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Evidence of a Preferred Kinetic Pathway in the Carnitine Acetyltransferase Reaction

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Abstract

Mammalian carnitine acetyltransferase (CrAT) is a mitochondrial enzyme that catalyzes the reversible transfer of an acetyl group from acetyl-CoA to carnitine. CrAT knockout studies have shown that this enzyme is critical to sustain metabolic flexibility, or the ability to switch between different fuel types, an underlying theme of the metabolic syndrome. These recent physiological findings imply that CrAT dysfunction, or its catalytic impairment, may lead to disease. To gain insight into the CrAT kinetic mechanism, we conducted stopped-flow experiments in various enzyme substrate/product conditions and analyzed full progress curves by global fitting. Simultaneous mixing of both substrates with CrAT produced relatively fast kinetics that follows an ordered bi bi mechanism. A great preference for ordered binding is supported by stopped-flow double mixing experiments such that premixed CrAT with acetyl-CoA or CoA demonstrated a biphasic decrease in initial rate that produces about a 100-fold attenuation in catalysis. Double mixing experiments also revealed that the CrAT initial rate is inhibited by 50% in approximately 8 s by either acetyl-CoA or CoA premixing. Analysis of available CrAT structures support a substrate conformational change between acetyl-CoA/CoA binary versus ternary complexes. Additional viscosity-based kinetic experiments yielded strong evidence that product release is the rate limiting step in the CrAT-catalyzed reaction.

Keywords

Enzyme kinetics; global fitting; mitochondrial metabolism; acetyl-CoA; acetylcarnitine

1. Introduction

Mammalian carnitine acetyltransferase [1] (CrAT) (E. C. 2.3.1.7) catalyzes the reversible transfer of an acetyl group between acetyl-CoA and L-carnitine, forming CoA and acetylcarnitine. CrAT is localized to the mitochondrial matrix [2] or peroxisomes [3], but

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Conflict of interest

The authors declare that they have no conflict of interest.

recent evidence also suggests a cytosolic CrAT [4]. In the mitochondrial matrix, CrAT-catalyzed consumption of acetyl-CoA and production of CoA is thought to be a critical acetyl-CoA/CoA buffer, for instance when the TCA cycle is overwhelmed as an acetyl-CoA sink [3]. Physiologically this scenario is more probable in times of high ATP demand, for example during exercise [5], where exercising muscle biopsies demonstrate increased acetyl-CoA and acetylcarnitine levels relative to resting muscle [6, 7].

CrAT has also been implicated in glucose sensitivity, with CrAT knockout mice demonstrating glucose insensitivity [8], underscoring the importance of this reaction to healthy metabolic function. CrAT function has also been linked to muscle endurance [5] and has been shown to be a positive metabolic regulator inhibited by lipid stress and obesity [9]. More broadly, CrAT mediated mitochondrial acetyl-CoA/CoA buffering is related to the balance between glucose oxidation (GO), fatty acid oxidation (FAO), and amino acid oxidation (AAO) – a central topic in metabolism termed fuel selection [8]. FAO and GO are the main sources of mitochondrial acetyl-CoA [10]. When mitochondrial acetyl-CoA levels exceed TCA cycle flux [11], excess acetyl-CoA may lead to protein acetylation [7], a ubiquitous metabolic regulator [12]. CrAT buffering of acetyl-CoA by the production of acetylcarnitine alleviates metabolic stress by reducing protein acetylation [13], and by replenishing the CoA pool. Thus, the capacity of CrAT to alleviate metabolic stress via acetyl-CoA/CoA buffering is of special interest to human health.

Our objective was to generate an accurate kinetic model of mammalian CrAT. To this end, we found early kinetic studies by Chase and Tubbs [14] that provided forward and reverse initial velocity and product inhibition data [15]. Using Lineweaver-Burk plot analysis, they concluded that CrAT, from pigeon breast muscle, followed a rapid equilibrium bi bi mechanism [14]. This method of kinetic analysis, however, does not fit the model itself, but rather predictions of linear patterns. Global analysis overcomes this issue by simultaneously fitting all of the data to the actual model [16–18].

Our efforts in global fitting CrAT literature data [14] have revealed deficiencies that can only be circumvented by producing a new collection of CrAT kinetic data. Thus, we have collected numerous CrAT forward and reverse full progress curves using a stopped-flow rapid mixing device utilizing various mixing strategies directly following the acetyl-CoA to CoA transition in the UV spectrum. Global analysis of full progress curve data with fluorescence intensity equilibrium titrations of substrates and double mixing experiments have revealed a preferred kinetic pathway in the mammalian CrAT reaction. This preferred pathway involves the ordered binding of substrates, with carnitine binding before acetyl-CoA in the forward direction and acetylcarnitine followed by CoA in the reverse direction. When this order is reversed, catalysis is decrease by about 100-fold.

We also conducted viscosity-based stopped-flow experiments that support product release as being rate limiting for the reaction. Available CrAT ligand bound structures show conformational differences between binary and ternary complexes that give further insight into our kinetic data. Additionally, we have shown here that CrAT does in fact catalyze the hydrolysis of acetyl-CoA, a hypothesis consistent with prior structural data [1].

2. Materials and Methods

2.1 Original kinetic data and modeling

Carnitine acetyltransferase, from pigeon breast muscle, was purchased from Sigma Aldrich and used without further purification based on SDS-PAGE analysis. L-Carnitine and acetylcarnitine were also purchased from Sigma Aldrich, while CoA and acetyl-CoA were purchased from COALA biosciences (Austin, TX). All kinetic assays were performed at ambient temperature in 50 mM Tris at a pH of 7.8 and 0.5 μ M CrAT. Solutions were also filtered with a 0.22 μ m filter before mixing.

