKAT activity – long-chain ketoacyl-CoA thiolase; Uchida *et al.* (1)).

"…comparative studies of the MTP β-subunit with monofunctional 3-ketoacyl-CoA thiolase indicate that three catalytic residues in these two enzymes are positioned similarly in the active sites." (32). This indicates that there should be no major surprises when looking at KACT activity in MTP.

KACT activity was optimal at pH = 9.5 (6)

* Unexplored kinetic implication: KACT substrate inhibition

The KACT activity of MTP is **inhibited by concentrations of its substrate >5 µM (Fig. 8B;** (6)**).** Hypothetically, this might be the beginnings of the detergent effect instead of a dedicated mechanism of substrate inhibition – since the membrane is an integral part of the channelling machinery of MTP (Fig. 5C, (5)), disruption of this structure might lead to disruption of MTP activity.

* Unexplored kinetic implication: KACT substrate inhibition

Levy *et al.* (33) found butyryl-CoA inhibits the ketothiolase function of MTP - this might be a way for β-oxidation to sense substrate overload in a way that is different to product inhibition - though the kinetics of this were not reported

For our modelling purposes, we assume that this effect is an artefact of high free lipid concentrations in the *in vitro* environment and not something which need be accounted for in the model.

* Unexplored kinetic implication: 2-methyl branched-chain fatty acids

Kiema *et al.* (31) mention that MTP is specific for 2-methyl branched-chain fatty acids in the mitochondrion. If these species are eventually added to the model, this activity should be included.

Metabolite channelling within MTP

Xia *et al.* (5) describe how the consecutive metabolites are channelled – mostly by means of electrostatic forces – from one active site to the next. In this process, the metabolites remain within the protein complex, wading along the mitochondrial inner-membrane by insertion of the hydrophobic acyl-group into the bilayer of the membrane. This underscores the importance of membrane association for the proper function of MTP.

[NAD^+]/[NADH]

In the presence of Tween-20, the enzyme appeared to be more sensitive to inhibition by a lower [NAD^+]/[NADH] ratio, whereas MTP in the absence of detergent was only inhibited by a [NAD^+]/[NADH] = 1 or less (8). This suggests that channeling effects brought about by colocalization at the mitochondrial inner membrane could be responsible for the maintenance of a stable flux through MTP and the ETC over a wider range of [NAD^+]/[NADH].

[CoA]/[Acetyl-CoA]

Eaton *et al.* (8) also found that MTP was mostly insensitive to the [CoA]/[acetyl-CoA] ratio at physiological conditions, while a decrease of the ratio to 0.1-0.5 led to an inhibition of the flux, with accumulating ketoacyl-CoAs.

*Unexplored kinetic implication: channelling between enzymes

There are also strong suggestions of metabolite channelling between this and other enzymes of mFAO, which might make a difference to the systemic impact of MTP's kinetics (4,9).

*Unexplored kinetic implication: Multiple NAD-NADH-binding sites

Uchida *et al.* (1) show data that they interpret as indicating that MTP has 4 NADH-binding sites. Though, the kinetic effects are not explored any further and I will therefore disregard this. However, Xu *et al.* (26) found clear evidence of only a single binding site per HACD dimer in *C. elegans*. Though this is a different enzyme and organism entirely, it provides some grounds for skepticism regarding the possibility of multiple binding sites in MTP for NADH/NAD^+.

Lack of 3-enoyl-CoA isomerase activity (ISO)

Middleton (4): "The lack of ISO activity should not prevent oxidation by the MTP of the most abundant unsaturated fatty acids because at least three cycles of oxidation are necessary before this auxiliary enzyme is needed and by then the acids would be shortened to medium chain length and could be acted on by β-oxidation enzymes located in the matrix."

Non-β-oxidation functions

α-subunit acylates cardiolipin

Independently of the subunit beta, the trifunctional enzyme subunit alpha/HADHA also has a monolysocardiolipin acyltransferase activity, which acylates cardiolipins – a major membrane building block (34) – which can affect cellular function in several ways.

** Unexplored kinetic implication: cardiolipin remodelling*

It might be worth considering the effect of cardiolipin remodelling on the behaviour of MTP or of the proposed VLCAD-MTP-ETC metabolon (9), as MTP does interact directly with cardiolipins in the membrane, and a change on their structure might have implications for the functioning of membrane-associated enzymes, or for the channelling behaviour between the enzymes.

α-subunit binds gastrin in cytosol

This was reported by Baldwin & Shulkes (35).

DISEASE

“Detailed studies suggest that MTP deficiency can be classified into two groups. In patients in group 1, α- and β-subunits are of a normal size and are present at the level found in the controls, and only the 3-hydroxyacyl-CoA dehydrogenase activity is deficient. In patients in group 2, trace amounts of the enzyme complex proteins are present, and all three activities are deficient.” (36).

MEASUREMENT CONDITIONS

Different reaction, different parameters

Vmax

The MTP multi-protein complex catalyses three reactions: ECH, HACD, and KACT. The intermediate metabolites of these reactions are channelled directly from one catalytic site to the next, allowing us to consider these reactions as a single step instead of as three separate reactions (see Van Eunen *et al.* (24)). The separate reactions, however, have different maximal capacities: Uchida *et al.* (1) found ECH activity of about 100 times *reverse* HACD activity and *reverse* HACD activity of about 3 times KACT activity. **Importantly,** HACD activity is seldom measured in the forward direction, since the reverse direction is thermodynamically preferred. From Middleton (4), who measured – on purified MTP – ECH, HACD, and KACT activity, forward HACD reaction is about 10 times slower than reverse HACD reaction.

Spiekerkötter *et al.* (37) also showed *reverse* HACD activity to be roughly 2-fold KACT activity in human fibroblasts and Carpenter *et al.* (2) also saw different specific activities for ECH which was about 10 times KACT activity.

ECH activity therefore seems to be the highest. Part of this phenomenon might be explained by the fact that the hydroxyacyl-CoA or ketoacyl-CoA metabolites have to enter the MTP "pipeline" further downstream, which might cause some steric hindrance that slows down the reaction when only an intermediate metabolite is provided. Part of it might simply be due to the inherent catalytic capacity of the enzyme. Since hydroxyacyl-CoA and ketoacyl-CoA can get into the catalytic machinery of MTP at all, and a substantial amount of the substrate channelling within MTP is by way of "surface crawling" in the membrane and not just intra-enzyme movement (5), we can assume that what we are seeing is off, but not too far off. Therefore, the fact that ECH activity is an order of magnitude larger than HACD activity, and HACD activity is an order of magnitude larger than KACT activity (Fig. 1, Kamijo *et al.* (3)) might be true reflections of the catalytic capacity of the enzyme for the different reactions.

KACT activity appears to be second highest, and HACD activity lowest (4).

So, how do we choose the right V_{max} for our enzyme?

Middleton (4) assayed the forward activity of each reaction individually on purified MTP, and found some substantial differences (Table 1, (4)).

Of the forward activities, HACD activity is the lowest, and the combined activity of the whole enzyme is equal to the HACD activity. **This suggests HACD as the rate-limiting activity in MTP**. Biochemically, it would mean that substrate flow through KACT is restricted by HACD, which makes KACT take place only as quickly as HACD allows, and for ECH it means that its activity is inhibited by the accumulation of product. Indeed, it is important to note that product accumulation exerts inhibition on the various activities and not some different effect, like the premature release of intermediates. Fould *et al.* (6) showed that the inactivation of the HACD or KACT led to equilibrium in the upstream enzymes, indicating feedback inhibition. When transfecting mammalian cells with the α- and then the β-subunit in a piecemeal way, Kamijo *et al*. (3) found HACD and KACT activity to be almost equal after

transfection with both subunits, while ECH activity is about 50% higher. This lends more credence to the idea that HACD and KACT activity could be inhibiting to the activity of ECH in the complex.

Uchida *et al.* (1) performed two HACD assays – starting from enoyl-CoA as substrate – first an assay pretreated with enoyl-CoA hydratase and second without, and in both cases saw similar reactions rates and similar substrate specificities – which were different from the rates and specificities seen if only ECH activity is measured. This suggests that HACD is the most important controlling factor with respect to the combined ECH-HACD rate: again, this reinforces the rate-limiting nature of HACD activity

** STRONG modelling decision: HACD is rate-limiting*

So, HACD can be considered rate limiting in the enzyme, and its forward V_{max} can be assumed as the V_{max} of the enzyme. Since the most reliable V_{max} values for HACD activity were measured in the reverse direction, you can adjust them by dividing them with 10, since Middleton (4) found human liver mitochondrial MTP's reverse HACD activity to be roughly 10 times smaller than its forward activity.