Kinetic UV absorption assays were performed on a KinTek SF-300X stopped-flow mixing device utilizing a deuterium lamp and followed using a multiwavelength CCD array detector. Averaged kinetic data (at 232 nm) were then imported into KinTek global kinetic explorer software (Austin, TX) and globally fit with a mechanism using non-linear least squares and numerical integration [19]. Confidence intervals were determined using KinTek explorer software, which systematically fixes parameters while allowing others to vary to find a range of parameters that fit the data. Ultimately all parameter sets that produce a chi

squared ratio $\left(\frac{\chi_{best}^2}{\chi_i^2}\right)$ threshold value within 0.833 of the best-fit value were used to

determine upper and lower parameter bounds [20]. The CrAT reaction was followed by observing the absorbance change at 232 nm between acetyl-CoA and CoA, which has a molar extinction difference of approximately 2 $\text{mM}^{-1} \text{cm}^{-1}$. CrAT-catalyzed acetyl-CoA hydrolysis experiments were conducted on a BioTek synergy 2 plate reader using a 96 well format with 0.5 μ M CrAT and 200 μ M acetyl-CoA in 50 mM Tris pH 7.8 with a 100 μ L total volume.

Equilibrium fluorescence titrations were performed by an auto-titration module accessory to the SF-300X instrument by exciting CrAT fluorescence at 295 nm and collecting fluorescence at wavelengths greater than 320 nm using an optical filter. In these experiments, the titrant was slowly injected, over the course of 5 min, into a cuvette with 200–250 μ L of a stirred CrAT solution (5 μ M) buffered in 50 mM Tris (pH = 7.8) at ambient temperature. Equilibrium fluorescent titration data were analyzed by global fitting and numerical integration along with all other kinetic data.

2.2 Solvent viscosity assays

Solvent viscosity assays were performed using various concentrations of sucrose or glycerol to produce relative solvent viscosities (η_{rel}) to water at 20 °C of 1X, 1.5X, 2X, and 3X. Corresponding concentrations of sucrose to produce 1.5X, 2X and 3X relative viscosities, were 0.41, 0.65, and 0.951 M [21]. Corresponding concentrations of glycerol to produce 1.5X, 2X and 3X relative viscosities, were 14.5, 24, and 35 % glycerol [21]. All solvent viscosity assays were buffered with 50 mM Tris at pH 7.8.

2.3 Etheno-CoA synthesis and fluorescence experiments

Etheno-CoA (e-CoA) was synthesized by first reacting 5 mM CoA with 10 mM 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) in 1 mL of 50 mM Tris, pH = 7.8, at ambient

temperature. The solution was then applied to a G-10 sephadex column (GE lifesciences), equilibrated with 100 mM citrate at pH 4, to separate free TNB from the CoA-TNB mixed disulfide. The CoA-TNB mixed disulfide, in 100 mM citrate (pH =4), was then reacted with approximately 3 M chloroacetaldehyde (Sigma Aldrich) at 37 °C to create the ϵ -CoA-TNB mixed disulfide.

The reaction was followed by scanning the UV-vis spectrum, observing evolving peaks at 265 and 275 nm until an equilibrium point was reached after about 3 days. The unreacted chloroacetaldehyde was then removed by applying the solution to a G-10 sephadex column. Purified ϵ -CoA-TNB mixed disulfide was then reduced to free ϵ -CoA and TNB using a 2-fold excess of tris(2-carboxyethyl)phosphine (TCEP). Free TNB was then removed by applying the solution to a G-10 sephadex column, equilibrated with water, yielding pure ϵ -CoA, assessed using LC-MS.

CrAT/ ϵ -CoA T-format fluorescence anisotropy experiments were carried out on a KinTek SF-300X stopped-flow mixing device. ϵ -CoA fluorescence was excited at 305 nm using a Glan-Taylor polarizer in the vertical position, and fluorescence was collected using vertical and horizontal polarizers in front of each photomultiplier tube along with 385 nm cutoff filters. Final concentrations after mixing were 20 and 4 μ M, for CrAT and ϵ -CoA, respectively. Anisotropy experiments were conducted at ambient temperature in 50 mM Tris pH 7.8.

2.4 Literature data and modeling

Double reciprocal plot data, for CrAT from Chase et. al., were extracted using Scanit software version 2.0.5.0 (AmsterCHEM) and then inverted to their original values. Data were fit using a global non-linear least squares approach that uses the Matlab function, `fmincon`, to minimize the error function [17, 18]. Simulated annealing, a global minimization routine, was also implemented. Initial parameter values were selected randomly between wide boundaries and used for fitting, a process repeated thousands of times to arrive at a best-fit. Data were fit with both the random rapid equilibrium bi bi (RRE) and ordered steady-state bi bi mechanism (SSB), the former being the published mechanism for these data [14]. Linear scaling factors were applied to the model, in an effort to better fit the data. In this respect, both RRE and SSB mechanisms were treated the same to maintain an equal fitting potential. Parameter confidence intervals (95%) were subsequently computed based on mainstream protocols found within Matlab software.

3. Results

3.1 CrAT literature kinetic data modeling

To establish a kinetic model for mammalian CrAT, with globally consistent parameters, we originally chose to analyze a comprehensive steady-state kinetic data set from Chase et. al. [14]. Their data was collected using CrAT from pigeon breast muscle, and based on Lineweaver-Burk (LB) plots the authors concluded that CrAT follows a random rapid equilibrium bi bi mechanism (RRE). Since classical LB analysis does not involve simultaneously fitting all the data (global fitting), we extracted these data [14] and globally

fit them to the RRE mechanism (Fig. S1) and to a steady-state ordered bi bi mechanism (SSB) (Fig. S1–S4). Here, we have delivered the best-fit parameters (Table S1), along with their 95% confidence intervals, and found that global fitting gives a lower error for the SSB mechanism ($\chi^2 = 1.495$) compared to the RRE mechanism ($\chi^2 = 1.797$). A F-test was conducted to determine if the SSB mechanism fits the data significantly better than the RRE mechanism, given the SSB mechanism adds another parameter. The F-test resulted in a calculated p-value being < 0.00001 , which indicates that the improved fit of the SSB mechanism is likely not random and therefore the more acceptable model.