Specificities

Different specificities characterize the different reactions of the MTP (ECH, HACD, KACT) both in rat and in human (1,3,38). For instance, Kamijo *et al.* (3) report the individual activities of each of the reactions in purified human MTP. As discussed earlier, there is no way to know that steric obstruction for getting to the appropriate catalytic site (in the case of HACD and KACT activity) or downstream feedback inhibition (in the case of ECH and HACD) isn't twisting the specificities seen in these assays.

However, since HACD is considered the rate-limiting step for the forward reaction, it seems likely that its substrate specificities would also be determinant for the substrate specificity of the enzyme as a whole. From Kobayashi *et al.* (29), we know that the substrate specificities of the forward and the reverse reactions are not necessarily the same.

** STRONG modelling decision: only forward specificity should be used*

Since the specificity factors that we are using are applied to a forward V_{max}, we will penalise reverse specificies

** Unexplored kinetic implication: different forward and reverse specificities*

Perhaps there lie some other assumptions in direction-specific substrate-specificity as reported by Kobayashi *et al.* (29), i.e. that equilibrium constants and K_m values should also adapt to changes in assay direction, but I will not explore this any more deeply for now.

pH

Uchida *et al.* (1) and Eaton *et al.* (8) showed that the % HACD activity seen *in vitro* with MTP can change with varying pH (Fig. 11, (1)). Increasing the pH from 7 to 8 can lower the rat liver HACD activity by about 50% in MTP. In contrast, human KACT activity has an optimum at pH = 9.5 (6). This strongly suggests that MTP should be treated with care, as it has different moving parts that respond differently to changing assay conditions.

What is more, this effect is different for different chain-lengths of substrate, meaning that the specificity of the enzyme for its substrates will also change with changing pH.

** Modelling decision: punish measurements outside 7.5 < pH < 8.5*

It would therefore be advisable to keep a narrow range of pH values when selecting parameters, for which **we will try to keep to between pH = 7.5 and pH = 8.5.**

Detergents

Uchida *et al.* (1) showed different %activities in the presence *versus* absence of Tween-20, also in a dose-dependent way: as much as 2-fold increase in reverse HACD activity in 0.1% of Tween-20.

Eaton *et al.* (8) also found the KACT activity to be stimulated by 726% by 0.3% Tween-20, while 3-hydroxyacyl-CoA dehydrogenase activity was not affected (reverse direction) and 2-enoyl-CoA hydratase activity was inhibited by 36%. In the presence of Tween-20, the enzyme also appeared to be more sensitive to inhibition by a lower $[NAD^+]/[NADH]$ ratio, whereas MTP in the absence of detergent was only inhibited by a $[NAD^+]/[NADH] = 1$ or less.

Since the membrane is an integral part of the channelling machinery of MTP (Fig. 5C, (5)), disruption of this structure might lead to disruption of MTP activity

** Modelling decision: penalise detergents*

Try to select parameters that were measured in the absence of detergents.

BSA

El-Fakhri and Middleton (39) report having seen stimulation of reverse HACD activity in the presence of BSA, with a peak at 0.3 mg/mL when assaying rat liver mitochondria for their activity towards palmitoyl-CoA; above 0.6 mg/mL the effect became inhibitory (Fig. 1, El-Fakhri and Middleton (39). Inhibition of long-chain ECH activity was also seen by Schulz (15), and this was abrogated by adding BSA. This effect was not seen in either source when the substrate was acetoacetyl-CoA, perhaps suggesting that it has something to do with disrupting micelle formation of longer chain fatty acids at low concentrations and oversequestering them at high concentrations, as was seen for CPT1 (40,41).

* Modelling decision: penalise BSA

Ionic strength

KACT activity in recombinant human MTP was found to be responsive to changes in ionic strength (6). El-Fakhri & Middleton (39) found the membrane-bound long-chain hydroxyacyl-CoA dehydrogenase to be solubilized by high salt concentrations. This indicates that MTP might lose some function by detaching from the membrane at high ionic strengths.

** Modelling decision: you can disregard small differences in buffer composition*

Since KACT activity is not the rate-limiting activity, this need not be a very strong consideration.

Labile in overnight storage

"The enzyme in the mitochondrial extract was labile, and the activity decreased by half or less when kept overnight at 4°C," Uchida *et al.* (1). Though they do not indicate explicitly any kinetic consequences of this, future researchers might explore this more complex kinetics of confirmed.

Tissue-specificity

"The presence of the trifunctional protein in various tissues was also confirmed by immunoblot analysis," suggesting that immunochemically identical proteins are present throughout the body's tissues (1).

El-Fakhri & Middleton (39) investigated the distribution of long-chain and short-chain hydroxyacyl-CoA dehydrogenase activity in rat tissues, and found almost identical distribution between the liver and the heart, whereas in the brain and kidney there was less of both activities. This constituted nice evidence that heart and liver parameters are interchangeable when looking at HCAD activity.

** STRONG modelling decision: accept all tissues, so long as they are protein-corrected*

Difference across species

Kobayashi *et al.* (29)'s novel long-chain HADH enzyme (28 kDa), was found only in horse, bovine, and sheep livers, with only weak signals in rat and dog livers, and no signal in human, rabbit, and pig livers. Kobayashi *et al.* (29) themselves mention that the presence of this second HACD enzyme suggests different substrate specificities in mFAO for bovine, rat, and human livers.

Though this does not pertain specifically to MTP, it does suggest that substrate specificity might be a characteristic of a species and not a ubiquitous feature of an enzyme that catalyses HACD.

** Modelling decision: penalise non-human sources*

Confounding effect of matrix-associated long-chain ECH

Jackson *et al.* (16) later report a matrix-associated long-chain enoyl-CoA hydratase enzyme which is distinct from MTP and Crotonase. They meticulously exclude both MTP (by both subfractionating human liver mitochondria into matrix and membrane fractions and by assaying cells from MTP-deficient patients) and crotonase activity (by immunoprecipitation of Crotonase). The presence of such an enzyme would have implications for the observed kinetics of crotonase: if crotonase was not explicitly isolated and detected (as in Jiang *et al.* (13)), then the presence of the long-chain ECH might be confounding the data.

* STRONG modelling decision

Only K_m values for enoyl-CoA for which MTP is isolated may be used, as there exists a real risk in homogenate that the effect of this possible third ECH enzyme could be confounding the data.

KINETICS

Specificity

Middleton (4): rat and human liver, pig heart MTP: all show highest activity towards C10-enoyl-CoA and longer, with lower activity towards C6-ketoacyl-CoA and zero affinity for C4-acyl-CoA (1–4).

Reaction mechanism

Since MTP catalyses three consecutive reactions, it is technically incorrect to speak about a single "reaction mechanism".

ECH

Enoyl-CoAs will enter the enzyme complex first, as they require no co-substrates.

HACD

We did not encounter any evidence suggesting an ordered binding mechanism for the HACD activity of MTP. However, according to Uchida *et al.* (1), their "data suggest that 1 mol of enzyme has four NADH-binding sites." Xu *et al.* (26) found clear evidence of only a single binding site per HACD dimer in *C. elegans*. Though this is a different enzyme and organism entirely, it provides some grounds for skepticism regarding the possibility of multiple binding sites in MTP for NADH/NAD^+.

**Arbitrary modelling decision: one NADH/NAD^+ site*

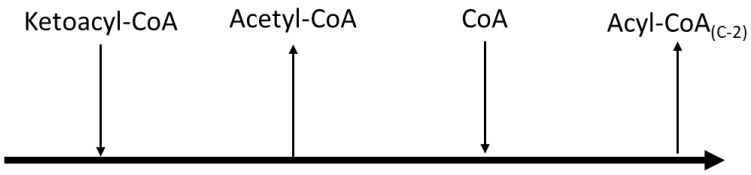
For simplicity, and as indicated by Uchida *et al.* (1), both NADH and NAD^+ will be given a single affinity for the enzyme.

** Unexplored kinetic implication: cooperative NADH/NAD^+ binding?*

The effect of multiple NAD-binding sites might be that the kinetics of NADH binding are more complicated than a single K_m value for both NAD^+ and NADH.

KACT

Liu *et al.* (32)'s observation that the "three catalytic residues" in the active sites of MCKAT and MTP's KACT activity suggests that the same mechanism should be valid for both. Miyazawa *et al.* (42) report a mechanism of ketoacyl-CoA thiolysis where an acetyl-CoA is first cleaved off the acyl-CoA substrate, after which a CoA enters the active site and binds to the shortened acyl-CoA. For this reason, they also report different K_m values for the CoA substrate of the reaction, depending on the ketoacyl-CoA substrate of which the acyl-group will still be occupying the active site:



** Arbitrary modelling decision: use MCKAT K_m for CoA*

K_m values for CoA that depend on the chain-length of the substrate are not available for MTP. However, according to Liu *et al.* (32)'s report of the similarity between the KACT active site in MTP and MCKAT, I will simply assume that the the K_m values of MCKAT for CoA are also applicable to MTP (42).

** Arbitrary modelling decision: use MCKAT K_m for AcetylCoA*

Assume K_m value to be the same as for MCKAT based on active site similarity (32).

** Arbitrary modelling decision: Haldane relation for only the KACT reaction to get K_m for AcylCoA*

Calculate Km values for the acyl-CoA products using the Haldane equation. I can assume some chain-length specific Km values for CoA and Acetyl-CoA from Miyazawa et al. (42) and Kiema et al. (31), respectively, based on active site similarity between MCKAT and MTP's KACT active site. I can also get K_m values and forward Vmaxes for the C4, C12 and C16 ketoacyl-CoA substrates in rat MTP (32) and equilibrium constants from eQuilibrator. Assume the reverse V_{max} to be in the same ratio to the forward V_{max} as the ratios of the thiolytic and synthetic k_{cat} values in Kiema *et al.* (31): 1.4 s^{-1} / 14.8 s^{-1} = 0.095.

If all of this is put together and **random bi-bi Michaelis-Menten kinetics are assumed**, I can calculate K_m values for the acyl-CoA products of MTP. Although these values are probably not purely a function of KACT activity and will be influenced by substrate stimulation spatial effects of being in the MTP protein complex, my assumption will be that this is the reductionist path to take to estimate chain-length specific acyl-CoAs. The values for C16 and C14 will be the same, the values for C10 and C12, and the values for C4 will be used for C6 and C8. MTP as a whole has no activity toward C4, probably due to the ECH and HACD active sites. The grouping is based on Middletons (4)'s observation that MTP has high affinity for C10 and longer chain substrates, with low affinity for C6, and zero for C4. This suggests that there is some clustering in the behaviour of the enzyme towards substrates of C10 and longer, and that those that are shorter can be clustered.

* Calculation

Haldane relation:

$$K_{m,MTP,C_{n-2}AcylCoA} = K_{eq,MTP} \cdot \frac{V_{MTP,r}}{V_{MTP,f}} \cdot \frac{K_{m,MTP,C_nKetoacylCoA} \cdot K_{m,MTP,C_nCoA}}{K_{m,MTP,AcetylCoA}}$$

C16 $K_{M,ACYL\text{-}COA(N\text{-}2)}$

PARAMETER	Value	Reference
$V_{MAX,F}$	151 µmol.min^{-1}.mg-$protein^{-1}$	From Liu *et al.* (32)
$V_{MAX,R}$	14 µmol.min^{-1}.mg-$protein^{-1}$	$V_{max,f}$ converted to $V_{max,r}$ using the ratio in Kiema *et al.* (31): x0.095
K_{EQ}	249 000	Estimated using eQuilibrator ((43); Ionic strength = 0.125 mM, pH = 8.0, temperature correction estimate using Van 't Hoff relation)
$K_{M,ACETYLCOA}$	250 µM	Assume MCKAT value from Kiema et al. (31)

$K_{M,COA}$	28.6 µM	Assume MCKAT value from Miyazawa *et al.* (42).
$K_{M,KETOACYL\text{-}COA}$	51 µM	Liu *et al.* (32)
$K_{M,ACYL\text{-}COA(N\text{-}2)}$	**12 4881 µM**	

C14 $K_{M,ACYL\text{-}COA(N\text{-}2)}$

PARAMETER	Value	Reference
$V_{MAX,F}$	151 µmol.min^{-1}.mg-protein^{-1}	From Liu *et al.* (32) (*assumed same as C16*)
$V_{MAX,R}$	14 µmol.min^{-1}.mg-protein^{-1}	$V_{max,f}$ converted to $V_{max,r}$ using the ratio in Kiema *et al.* (31): x0.095
K_{EQ}	249 000	Estimated using eQuilibrator ((43); Ionic strength = 0.125 mM, pH = 8.0, temperature correction estimate using Van 't Hoff relation)
$K_{M,ACETYLCOA}$	250 µM	Assume MCKAT value from Kiema et al. (31)
$K_{M,COA}$	33.5 µM	Assume MCKAT value from intrapolation of Miyazawa *et al.* (42).
$K_{M,KETOACYL\text{-}COA}$	51 µM	Liu *et al.* (32) *(assumed same as C16*)
$K_{M,ACYL\text{-}COA(N\text{-}2)}$	**14 6277 µM**	

C12 $K_{M,ACYL\text{-}COA(N\text{-}2)}$

PARAMETER	Value	Reference
$V_{MAX,F}$	98 µmol.min^{-1}.mg-protein^{-1}	From Liu *et al.* (32)
$V_{MAX,R}$	9.31 µmol.min^{-1}.mg-protein^{-1}	$V_{max,f}$ converted to $V_{max,r}$ using the ratio in Kiema *et al.* (31): x0.095
K_{EQ}	249 000	Estimated using eQuilibrator ((43); Ionic strength = 0.125 mM, pH = 8.0, temperature correction estimate using Van 't Hoff relation)
$K_{M,ACETYLCOA}$	250 µM	Assume MCKAT value from Kiema et al. (31)
$K_{M,COA}$	38.4 µM	Assume MCKAT value from Miyazawa *et al.* (42).
$K_{M,KETOACYL\text{-}COA}$	58 µM	Liu *et al.* (32)
$K_{M,ACYL\text{-}COA(N\text{-}2)}$	**20 0201 µM**	

C10 $K_{M,ACYL-COA(N-2)}$

PARAMETER	Value	Reference
$V_{MAX,F}$	98 µmol.min^{-1}.mg-protein^{-1}	From Liu *et al.* (32) *(assumed same as C12*)
$V_{MAX,R}$	9.31 µmol.min^{-1}.mg-protein^{-1}	$V_{max,f}$ converted to $V_{max,r}$ using the ratio in Kiema *et al.* (31): x0.095
K_{EQ}	249 000	Estimated using eQuilibrator ((43); Ionic strength = 0.125 mM, pH = 8.0, temperature correction estimate using Van 't Hoff relation)
$K_{M,ACETYLCOA}$	250 µM	Assume MCKAT value from Kiema et al. (31)
$K_{M,COA}$	37.5 µM	Assume MCKAT value from Miyazawa *et al.* (42).
$K_{M,KETOACYL-COA}$	58 µM	Liu *et al.* (32) *(assumed same as C12*)
$K_{M,ACYL-COA(N-2)}$	**19 5509 µM**	

C8 $K_{M,ACYL-COA(N-2)}$

PARAMETER	Value	Reference
$V_{MAX,F}$	51 µmol.min^{-1}.mg-protein^{-1}	From Liu *et al.* (32) *(assumed same as C4*)
$V_{MAX,R}$	4.85 µmol.min^{-1}.mg-protein^{-1}	$V_{max,f}$ converted to $V_{max,r}$ using the ratio in Kiema *et al.* (31): x0.095
K_{EQ}	249 000	Estimated using eQuilibrator ((43); Ionic strength = 0.125 mM, pH = 8.0, temperature correction estimate using Van 't Hoff relation)
$K_{M,ACETYLCOA}$	250 µM	Assume MCKAT value from Kiema et al. (31)
$K_{M,COA}$	35.5 µM	Assume MCKAT value from Miyazawa *et al.* (42).
$K_{M,KETOACYL-COA}$	71 µM	Liu *et al.* (32) *(assumed same as C4*)
$K_{M,ACYL-COA(N-2)}$	**22 7033 µM**	

C6 $K_{M,ACYL-COA(N-2)}$

PARAMETER	Value	Reference
$V_{MAX,F}$	51 µmol.min^{-1}.mg-protein^{-1}	From Liu *et al.* (32) *(assumed same as C4*)
$V_{MAX,R}$	4.85 µmol.min^{-1}.mg-protein^{-1}	$V_{max,f}$ converted to $V_{max,r}$ using the ratio in Kiema *et al.* (31): x0.095
K_{EQ}	699 000	Estimated using eQuilibrator ((43); Ionic strength = 0.125 mM, pH = 8.0, temperature correction estimate using Van 't Hoff relation)
$K_{M,ACETYLCOA}$	250 µM	Assume MCKAT value from Kiema et al. (31).
$K_{M,COA}$	18.9 µM	Assume MCKAT value from Miyazawa *et al.* (42).
$K_{M,KETOACYL-COA}$	71 µM	Liu *et al.* (32) *(assumed same as C4*)
$K_{M,ACYL-COA(N-2)}$	**33 9313 µM**	

** Arbitrary modelling decision: whole-complex reversibility*

Note that modelling the reaction as a random-order Tri-Tri reversible Michaelis-Menten equation, implicitly assumes that the entire MTP reaction is reversible. Considering the channelling dynamics of the protein complex, this is questionable. Future research might shed some light on this matter.