3.2 CrAT stopped-flow without premixing

CrAT kinetic data collected using purified protein from pigeon breast muscle were collected on a stopped-flow rapid mixing device using a CCD multiwavelength detector by following the decrease in UV absorbance of acetyl-CoA to CoA (Fig. 1A and B), in the forward CrAT direction, or an increase in UV absorbance of CoA to acetyl-CoA in the reverse direction (Fig. 1C and D).

We first implemented a stopped-flow mixing strategy where CrAT was separated from substrates before mixing (Fig. 2). In these experiments we varied all substrates for the forward (Fig. 2A and B) and reverse reaction (Fig. 2C and D). These data demonstrated fast kinetics in either direction. Full progress curves were simulated by numerical integration and non-linear least squares global fitting (Fig. 2A–D) of the steady-state ordered bi bi mechanism (SSB) using molar absorptivities (Fig. S5). Therefore, the SSB mechanism can very well describe these progress curve data.

3.3 CrAT tryptophan fluorescence quenching

Equilibrium binding data with carnitine (Fig. 3B) and acetylcarnitine (Fig. 3C) were collected by tryptophan fluorescence quenching experiments conducted on an auto-titration module. These data were incorporated into the global fitting procedure, to model all data collectively, by simulating the binding experiments through numerical integration of the model. Although tryptophan fluorescence quenching data was collected with acetyl-CoA and CoA, these titrations lacked good signal following inner filter effect corrections [22]. Together these results are consistent with structural data [23] (Fig. 3A) that shows tryptophan residues only located near the carnitine binding site, but not the CoA binding site.

3.4 CrAT stopped-flow fluorescence anisotropy

To circumvent lack of fluorescence signal for acetyl-CoA and CoA binding, we synthesized the fluorescent CoA derivative etheno-CoA (e-CoA) (Fig. S6A–C), which is not commercially available [24, 25]. We attempted to monitor e-CoA binding to CrAT using equilibrium fluorescence anisotropy by titrating CrAT into a solution of e-CoA but these experiments were confounded by light scattering from the high concentration of CrAT needed for appreciable binding (Not shown). Therefore, we conducted stopped-flow

fluorescence anisotropy (Fig. S6D–F), time dependent experiments, to help ameliorate light scattering. However, the fluorescence anisotropy change was negligible compared to buffer (Fig. S6F). Simulation of the Perrin equation [22], which enables one to predict anisotropy changes based on the sizes of the interacting molecules and fluorescent lifetime, demonstrated (Not shown) that the K_d for ϵ -CoA is likely $\sim 100 \mu\text{M}$. This result is corroborated by a K_i for CoA of $102 \mu\text{M}$ (Table 1), which is equaled to the equilibrium dissociation constant of CoA.

3.5 CrAT ordered bi bi kinetic constant determination

Rate constants for the ordered bi bi mechanism were determined by numerical integration of the model and global fitting of kinetic (Fig. 2) and equilibrium data (Fig. 3). Since rate constants are known to be difficult to constrain using steady-state kinetic data [20, 26], we used the rate constants to calculate the various steady-state kinetic parameters according to their definitions (Eqn. S3). Parameter bounds were determined using Kintek Explorer software [19, 20], which finds an upper and lower boundary level for each parameter while maintaining a good fit with the data. Steady-state parameter values and their associated boundaries are given in Table 1.

3.6 CrAT stopped-flow with premixing

We also implemented a mixing strategy where we premixed CrAT with one of its four reactants, before then rapidly mixing it with the other substrate to produce either forward (Fig. 4A and B) or reverse activity (Fig. 4C and D). This mixing strategy demonstrated severe kinetic inhibition when CrAT was premixed with either CoA (Fig. 4C) or acetyl-CoA (Fig. 4A), relative to no CoA (Fig. 2C) or acetyl-CoA (Fig. 2A) premixing. Thus, premixing CoA or acetyl-CoA (Fig. 4C and A) progress curves follow a much slower time scale compared to simultaneous substrate mixing (Fig. 2C and A). This result suggests that CrAT from pigeon muscle prefers to bind carnitine first, in the forward direction, and acetylcarnitine first in the reverse direction, rather than acetyl-CoA or CoA, respectively. We also observed that when CrAT was premixed with CoA (Fig. 4C), increasing amounts of acetylcarnitine beyond 0.25 mM , caused acetylcarnitine induced substrate inhibition. Data sets in Fig. 4B and D can be globally fit along with the previously discussed kinetic data to a simple ordered bi bi mechanism. However, data sets in Fig. 4A and C required a modification of the mechanism, described below. Additionally, individual fitting of the data sets in Fig. 4A and C yield an apparent k_{cat} about 100-fold below when acetyl-CoA or CoA are not premixed.

3.7 CrAT stopped-flow double mixing

To examine the short-time dependency of the inhibitory nature of acetyl-CoA and CoA premixing, we conducted stopped-flow double mixing experiments (Fig. 5) by varying the incubation time of acetyl-CoA or CoA with CrAT before then rapidly mixing with the other substrate. CrAT was premixed with either CoA from 0.023 s to 60 min (Fig. 5C), or acetyl-CoA for 0.023 s to 10 min (Fig. 5A), before rapid mixing with either acetylcarnitine or carnitine, respectively. These experiments revealed the CrAT initial rate is inhibited by 50% in approximately 8 s by either acetyl-CoA or CoA premixing. Initial rates of the progress curves were determined and normalized to the double mixing dead-time of the instrument

(23 msec) and fit with both a single and double exponential equation (Fig. 5B and D; Table 2). The progressive decrease in initial rates with premixing time demonstrates both the short and long-time inhibitory nature of acetyl-CoA and CoA binding, prior to the respective binding of carnitine or acetylcarnitine.

Premixing with either CoA or acetyl-CoA demonstrates a biphasic pattern of initial rates (Fig. 5B and D), determined by a much better fit of these data with a double exponential equation (Fig. 5B and D, solid line; Table 2) relative to a single exponential equation (Fig. 5B and D, dashed line; Table 2). Biphasic kinetics implies at least two kinetically distinguishable steps in the out-of-order inhibitory mechanism of acetyl-CoA and CoA binding. The observed rate constants for acetyl-CoA inhibition ($k_{\text{obs}(1)} = 61.7 \text{ min}^{-1}$, $k_{\text{obs}(2)} = 0.918 \text{ min}^{-1}$) are larger than for CoA ($k_{\text{obs}(1)} = 12.1 \text{ min}^{-1}$, $k_{\text{obs}(2)} = 0.1 \text{ min}^{-1}$), suggesting a faster acting CrAT premixing inhibition with acetyl-CoA relative to CoA.