** STRONG modelling decision: AcetylCoA and CoA compete*

Note that modelling the reaction as a random-order Tri-Tri reversible Michaelis-Menten equation, implicitly assumes that CoA and acetyl-CoA compete for a binding site. For the soluble ketoacyl-CoA thiolase, both a synthetic and a degradative reaction are seen, and since acetyl-CoA and CoA occupy more or less the same binding site, this is a justifiable assumption (31).

** Arbitrary modelling decision: acyl-CoA and enoyl-CoA compete*

Note that modelling the reaction as a random-order Tri-Tri reversible Michaelis-Menten equation, implicitly assumes that the enoyl-CoA and the two-carbons-shorter acyl-CoA compete for binding to the enzyme. This is probably not the case, since the acyl-CoA interacts with a completely different part of the enzyme complex that enoyl-CoA. The justification for this might be that the "compete" insofar as having downstream acyl-CoA bound to the KACT site due to overabundance of acyl-CoA might inhibit the rate of enoyl-CoA conversion as a form of product inhibition, which has been observed (6). However, this is surely not mechanistically equivalent to competitive inhibition.

Rate equation

For the reaction as a whole, I will assume a random order Tri-Tri reaction mechanism, based on the argument of Rohwer *et al.* (44) that a random order Bi-Bi reversible Michaelis-Menten can be used to described most Bi-Bi reactions. The rate equation will therefore be indistinguishable from what was used by Van Eunen *et al.* (24).

For n = {6, 8, 10, 12, 14, 16}:

$$vmtpC_n = \frac{sfmtpC_n \cdot \frac{Vmtp}{VMAT} \cdot \left(\frac{C_nEnoylCoAMAT[t] \cdot NADMAT \cdot CoAMAT[t]}{K_mmtpC_nEnoylCoAMAT \cdot K_mmtpNADMAT \cdot K_mmtpCoAMAT} - \frac{AcetylCoAMAT \cdot C_{n-2}AcylCoAMAT[t] \cdot NADHMAT}{K_{eq}mtpC_n \cdot K_mmtpC_nEnoylCoAMAT \cdot K_mmtpNADMAT \cdot K_mmtpCoAMAT} \right)}{(1 + \frac{AcetylCoAMAT[t]}{K_mmtpAcetylCoAMAT} + \frac{CoAMAT[t]}{K_mmtpC_nCoAMAT}) \cdot (1 + \frac{NADMAT}{K_mmtpNADMAT} + \frac{NADHMAT}{K_mmtpNADHMAT}) \cdot (1 + \sum_{n=6}^{n=16} (\frac{C_nEnoylCoAMAT[t]}{K_mmtpC_nEnoylCoAMAT} + \frac{C_{n-2}AcylCoAMAT[t]}{K_mmtpC_{n-2}AcylCoAMAT}))}$$

where:

$$NADMAT = \left(\frac{NAD_tMAT}{\frac{NAD^+}{NADH} + 1}\right) \cdot \frac{NAD^+}{NADH}$$

$$NADHMAT = \left(\frac{NAD_tMAT}{\frac{NAD^+}{NADH} + 1}\right)$$

Variables == initial values

Enoyl-CoAs	Acylcarnitines	AcetylCoA
C16EnoylCoAMAT[t] == 0 µM		AcetylCoA[t] == 700 µM
C14EnoylCoAMAT[t] == 0 µM	C14AcylCoAMAT[t] == 0 µM	
C12EnoylCoAMAT[t] == 0 µM	C12AcylCoAMAT [t] == 0 µM	
C10EnoylCoAMAT[t] == 0 µM	C10AcylCoAMAT [t] == 0 µM	
C8EnoylCoAMAT[t] == 0 µM	C8AcylCoAMAT [t] == 0 µM	
C6EnoylCoAMAT[t] == 0 µM	C6AcylCoAMAT [t] == 0 µM	

Parameters

Weighting rule

I give the parameters weights based on my subjective evaluation. There will be four categories.

1 = credible measurement

0.9 = just short of perfect (e.g. wrong tissue and had to be adjusted, 30°C instead of 37°C)

0.5 = uncertain

0.1 = “I probably wouldn’t choose this if I had another option”

Using the weights, I will reduce the impact of poor measurements.

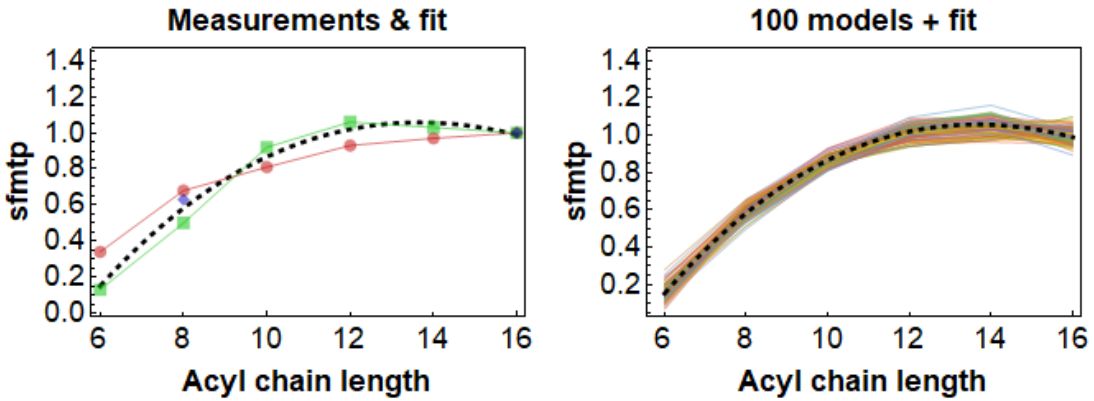
Weights are given in curly brackets next to parameter values: {} with short reasons

sfmtp

Satisfactory values found

Parameter		Chosen value [range]	Alternatives		Comments
sfmtp		Kamijo et al. (1994, (3)) Purified human liver MTP, reverse HACD activity, 50 mM KPi, pH = 7.5, 0.1 mM NADH, 25 uM ketoacyl-CoA, 1 mM acetoacetyl pantetheine, no temp given; for C14, C10, and C6, the means of the two surrounding values were chosen as intrapolation technique.	Uchida et al. (1992, (1)) rat liver mitochondrial extract, forward HACD activity, 30°C, pH = 7.5, 100 mM Tris, 100 mM KCl, enoyl-CoA as substrate, not preincubated with ECH, 200uM NAD	Liu et al. (2008, (32)) recombinant rat MTP, reverse HACD activity, 100 mM KPi, pH = 6.5, 0.1 mg/mL BSA, 0.1 mM DTT, 40 uM ketoacyl-CoA, 100 uM NADH, 37°C	1) Different reactions of the MTP (ECH, HACD, KACT), both in rat and in human, have different substrate specificities (1,3,38). Fould et al. (6) also showed substrate inhibition *within* MTP. Therefore, forward specificity of the rate-limiting step seems apt for our model, as it modifies a forward Vmax. We repeatedly saw forward HACD activity to be the lowest (1–3,37) - and considering Middleton (4)'s finding that HACD activity is rate-limiting - we assume hydroxyacyl-CoA dehydrogenase (HACD) activity as the Vmax for MTP. 2) Reverse and forward specificities of HACD reactions have been seen to differ (29). However, from the data seen here, the forward rat HACD specificity (starting from enoyl-CoA) shows a very similar trend to the reverse rat HACD specificity from Kamijo et al. (3) and Liu et al. (32). This suggests that MTP's HACD substrate specificity is the same in both directions. 3) Middleton (4): rat and human liver, pig heart MTP: all show highest activity towards C10 and higher. This is good confirmation of my parameter.
	sfmtpC16	1.0 *{0.1, no temp + only for HACD activity + reverse}*	1.0 *{0.5, temp + only for HACD activity}*	1.0 *{0.1, rat + pH + only for HACD activity + reverse + BSA}*	
	sfmtpC14	0.97 *{0.1, no temp + only for HACD activity + reverse }* [0.97 – 1.03]	1.03 *{0.5, temp + only for HACD activity}*		
	sfmtpC12	0.93 *{0.1, no temp + only for HACD activity + reverse }* [0.93 – 1.06]	1.06 *{0.5, temp + only for HACD activity}*		
	sfmtpC10	0.81 *{0.1, no temp + only for HACD activity + reverse }* [0.81 – 0.92]	0.92 *{0.5, temp + only for HACD activity}*		
	sfmtpC8	0.68 *{0.1, no temp + only for HACD activity + reverse }* [0.5 – 0.68]	0.5 *{0.5, temp + only for HACD activity}*	0.63 *{0.1, rat + pH + only for HACD activity + reverse }*	
	sfmtpC6	0.34 *{0.1, no temp + only for HACD activity + reverse }* [0.13 – 0.34]	0.13 *{0.5, temp + only for HACD activity}*		

Cubic polynomial (multinormal distribution)	
Formula	$a * x^3 + b * x^2 + c * x + d$
Best fit parameters	a = 0.000304648 b = -0.0256073 c = 0.528999 d = -2.16707
R^2	0.99
Covariance matrix	{{4.8508*10^{-7}, -0.000016035, 0.000166874, -0.000541432}, {-0.000016035, 0.000533481, -0.00559211, 0.0182772}, {0.000166874, -0.00559211, 0.0591166, -0.194983}, {-0.000541432, 0.0182772, -0.194983, 0.650192}}
Bound	{{"bounds: C6", 0.034, 3.4}, {"bounds: C8", 0.068, 6.8}, {"bounds: C10", 0.081, 8.1}, {"bounds: C12", 0.093, 9.3}, {"bounds: C14", 0.097, 9.7}, {"bounds: C16", 0.1, 10.}}



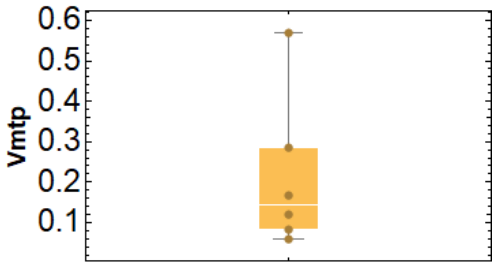
Comments: n/a

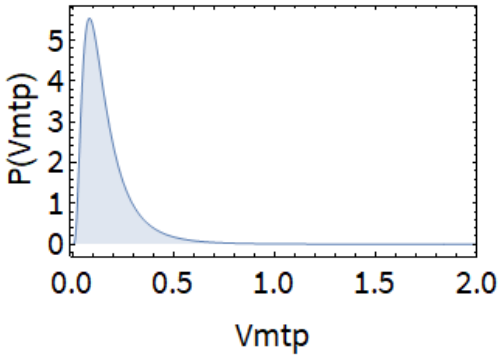
Vmtp

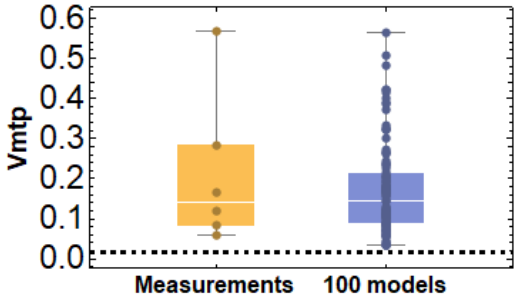
Semi-satisfactory value proposed. We would prefer one that is directly measured instead of one that is converted two times, as below.

	Parameter	Chosen value [range]	Alternatives		Comments
Vmtp		Oey *et al* (2005, (45)): Thawed human baby liver tissue, 6 weeks age, measured in reverse direction according to Wanders et al. 1990, at 37°C, 50 mM L 2-(N-morpholino)-ethanesulpheric acid, 100 mM KPi, 0.1 mM DTT, 0.1% Triton X-100, 100 uM NADH (final pH = 6.16) and homogenate. Substrate: C16-ketoacyl-CoA) To convert to forward rate: divide by 10, according to the finding of Middleton (4): and multiply by 4 to scale to the mitochondrial protein (46)	Uchida et al. (1992, (1)) rat liver mitochondrial extract, hydroxyacyl-CoA dehydrogenase activity, 30°C, pH = 7.5, 100 mM Tris, 100 mM KCl reverse with C16-ketoacyl-CoA as substrate, NADH (???) - assuming that - like in human (4) - rat HACD activity is 10 times higher in the reverse direction, we rescale this by dividing by 10 - no rescaling to mitochondria necessary, as it is already mitochondrial extract	El-Fakhri and Middleton (1982, (39)): rat liver mitochondrial extract, reverse activity, 100 mM KPi, pH = 7.0, 40 uM 3-ketoacyl-CoA, 0.1 mg/mL NADH, 30°C),converted to forward rate according to Middleton (4) (x0.1)	**1)** The value proposed by Uchida *et al.* (1) in rat liver mitochondrial extract is about 4 times larger than our chosen parameter, but at least it confirms that if you take the forward HACD activity as the V_{max} of MTP, you get something way smaller than what Van Eunen *et al.* (24) proposed. **2)** Converting the value measured by El-Fakhri and Middleton (39) in rat liver mitochondria to the forward rate according to the forward/reverse (1/10) ratio given by Middleton (4) gives us 0.06µmol.min^{-1}.mg-mito-prot^{-1}, which is very close to out chosen value. This is good support for our chosen parameter value. **3)** Kispel *et al.* (10) saw the long-chain-specific HACD activity of pig heart increasing 2-fold in the presence of the binding protein that anchors it to the membrane**. If the Vmax of MTP proves important for the model, you can consider doubling these values accordingly.**
	Vmtp	0.0828 µmol.min^{-1}.mg-mito-Protein^{-1} *{0.5, pH + technically only measuring HACD activity + calculation}* [0.06 – 0.284] **0.1656 µmol.min^{-1}.mg-mito-Protein^{-1} (if doubled according to Kispel *et al.* (10) assuming the presence of anchoring proteins)** ***{0.1, pH + NADH measured, so unsure if it's all three MTP activities + calculation + detergents}*** ([0.06 – 0.568] if you consider the activity doubling due to the presence of binding proteins (10))	0.284 µmol.min^{-1}.mg-liver-protein^{-1} *{0.1 rat + temp + only HACD activity}* **0.568 µmol.min^{-1}.mg-mito-Protein^{-1} (if doubled according to Kispel *et al.* (10) assuming the presence of anchoring proteins)** ***{0.1 rat + temp + pH + only HACD activity + calculation}***	0.06 µmol.min^{-1}.mg-mito-prot^{-1} *{0.1, rat + temp + pH + only HACD activity}* **0.12 µmol.min^{-1}.mg-mito-Protein^{-1} (if doubled according to Kispel *et al.* (10) assuming the presence of anchoring proteins)** ***{0.1 rat + temp + pH + only HACD activity + calculation}***	

Log-normal distribution	
Parameters **(of the normal distribution)**	μ = -2.01469 σ = 0.680036
Bound	{"bounds", 0.01656, 1.656}







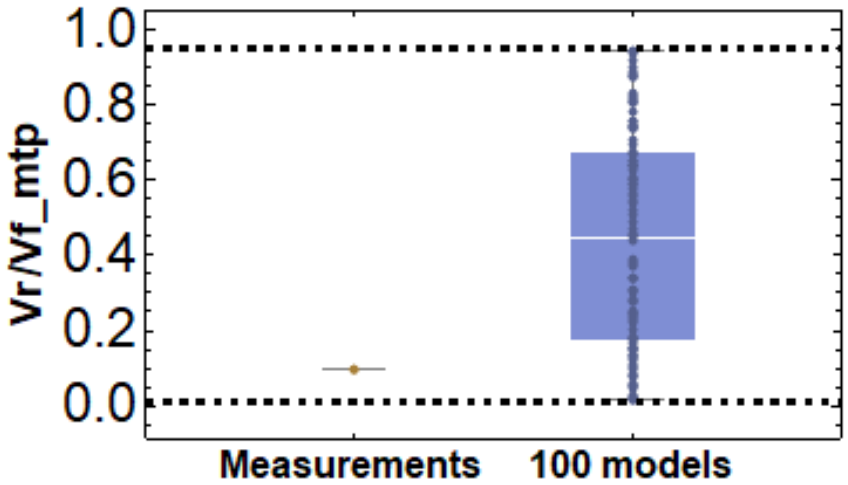
Comments: n/a

Vr/Vf_mtp_KACT

No satisfactory value found. Assumed the same as MCKAT.