3.8 CrAT stopped-flow global fitting and mechanism

Double mixing experiments were also analyzed by globally fitting the short-time double mixing progress curve data (Fig. 6A and B) with all prior data. These data can generally be described by a kinetic mechanism that includes a slow pathway (Fig. 6D; red), beginning with the binding of acetyl-CoA or CoA. Otherwise, the fast pathway (Fig. 6D; green) involves either carnitine or acetylcarnitine binding first. We also needed to add a dead-end inhibition step (Fig. 6D; red) to fit the substrate inhibition observed when CrAT was premixed with CoA, while acetylcarnitine was varied (Fig. 4C).

Despite being able to fit the short-time double mixing data with all others, this mechanism has difficulty fitting double mixing data collected during the intermediate time period of collection (several min of age time). But this mechanism does ultimately match the data in Fig. 4A and C, where premixing time was not monitored but was at least 10 min. The double exponential fitting, or two-phase initial rate data, suggests perhaps another enzyme intermediate state is involved in the slow pathway. Although this may be the case, the mechanism is already too complex to constrain kinetic constants, other than simple analytical fitting (Table 2). Therefore, we did not pursue more complex mechanisms. However, to shed more light on the inhibitory interaction of CrAT with acetyl-CoA and CoA, we report the resulting off rate constants with the free enzyme from the global fitting to be on the order of 1 s^{-1} and 0.00315 s^{-1} , for acetyl-CoA and CoA respectively. Confidence intervals for these rate constants were 0.00102 to 22.3 s^{-1} for acetyl-CoA, and 0.000424 to 0.05 s^{-1} for CoA.

X-ray crystallographic observations of acetyl-CoA soaked CrAT crystals revealed the apparent ability of CrAT to catalyze acetyl-CoA hydrolysis, generating CoA and acetate, although acetate was unresolved in the structure [1]. The ability of CrAT to catalyze the hydrolysis of acetyl-CoA to CoA creates the possibility that prior incubations of CrAT and acetyl-CoA alone could simply inhibit the transfer of the acetyl group from acetyl-CoA to carnitine by diminishment of the acetyl-CoA concentration. To address this, we followed the hydrolysis of acetyl-CoA by CrAT at 232 nm (Fig. 6C).

Our data (Fig. 6C) shows that CrAT does indeed catalyze the hydrolysis of acetyl-CoA to CoA relative to the non-enzymatic hydrolysis of acetyl-CoA to CoA (Fig. 6C); a 5-fold increase in initial rate from non-enzymatic to enzymatic rates, in these assay conditions. Despite this, our data also demonstrate how slow this reaction is compared to the fast-time inhibitory nature of acetyl-CoA incubation with CrAT (Fig. 6A). Thus, this side reaction does not explain the inhibition of CrAT by CrAT/acetyl-CoA preincubation, at least at incubation times within min as opposed to hours. Furthermore, inhibition of CrAT catalysis by the initial binding of CoA is also consistent with this conclusion.

3.9 CrAT structural analysis

X-ray crystallographic data of CrAT with several binary and ternary complexes are available [1, 23], and can be used to investigate structural discrepancies between these complexes to help understand the observed kinetic differences in binding order. A prior detailed analysis concluded that the active site residues in these complexes have generally the same conformation [23]. Thus, little changes in amino acid residue main chain and side chain position are observed from one complex to another.

To demonstrate binary and ternary complex differences, we aligned four different CrAT structures available from the protein data bank (Fig. 7). To simplify the view (Fig. 7), CrAT complex alignments show only different conformations of the CoA/acetyl-CoA ligand, as carnitine and the two active site residues shown, serine 554 and histidine 343, are essentially static. The major difference lies between the binary-CoA (cyan carbons) conformation and the conformation of all other CoA/acetyl-CoA ligands. The structure designated here as binary-CoA is a structure obtained without carnitine, while the ternary structures have been obtained with carnitine or an acyl-carnitine (not shown for clarity).

The thiol of the binary-CoA (Fig. 7A; cyan carbons) has a linear distance of 1.2 Å from the ternary-CoA (Fig. 7A; green carbons), as with the ternary-acetyl-CoA (Fig. 7A; blue carbons). Moving the sulfur-acetyl moiety of the ternary-acetyl-CoA to the position of the binary-CoA sulfur, with PyMOL [27] software, puts the carbonyl oxygen about 0.5 Å closer to the S554 oxygen (Fig. 7B). Furthermore, the sulfur in the binary-CoA position is only 2.0 Å away from the carnitine hydroxyl, which could alter catalysis. The position of the CoA thiol(ate) in the binary structure may be more freely rotatable compared to the ternary structure, which allows the thiol(ate) to be repositioned to potentially alter catalysis, discussed further below.

3.10 CrAT stopped-flow solvent viscosity assays

To identify the rate limiting step in the CrAT-catalyzed reaction, we monitored the forward reaction by varying acetyl-CoA and solvent viscosity using glycerol as a viscogen (Fig. 8A–C). The full progress curve data were globally fit with either a simple uncompetitive inhibition model (solid lines; Fig. 8A–C (χ^2 fitness = 0.43)) or a competitive inhibition model (dashed lines; Fig. 8A–C (χ^2 fitness = 2.80)) using numerical integration and global fitting. The uncompetitive inhibition model simulates the viscogen's ability to attenuate the k_{cat} alone, whereas the competitive inhibition model simulates its ability to attenuate k_{cat}/K_m [28].