	Parameter	Chosen value [range]	Alternatives		Comments
Vr/Vf_mtp_KACT		Kiema *et al.* (2014, (31)): human liver MCKAT expressed in E. coli, 25°C, pH = 7.8, 25 mM MgCl2, 50 mM Tris, 60 µM CoA			**1)** Assumed the same as MCKAT. 2) Used to calculate KMmtpAcylCoAMAT
	Vr/Vf_mtp_KACT	0.095 *{0.1, taken from MCKAT + temp}*			

uniform distribution	
Range	{"bounds", 0.0095, 0.95}



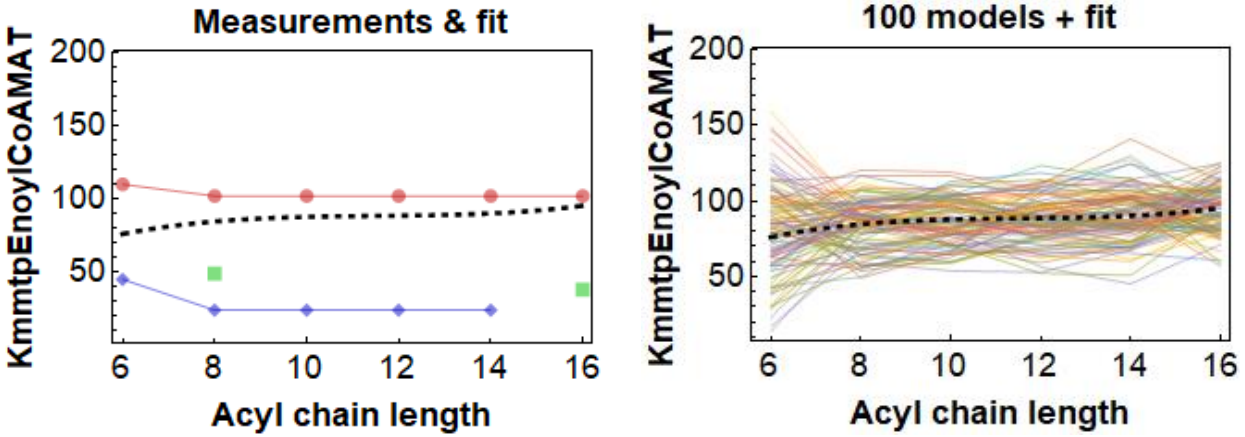
Comments: n/a

KmmtpEnoylCoAMAT

Semi-satisfactory values. We would have liked more directly measured parameters, but this isn't bad.

	Parameter	Chosen value (range)	Alternatives		Comments
KmmtpAnoylCoACYT		**C16, and assumed for C14, C12, C10, C8:** Liang et al. (2018, (7)) full MTP reaction - enoyl-Coa -> acyl-CoA, recombinant human TFP expressed in E. coli, 37°C, 50 mM Tris, pH = 8.5, 100 mM KCl, 100 mM MgCl2, 1 mM CoA, 1 mM NAD, 0.1 mg/mL BSA) very good parameter **C6:** Jiang *et al.* (1996) human MTP, 30°C, pH = 7.5, 50 mM KPi, 2 mM mercaptoethanol, ECH activity	Liu et al. (2008, (32)) ECH activity on recombinant rat MTP expressed in BL21 (DE3) cells, 200 mM KPi, pH = 7.5, 50 mM KCl, 100 uM DTT, 10 uM C8-enoyl-CoA, temp not given	Schulz (1974, (15)): measured as ECH activity before we knew about MTP, we assume that it is MTP because it show multiple characteristics of MTP: highest ECH activity for C8-enoly-CoA, a pH optimum around 8.5, no affinity for crotonase; bovine heart homogenate, 83.3 mM Kpi, pH - 8.0, 50 ug BSA, 15 nmol, enoyl-CoA, 0.6 mL assay volume, no temp given).	**1)** There is quite some scatter in the parameters that we managed to get from literature - more than a fold difference for parameters regarding ECH activity. This might be because of downstream activity of the MTP, or perhaps due to species-specific effects. However, it is at least mildly encouraging that the alternative values from Liu et al. (32) are also 100 µM or lower. 2) Note that Jiang et al. (13) report non-linear binding kinetics when measuring MTP affinity for enoyl-CoA with ECH activity. This might be because of downstream activity. It is worth keeping in mind in future measurements to see if this is a reproducible, kinetic feature of the enzyme or just a consequence of the complexity of its kinetics. **3)** The measurements of Schulz (15) give a precedent for assuming that the the K_m values of longer-chain substrates are the same or similar (C8 - C16). Though they are lower than the human enzyme in Liang *et al.* (7) they are in the same order of magnitude and consistent, with slightly lower affinity for the C6-substrate.
	KmmtpC16EnoylCoAMAT	102.05 µM *{0.9, BSA}* [38 – 102.05]	38 µM *{0.1, ECH activity + temp + rat}*		
	KmmtpC14EnoylCoAMAT	102.05 µM *{0.5, assumed from C16 + BSA}* [24 – 102.05]		24 µM *{0.1, ECH activity + temp + bovine + BSA }*	
	KmmtpC12EnoylCoAMAT	102.05 µM *{0.5, assumed from C16 + BSA }* [24 – 102.05]		24 µM *{0.1, ECH activity + temp + bovine + BSA }*	
	KmmtpC10EnoylCoAMAT	102.05 µM *{0.5, assumed from C16 + BSA }* [24 – 102.05]		24 µM *{0.1, ECH activity + temp + bovine + BSA }*	
	KmmtpC8EnoylCoAMAT	102.05 µM *{0.5, assumed from C16 + BSA }* [24 – 102.05]	49 µM *{0.1, ECH activity + temp + pH + rat}*	24 µM *{0.1, ECH activity + temp + bovine + BSA }*	
	KmmtpC6EnoylCoAMAT	110 µM *{0.1, ECH activity + temp + BSA }* [45 – 110]		45 µM *{0.1, ECH activity + temp + bovine + BSA }*	

Cubic polynomial (multinormal distribution)	
Formula	$a * x^3 + b * x^2 + c * x + d$
Best fit parameters	a = 0.0632976 b = -2.19018 c = 25.5573 d = -12.0879
R^2	0.91
Covariance matrix	{{0.143434, -4.88199, 52.7657, -180.089}, {-4.88199, 167.244, -1821.18, 6265.51}, {52.7657, -1821.18, 20008.7, -69522.5}, {-180.089, 6265.51, -69522.5, 244450.}}
Bounds	{{"bounds: C6", 11, 1100}, {"bounds: C8", 10.205, 1020.5}, {"bounds: C10", 10.205, 1020.5}, {"bounds: C12", 10.205, 1020.5}, {"bounds: C14", 10.205, 1020.5}, {"bounds: C16", 10.205, 1020.5}}



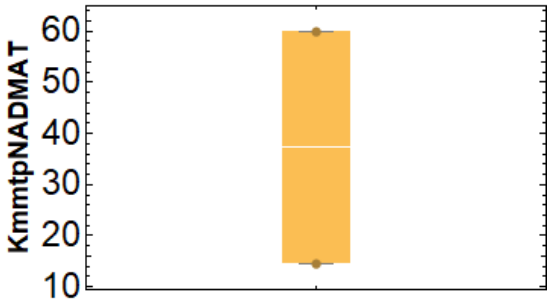
Comments: n/a

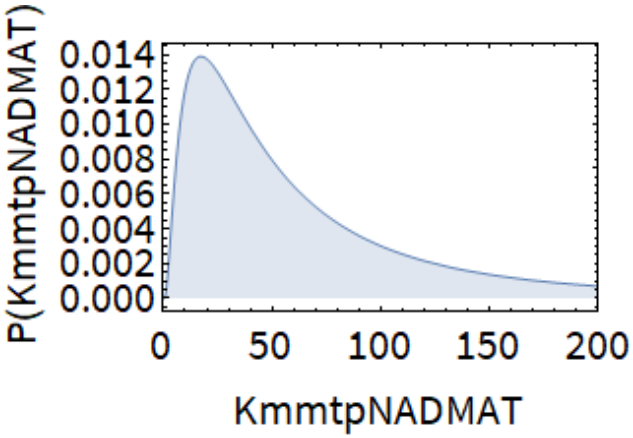
KmmtpNADMAT

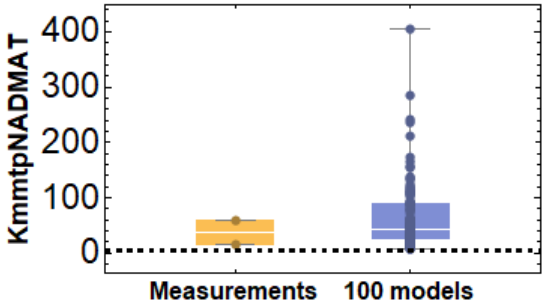
Semi-satisfactory value: though this parameter is not human, it is at least a direct measurement.