The same fitting procedure was performed on reverse data by varying CoA (Fig. 8D–F), also yielding a better fit with the uncompetitive inhibition model (Fig. 8D–F (solid lines; χ^2 fitness = 0.344)) compared to the competitive inhibition model (Fig. 8D–F (dashed lines; χ^2 fitness = 2.82)). Attempts to model the data using a mixed inhibition model that combines both k_{cat} and k_{cat}/K_m inhibition did not improve the fitting (not shown). The same global fitting procedure on progress curve data using sucrose as a viscogen yielded a similar result (Fig. S7).

Solvent viscosity data were also analyzed using the more traditional initial velocity data (Fig. 9), by fitting the solvent viscosity modified version of the Michaelis-Menten equation (Eqn. 1), where solvent viscosity impacts both k_{cat} and k_{cat}/K_m (Eqn. 1), or k_{cat} alone (Eqn. 2).

$$\frac{v}{E_T} = \frac{k_{\text{cat}}S}{K_m(1 + m(\eta_{\text{rel}} - 1)) + S(1 + n(\eta_{\text{rel}} - 1))} \quad (1)$$

$$\frac{v}{E_T} = \frac{k_{\text{cat}}S}{K_m + S(1 + n(\eta_{\text{rel}} - 1))} \quad (2)$$

This analysis yielded a similar result to full progress curve fitting demonstrating that k_{cat} is greatly impacted by increasing solvent viscosity. By fitting Eqn. 1 (Fig. 9; solid lines) and Eqn. 2 (Fig. 9; dashed lines) separately, we demonstrated little need to invoke changes in k_{cat}/K_m with solvent viscosity to fit the data. Furthermore, inspection of the optimized parameters to Eqn. 1 in Table 3, demonstrates the lack of a lower bound on the parameter (m) associated with the k_{cat}/K_m effect, also suggesting that this parameter is not required to fit the data. Minor effects of solvent viscosity on k_{cat}/K_m may impact reverse catalysis as shown with variation of CoA (Fig. 9D).

Overall, these experiments and modeling demonstrate that the viscosity effect on CrAT catalysis can be modeled by its effect on the k_{cat} alone and not k_{cat}/K_m . An attenuation of k_{cat} by increased relative viscosity can either be explained by an enzyme catalyzed reaction limited by product release or a large conformational change required for catalysis, each impacted by diffusion [28]. With no clear evidence of any large conformational changes in any of the many available X-ray crystal structures [1, 23], and CrAT being referred to as a lock and key mechanism by others [1, 23], we infer that CrAT catalysis is limited by product release. Collectively, the data and fitting suggest that CrAT is limited by acetyl-CoA or CoA release, rather than acetylcarnitine or carnitine.

Discussion and Conclusions

Here we have determined that mammalian CrAT generally follows an ordered bi bi mechanism by performing various full progress curve stopped-flow experiments, coupled with numerical integration and global fitting. Importantly, we followed the CrAT reaction more directly, using the acetyl-CoA/CoA UV spectral transition, rather than the more indirect method of reaction coupling, which has the potential to confound the kinetics. This

process yielded steady-state constants with rigorously determined boundaries, with carnitine or acetylcarnitine binding first in the forward and reverse direction, respectively.

Throughout this experimental routine we discovered that mammalian CrAT is very sensitive to acetyl-CoA or CoA premixing, which greatly inhibits catalysis. Inhibition of CrAT catalysis due to acetyl-CoA or CoA premixing displayed biphasic kinetics, leading to about a 100-fold decrease in k_{cat} . Stopped-flow double mixing experiments revealed that CrAT catalysis is inhibited by 50% in about 8 s of acetyl-CoA and CoA premixing, demonstrating its short-time efficacy. Therefore, CrAT has a very strong catalytic preference to bind carnitine or acetylcarnitine first. Available murine CrAT binary and ternary structures support the idea that when CoA binary complexes are determined, they reveal a perturbed CoA orientation relative to ternary structures with pre-bound carnitine. Authors of these crystallographic data sets [23] explained that the binary-CoA crystals may have received some damage during the data collection process. However, all other aspects of this structure were reported to be consistent with the others [1], providing evidence of binary-CoA/acetyl-CoA structural variations. These variations could then lead to the kinetic differences observed here when either acetyl-CoA or CoA are allowed to bind first.

The perturbed CoA conformation results in a misoriented thiol(ate), or thioester in acetyl-CoA. In the forward direction, if acetyl-CoA binds before carnitine, we propose acetyl-CoA adopts the same conformation as CoA in the binary structure. Therefore, the sulfur of acetyl-CoA is 0.7 Å closer to the carnitine hydroxyl as is inferred from the CoA binary structure. This alternative conformation places the acetyl group of acetyl-CoA about 0.5 Å closer to serine 554. Mechanistically, serine 554 is thought to be a part of an oxyanion hole that stabilizes a tetrahedral transition state [23]. When mutated to alanine, the k_{cat} is decreased by 10-fold with a corresponding 5-fold increase in acetyl-CoA K_m [23, 29]. Closer proximity of the acetyl moiety, due to CoA/acetyl-CoA binary complex formation, could stabilize the transition state intermediate to ultimately impact the chemical step or product release. Even after the acetyl group has been transferred, the 0.7 Å tighter interaction between the CoA thiol to acetylcarnitine oxygen lone pair could potentially decrease the rate of CoA release. This mechanism of catalytic impairment is consistent with the idea that CoA product release is rate limiting in the forward direction, corroborated by our solvent viscosity assays.

In the reverse direction, product release of acetyl-CoA may also be affected if the acetyl group interacts with serine 554 more strongly due to the 0.5 Å closer proximity. The greater proximity (0.7 Å) between the carnitine hydroxyl and the acetyl-CoA sulfur may also stabilize the ternary complex deterring acetyl-CoA product release and thus slowing the reverse reaction. Our solvent viscosity assays demonstrate the potential for acetyl-CoA product release being rate limiting. Therefore, if product released is slowed, it would decrease the catalytic rate.

The physiological significance of CrAT inhibition due to out-of-order binding is not completely clear but does open the possibility that high concentrations of acetyl-CoA with depleted carnitine could yield a compromised CrAT ternary complex. This situation could become more relevant as such a complex may form over an extended period in these

conditions. Notably, carnitine supplementation has been shown to have a beneficial impact on cardiovascular glucose oxidation by increased pyruvate dehydrogenase complex flux [30], important to stave off heart failure [31].

Patients suffering from CrAT deficiency have been shown to have missense mutations [32] (Y110C and V569M) that are near the carnitine/acetylcarnitine binding site and are thus proposed to affect binding affinity [32]. Kinetic assays demonstrate that these mutations impact both CrAT Vmax and carnitine Km. Considering our findings, it is reasonable that clinical mutations not only impact simple carnitine binding but may also increase the probability of out-of-order binding leading to the misoriented conformation. This is consistent with these mutations having an impact on Vmax which gives the theoretical rate with substrates completely bound. Further kinetic analysis of these mutants could reveal their tendency to deviate from the wildtype preferred substrate binding order.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Jogl G, Tong L, Crystal structure of carnitine acetyltransferase and implications for the catalytic mechanism and fatty acid transport, *Cell* 112(1) (2003) 11–22. [PubMed: 12526789]
- [2]. Norum KR, Bremer J, The localization of acyl coenzyme A-carnitine acyltransferases in rat liver cells, *J Biol Chem* 242(3) (1967) 407–11. [PubMed: 4290216]
- [3]. Ramsay RR, Zammit VA, Carnitine acyltransferases and their influence on CoA pools in health and disease, *Mol Aspects Med* 25(5–6) (2004) 475–93. [PubMed: 15363637]
- [4]. Altamimi TR, Thomas PD, Darwesh AM, Fillmore N, Mahmoud MU, Zhang L, Gupta A, Al Batran R, Seubert JM, Lopaschuk GD, Cytosolic carnitine acetyltransferase as a source of cytosolic acetyl-CoA: a possible mechanism for regulation of cardiac energy metabolism, *Biochem J* 475(5) (2018) 959–976. [PubMed: 29438065]
- [5]. Seiler SE, Koves TR, Gooding JR, Wong KE, Stevens RD, Ilkayeva OR, Wittmann AH, DeBalsi KL, Davies MN, Lindeboom L, Schrauwen P, Schrauwen-Hinderling VB, Muoio DM, Carnitine Acetyltransferase Mitigates Metabolic Inertia and Muscle Fatigue during Exercise, *Cell Metab* 22(1) (2015) 65–76. [PubMed: 26154055]
- [6]. Carter AL, Lennon DL, Stratman FW, Increased acetyl carnitine in rat skeletal muscle as a result of high-intensity short-duration exercise. Implications in the control of pyruvate dehydrogenase activity, *FEBS Lett* 126(1) (1981) 21–4. [PubMed: 7238861]
- [7]. Overmyer KA, Evans CR, Qi NR, Minogue CE, Carson JJ, Chermide-Scabbo CJ, Koch LG, Britton SL, Pagliarini DJ, Coon JJ, Burant CF, Maximal oxidative capacity during exercise is associated with skeletal muscle fuel selection and dynamic changes in mitochondrial protein acetylation, *Cell Metab* 21(3) (2015) 468–78. [PubMed: 25738461]
- [8]. Muoio DM, Noland RC, Kovalik JP, Seiler SE, Davies MN, DeBalsi KL, Ilkayeva OR, Stevens RD, Kheterpal I, Zhang J, Covington JD, Bajpeyi S, Ravussin E, Kraus W, Koves TR, Mynatt RL, Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility, *Cell Metab* 15(5) (2012) 764–77. [PubMed: 22560225]