	Parameter	Chosen value [range]	Alternatives	Comments
KmmtpNADMAT		Uchida *et al.* (1992, (1)) rat liver mitochondria, 30°C, pH = 7.5, 1000 mM Tris, 100 mM KCl.	He et al. (1989, (47)) pig heart homogenate, assume C16 parameters belonging to MTP, as they do not discern between MTP and M/SCHAD, 25°C, 200 mM KPi, pH = 8.0, 0.2 mg/mL BSA, 0.5 mM NAD, 0.25 mM CoASH, 79 mU pig heart 3-ketothiolase; *it might be worth noting that they find a much larger Vmax (5.8x4 = 23.2 umol.min^{-1}.mg-mito-prot^{-1}) than I expect, especially since El Fakhri and Middleton* (39) *saw heart and liver HCAD activity to be more or less identically distributed*	**1)** Since the V_{max} values measured by He *et al.* (1988) were bizarrely large, for both MTP and M/SCHAD activity, I will prefer the value measured by Uchida *et al.* (1). **2)** Changing NADH/NAD concentrations seems to be less effective at modulating the activity of MTP in the absence of detergents, suggesting a channelling effect of NADH\NAD between MTP and the ETC which renders this branch of mFAO more robust to changes in NADH/NAD (8) **3)** "The data suggest that 1 mol of enzyme has four NADH-binding sites" (1). **4)** This is a factor of 4 larger than the Km value suggested by He *et al.* (22), but it is at least in the same order of magnitude, which suggests some reliability.
	KmmtpNADMAT	60 µM *{0.5, rat + temp}* [14.5 – 60]	14.5 µM *{0.1, pig heart + temp + bizarrely large Vmax from this paper renders this parameter suspect}*	

Log-normal distribution	
Parameters (of the normal distribution)	μ = 3.85765 σ = 1.00423
Bound	{"bounds", 6, 600}







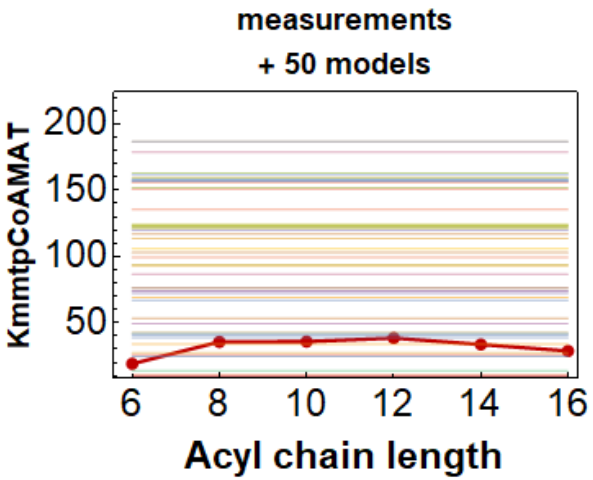
Comments: n/a

KmmtpCoAMAT

No satisfactory values found pertaining to MTP specifically.

	Parameter	Chosen value	Alternatives	Comments
KmmtpCoAMAT		Miyazawa *et al.* (1981, (42)) **The K_m values of CoA for MCKAT are assumed applicable to the KACT site of MTP, based on Liu *et al.* (2008, (32))'s observation that the sites are similar in composition.** rat liver, 25°C, pH = 8.3, 100 mM Tris-Cl, 25 mM MgCl2, 50 mM KCl		**1)** Liu et al. (32)'s observation that the "three catalytic residues" in the active sites of MCKAT and MTP's KACT activity suggests that the same mechanism should be valid for both. Miyazawa et al. (42) report a mechanism of ketoacyl-CoA thiolysis where an acetyl-CoA is first cleaved off the acyl-CoA substrate, after which a CoA enters the active site and binds to the shortened acyl-CoA. For this reason, they also report different Km values for the CoA substrate of the reaction, depending on the ketoacyl-CoA substrate of which the acyl-group will still be occupying the active site. This suggests that CoA values should also change depending on the lengths of the acyl-group of the other substrate. **2)** For this reason, we take over the values chosen for MCKAT directly, since there is no experimental value available specifically for MTP according to our knowledge.
	KmmtpC16CoAMAT	28.6 µM *{0.1, assumed from MCKAT + rat + temp}*		
	KmmtpC14CoAMAT	33.5 µM *{0.1, assumed from MCKAT + rat + temp}*		
	KmmtpC12CoAMAT	38.4 µM *{0.1, assumed from MCKAT + rat + temp}*		
	KmmtpC10CoAMAT	35.7 µM *{0.1, assumed from MCKAT + rat + temp}*		
	KmmtpC8CoAMAT	35.5 µM *{0.1, assumed from MCKAT + rat + temp}*		
	KmmtpC6CoAMAT	18.9 µM *{0.1, assumed from MCKAT + rat + temp}*		

Linear (flat - pick one value for all the chain-lengths)	
Formula	c
Chosen parameters	a = {0,0} b = 0 c = {0, 200}
R^2	n/a
Covariance matrix	n/a
Bounds	{{"bounds: C6", 1.89, 189.}, {"bounds: C8", 3.55, 355.}, {"bounds: C10", 3.57, 357.}, {"bounds: C12", 3.84, 384.}, {"bounds: C14", 3.35, 335.}, {"bounds: C16", 2.86, 286.}}



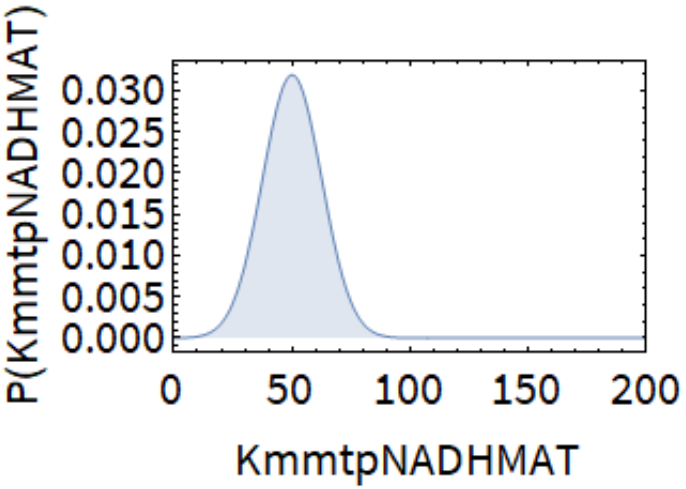
Comments: I have no certainty even about any one of the values. This is quite an important parameter, since the depletion of CoA is a key part of our analysis. Therefore, I chose a range of values up to 200 µM to widen the chance that I find something interesting if the MTP happens to bind to CoA with higher affinity.

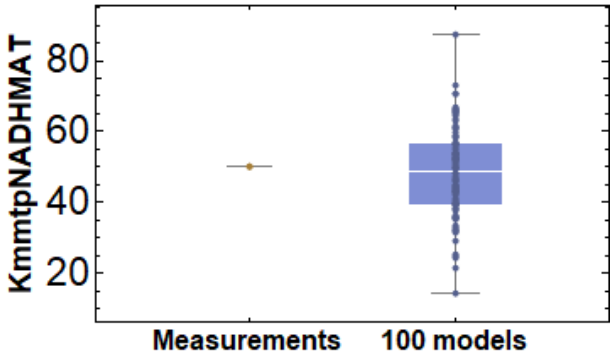
KmmtpNADHMAT

Semi-satisfactory value: though this parameter is not human, it is at least a direct measurement.