- [9]. Seiler SE, Martin OJ, Noland RC, Slentz DH, DeBalsi KL, Ilkayeva OR, An J, Newgard CB, Koves TR, Muoio DM, Obesity and lipid stress inhibit carnitine acetyltransferase activity, *J Lipid Res* 55(4) (2014) 635–44. [PubMed: 24395925]
- [10]. Pougovkina O, te Brinke H, Ofman R, van Cruchten AG, Kulik W, Wanders RJ, Houten SM, de Boer VC, Mitochondrial protein acetylation is driven by acetyl-CoA from fatty acid oxidation, *Hum Mol Genet* 23(13) (2014) 3513–22. [PubMed: 24516071]
- [11]. Moxley MA, Vinnakota KC, Bazil JN, Qi NR, Beard DA, Systems-level computational modeling demonstrates fuel selection switching in high capacity running and low capacity running rats, *PLoS Comput Biol* 14(2) (2018) e1005982. [PubMed: 29474500]
- [12]. Zhao S, Xu W, Jiang W, Yu W, Lin Y, Zhang T, Yao J, Zhou L, Zeng Y, Li H, Li Y, Shi J, An W, Hancock SM, He F, Qin L, Chin J, Yang P, Chen X, Lei Q, Xiong Y, Guan KL, Regulation of cellular metabolism by protein lysine acetylation, *Science* 327(5968) (2010) 1000–4. [PubMed: 20167786]
- [13]. Davies MN, Kjalarsdottir L, Thompson JW, Dubois LG, Stevens RD, Ilkayeva OR, Brosnan MJ, Rolph TP, Grimsrud PA, Muoio DM, The Acetyl Group Buffering Action of Carnitine Acetyltransferase Offsets Macronutrient-Induced Lysine Acetylation of Mitochondrial Proteins, *Cell Rep* 14(2) (2016) 243–54. [PubMed: 26748706]
- [14]. Chase JF, Tubbs PK, Some kinetic studies on the mechanism of action of carnitine acetyltransferase, *Biochem J* 99(1) (1966) 32–40. [PubMed: 5965344]
- [15]. Segel IH, *Enzyme kinetics : behavior and analysis of rapid equilibrium and steady state enzyme systems*, Wiley, New York, 1975.
- [16]. Johnson KA, A century of enzyme kinetic analysis, 1913 to 2013, *FEBS Lett* 587(17) (2013) 2753–66. [PubMed: 23850893]
- [17]. Moxley MA, Beard DA, Bazil JN, A pH-dependent kinetic model of dihydrolipoamide dehydrogenase from multiple organisms, *Biophys J* 107(12) (2014) 2993–3007. [PubMed: 25517164]
- [18]. Moxley MA, Beard DA, Bazil JN, Global Kinetic Analysis of Mammalian E3 Reveals pH-dependent NAD⁺/NADH Regulation, Physiological Kinetic Reversibility, and Catalytic Optimum, *J Biol Chem* 291(6) (2016) 2712–30. [PubMed: 26644471]
- [19]. Johnson KA, Simpson ZB, Blom T, Global kinetic explorer: a new computer program for dynamic simulation and fitting of kinetic data, *Anal Biochem* 387(1) (2009) 20–9. [PubMed: 19154726]
- [20]. Johnson KA, Simpson ZB, Blom T, FitSpace explorer: an algorithm to evaluate multidimensional parameter space in fitting kinetic data, *Anal Biochem* 387(1) (2009) 30–41. [PubMed: 19168024]
- [21]. Haynes WM, *CRC Handbook of Chemistry and Physics: A Ready-Reference Book of Chemical and Physical Data*, (2014–2015).
- [22]. Lakowicz JR, *Principles of fluorescence spectroscopy*, 3rd ed., Springer, New York, 2006.
- [23]. Hsiao YS, Jogl G, Tong L, Crystal structures of murine carnitine acetyltransferase in ternary complexes with its substrates, *J Biol Chem* 281(38) (2006) 28480–7. [PubMed: 16870616]
- [24]. Secrist JA 3rd, Barrio JR, Leonard NJ, A fluorescent modification of adenosine triphosphate with activity in enzyme systems: 1,N 6-ethenoadenosine triphosphate, *Science* 175(4022) (1972) 646–7. [PubMed: 4257930]
- [25]. Hsu KH, Powell GL, Inhibition of citrate synthase by oleoyl-CoA: a regulatory phenomenon, *Proc Natl Acad Sci U S A* 72(12) (1975) 4729–33. [PubMed: 1061066]
- [26]. Johnson KA, Fitting enzyme kinetic data with KinTek Global Kinetic Explorer, *Methods Enzymol* 467 (2009) 601–626. [PubMed: 19897109]
- [27]. The PyMOL Molecular Graphics System, Version 1.7.2 Schrödinger, LLC.
- [28]. Gadda G, Sobrado P, Kinetic Solvent Viscosity Effects as Probes for Studying the Mechanisms of Enzyme Action, *Biochemistry* 57(25) (2018) 3445–3453. [PubMed: 29874467]
- [29]. Cronin CN, The conserved serine-threonine-serine motif of the carnitine acyltransferases is involved in carnitine binding and transition-state stabilization: a site-directed mutagenesis study, *Biochem Biophys Res Commun* 238(3) (1997) 784–9. [PubMed: 9325168]
- [30]. Broderick TL, Quinney HA, Barker CC, Lopaschuk GD, Beneficial effect of carnitine on mechanical recovery of rat hearts reperfused after a transient period of global ischemia is

accompanied by a stimulation of glucose oxidation, *Circulation* 87(3) (1993) 972–81. [PubMed: 8443916]

- [31]. Sun W, Liu Q, Leng J, Zheng Y, Li J, The role of Pyruvate Dehydrogenase Complex in cardiovascular diseases, *Life Sci* 121 (2015) 97–103. [PubMed: 25498896]
- [32]. Laera L, Punzi G, Porcelli V, Gambacorta N, Trisolini L, Pierri CL, De Grassi A, CRAT missense variants cause abnormal carnitine acetyltransferase function in an early-onset case of Leigh syndrome, *Hum Mutat* 41(1) (2020) 110–114. [PubMed: 31448845]

Highlights

- Carnitine acetyltransferase, fast catalysis, requires an ordered kinetic mechanism
- Kinetic constants were determined by global fitting and kinetic simulation
- Coenzyme A pre-binding imparts a 100-fold decrease in catalysis
- Inhibition of catalysis induced by Coenzyme A pre-binding is biphasic
- Solvent viscosity experiments support product release as the rate limiting step

Figure 1. Multiwavelength monitored stopped-flow forward and reverse CrAT kinetics.

A) The forward CrAT reaction at pH 7.8 was monitored, where CrAT (1 μ M before mixing) was premixed with carnitine (2 mM before mixing) in the same syringe, and then rapidly mixed with acetyl-CoA (250 μ M after mixing) on a stopped-flow device equipped with a CCD array multiwavelength detector. **B)** The maximum difference in absorbance spectra at 232 nm is shown as a function of time for the forward direction. **C)** The reverse CrAT reaction at pH 7.8 was monitored, where CrAT (1 μ M before mixing) was premixed with acetylcarnitine (2 mM before mixing) in the same syringe, and then rapidly mixed with CoA (125 μ M after mixing) on a stopped-flow device equipped with a CCD array multiwavelength detector. **D)** The maximum difference in absorbance spectra at 232 nm is shown as a function of time for the reverse direction.

Figure 2. Rapid forward and reverse CrAT progress curves as a function of substrate concentration without premixing enzyme and substrate.

Data are circles and lines are the simulated model. Starting absorbances were normalized to the highest starting absorbance to better view curves and data. **A)** Forward CrAT progress curves as a function of carnitine, where CrAT (0.5 μM after mixing) was rapidly mixed with carnitine and a fixed concentration of acetyl-CoA (200 μM after mixing) on a stopped-flow device. **B)** Forward CrAT progress curves as a function of acetyl-CoA, where CrAT (0.5 μM after mixing) was rapidly mixed with acetyl-CoA and a fixed concentration of carnitine (1 mM after mixing). **C)** Reverse CrAT progress curves as a function of acetylcarnitine concentration, where CrAT (0.5 μM after mixing) was rapidly mixed with acetylcarnitine and a fixed concentration of CoA (200 μM after mixing) on a stopped-flow device. **D)** Reverse CrAT progress curves as a function of CoA concentration, where CrAT (0.5 μM after mixing) was rapidly mixed with CoA and a fixed concentration of acetylcarnitine (1 mM after mixing) on a stopped-flow device.