	Parameter	Chosen value [range]	Alternatives	Comments
KmmtpNADHMAT		Uchida *et al.* (1992, (1)) rat liver mitochondria, 30°C, pH = 7.5, 1000 mM Tris, 100 mM KCl.		**1)** This is the only measurement of this parameter that we found in literature. **2)** Changing NADH/NAD concentrations seems to be less effective at modulating the activity of MTP in the absence of detergents, suggesting a channelling effect of NADH\NAD between MTP and the ETC which renders this branch of mFAO more robust to changes in NADH/NAD (8) **3)** "The data suggest that 1 mol of enzyme has four NADH-binding sites" (1).
	KmmtpNADHMAT	50 µM *{0.5, rat + temp}*		

Normal distribution (constructed)	
Parameters **(of the normal distribution)**	μ = 50 σ = 12.5
Bounds	{"bounds", 5, 500}





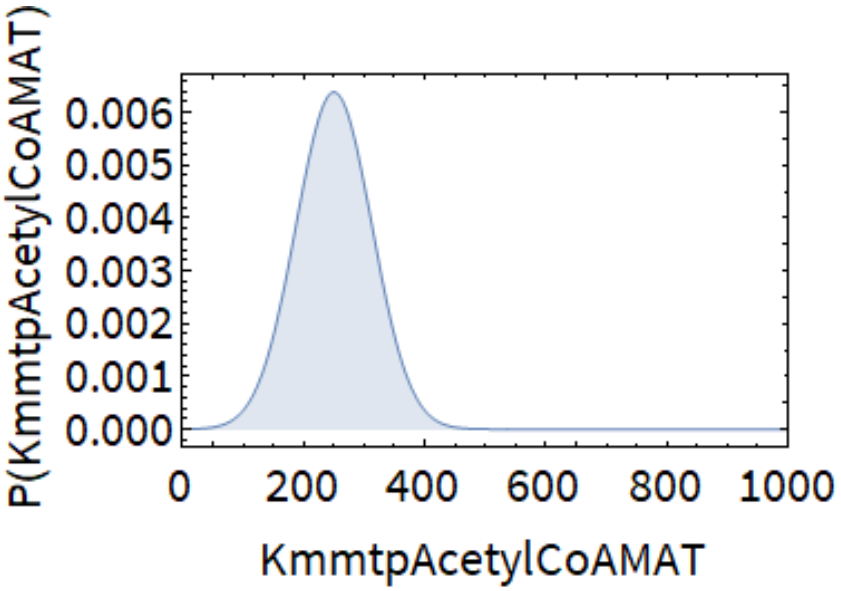
Comments: n/a

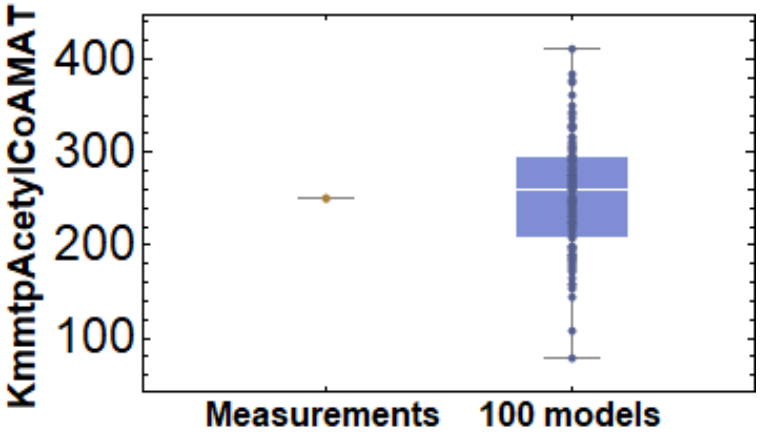
KmmtpAcetylCoAMAT

Semi-satisfactory value: though this parameter is not human, it is at least a direct measurement.

	Parameter	Chosen value [range]	Alternatives	Comments
KmmtpAcetylCoAMAT		Kiema *et al.* (2014, (31)) MCKAT parameter, Human liver, 25°C, pH = 7.8, 25 mM MgCl2, 50 mM Tris, 60 µM CoA, 100 µM OctCoA		**1)** Assume Km- value to be the same as for MCKAT based on active site similarity (32).
	KmmtpAcetylCoAMAT	250 µM *{0.5, MCKAT parameter + temp}*		

normal distribution (constructed)	
Parameters **(of the normal distribution)**	μ = 250 σ = 62.5
Bounds	{"bounds", 25, 2500}





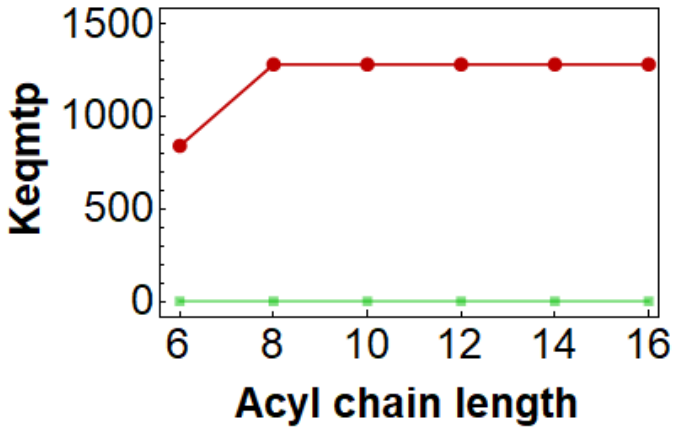
Comments: n/a

Keqmtp

eQuilibrator yields reliable and systematically determined values

	Parameter	Chosen value	Alternatives	Comments
Keqmtp		Estimated using eQuilibrator (43) Ionic strength = 0.125 mM, pH = 8.0, temperature correction estimate using Van 't Hoff relation	Kohn & Garfinkel (1983, (48)) *Conditions unknown*	
	KeqmtpC16	1280 *{1.0}* [0.71 – 1280]		**1)** Quite different from the value in Kohn and Garfinkel (48), but it doesn't seem to affect the model too much, and I cannot think of any reason why it would be different from theirs. **2)** The product of the individual K_{eq} value from each of the crotonase-branch reactions (ECH, HACD, KACT) equal the K_{eq} values of the MTP branch: this is good confirmation that the model is thermodynamically sound.
	KeqmtpC14	1280 *{1.0}*		
	KeqmtpC12	1280 *{1.0}*	0.71 *{0.1, conditions unknown}*	
	KeqmtpC10	1280 *{1.0}*		
	KeqmtpC8	1280 *{1.0}*		
	KeqmtpC6	840 *{1.0}*		

Unique	
Values	{{6, 840}, {8, 1280}, {10, 1280}, {12, 1280}, {14, 1280}, {16, 1280}}



Comments: No variation allowed, unique values from eQuilibrator.

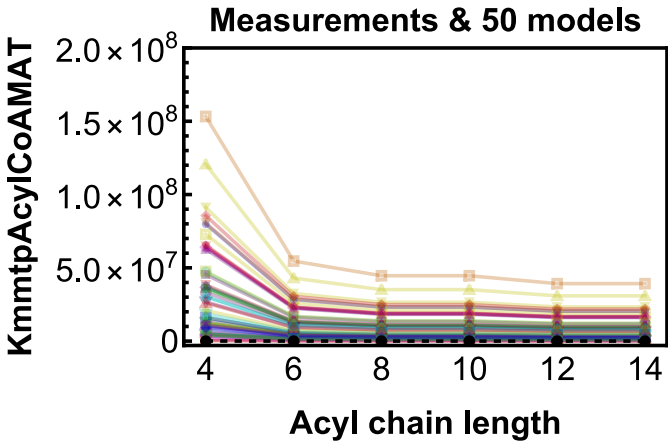
KmmtpAcylCoAMAT

No satisfactory, measured values found in literature.

	Parameter	Chosen value (range)	Alternatives	Comments
KmmtpAcylCoACYT		(estimated assuming random-order Bi-Bi Michaelis-Menten Kinetics according to Rohwer et al. (44), which then allows us to use the Haldane relation; other parameter values based on Miyazawa et al. (42); Kiema et al. (31), Liu et al, (32), Flamholz et al. (43), Middleton et al. (4))		**1)** Calculated as described in the section on the reaction mechanism. **2)** No measured value found in literature.
	KmmtpC14AcylCoAMAT	12 2881 µM *{0.1, very rough estimate}*		
	KmmtpC12AcylCoAMAT	14 6277 µM *{0.1, very rough estimate}*		
	KmmtpC10AcylCoAMAT	20 0201 µM *{0.1, very rough estimate}*		
	KmmtpC8AcylCoAMAT	19 5509 µM *{0.1, very rough estimate}*		
	KmmtpC6AcylCoAMAT	22 7033 µM *{0.1, very rough estimate}*		
	KmmtpC4AcylCoAMAT	33 9313 µM *{0.1, very rough estimate}*		

To maintain thermodynamic consistency, KmmtpAcylCoAMAT is calculated using this value and the other ones according to the formula:

$$K_{m,MTP,C_{n-2}AcylCoA} = K_{eq,MTP} \cdot \frac{V_{MTP,f}}{V_{MTP,r}} \cdot \frac{K_{m,MTP,C_nKetoacylCoA} \cdot K_{m,MTP,C_nCoA}}{K_{m,MTP,AcetylCoA}}$$



Comments: Very high values, as calculated before also.

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