Figure 3. CrAT active site with bound CoA/carnitine and nearby tryptophan residues.

A) The CrAT crystal structure (PDB: 2H3U) shows a ternary complex between CoA and carnitine. Tryptophan residues are highlighted in green to demonstrate their proximity to the carnitine binding site. **B)** A carnitine stock solution was auto-titrated into a CrAT a solution (5 μ M) with protein fluorescence excited at 295 nm and fluorescence collected using a long pass filter (> 320 nm). Data (green circles) were fit by simulation (black line) using KinTek Global Explorer software yielding a K_d of 0.470 mM. **C)** As in (B) an acetylcarnitine stock was auto-titrated into a CrAT solution, and yielded a K_d of 1.9 mM. Panel A was made with PyMOL 2.3.3 [27].

Figure 4. Rapid forward and reverse CrAT progress curves as a function of substrate concentration with CrAT/substrate premixing.

Data are circles and lines are the simulated model. **A)** Forward CrAT progress curves as a function of carnitine concentration, where CrAT (0.5 μM after mixing) was premixed with acetyl-CoA (200 μM after mixing) in the same syringe, and then rapidly mixed with carnitine on a stopped-flow device. **B)** Forward CrAT progress curves as a function of acetyl-CoA concentration, where CrAT (0.5 μM after mixing) was premixed with carnitine (1 mM after mixing) in the same syringe, and then rapidly mixed with acetyl-CoA on a stopped-flow device. **C)** Reverse CrAT progress curves as a function of acetylcarnitine concentration, where CrAT (0.5 μM after mixing) was premixed CoA (200 μM after mixing) in the same syringe, and then rapidly mixed with acetylcarnitine on a stopped-flow device. **D)** Reverse CrAT progress curves as a function of CoA concentration, where CrAT (0.5 μM after mixing) was premixed with acetylcarnitine (1 mM after mixing) in the same syringe, and then rapidly mixed with CoA on a stopped-flow device. Starting absorbances were adjusted to better visual multiple curves in a single plot.

Figure 5. Double mixing CrAT progress curves and initial rates with acetyl-CoA or CoA pre-mixing time.

A) Forward CrAT progress curves as a function of pre-mixing time, where CrAT ($0.25 \mu\text{M}$ after mixing) was premixed with acetyl-CoA ($100 \mu\text{M}$ after mixing), and then rapidly mixed with carnitine (1mM after mixing) on a stopped-flow device. **B)** Initial rates from the experiment in (A) were determined and fit with a single exponential (dashed lines) or double (solid line) exponential equation. Parameters are given in Table 2. **C)** Reverse CrAT progress curves as a function of pre-mixing time, where CrAT ($0.25 \mu\text{M}$ after mixing) was premixed with CoA ($100 \mu\text{M}$ after mixing), and then rapidly mixed with acetylcarnitine (1mM after mixing) on a stopped-flow device. **D)** Initial rates from the experiment in (C) were determined and fitted to a single exponential (dashed lines) or double (solid line) exponential equation. Parameters are given in Table 2.

Figure 6. Global fitting double mixing CrAT progress curves and CrAT-catalyzed acetyl-CoA hydrolysis.

Data are circles and lines are the simulated model. **A)** Forward CrAT progress curves as a function of pre-mixing time, where CrAT (0.25 μ M after mixing) was pre-mixed with acetyl-CoA (100 μ M after mixing), and then rapidly mixed with carnitine on a stopped-flow device. From bottom to top: 0.023, 0.5, 1, 3, and 6 s pre-mixing time. **B)** Reverse CrAT progress curves as a function of pre-mixing time, where CrAT (0.25 μ M after mixing) was pre-mixed with CoA (100 μ M after mixing), and then rapidly mixed with acetylcarnitine on a stopped-flow device. From top to bottom: 0.023, 0.5, 1, 3, and 6 s pre-mixing time. **C)** CrAT (0.5 μ M) was mixed with acetyl-CoA (200 μ M) (magenta), in 50 mM Tris pH 7.8 and followed at 232 nm. Acetyl-CoA in 50 mM Tris pH 7.8 was followed at 232 nm (cyan) without CrAT. Assays were performed in triplicate on a plate reader. **D)** CrAT generally follows an order bi bi mechanism (green cycle) but when allowed to pre-incubate with either acetyl-CoA or CoA, slow kinetics are observed (red cycle).

Figure 7. Structural alignment of binary and ternary CrAT complexes.

A) Four CrAT complexes were aligned together. These include three CrAT ternary structures: 1) PDB ID: 2H3U (Carnitine (blue carbons) and CoA (green carbons)), 2) PDB ID: 2H3P (Carnitine (blue carbons) and acetyl-CoA (blue carbons)), 3) PDB ID: 2H3W (CoA (magenta carbons) and acyl-carnitine not shown). One binary structure PDB ID: 1NDI (CoA only; cyan carbons). Active site residues serine 554 and histidine 343 are also shown.

B) The binary-CoA (cyan carbons) in (A) was modified by adding an acetyl group to model acetyl-CoA in the same position of the binary-CoA. Distances are shown in Angstroms.

Figure was made with PyMOL 2.3.3 [27].

Figure 8. Forward and reverse CrAT progress curves as a function of solvent viscosity using glycerol as a viscogen.

Data are circles and solid lines are the uncompetitive inhibition model simulation and the dashed lines are the competitive inhibition model simulation. Forward CrAT progress curves in **A)** 1X, **B)** 2X (25% glycerol), and **C)** 3X (35% glycerol) relative solvent viscosity.

Experiments plotted in panels A-C contained a fixed amount of 1 mM carnitine and acetyl-CoA varied at 15, 30, 60, 120, 240, and 340 μM . Reverse CrAT progress curves in **D)** 1X, **E)** 2X (25% glycerol), and **F)** 3X (35% glycerol) relative solvent viscosity. Experiments plotted in panels D-F contained a fixed amount of 1 mM acetylcarnitine and CoA varied at 19, 38, 75, 125, 200, and 250 μM .

Figure 9. Initial velocities as a function of solvent viscosity.

All data were fit with Eqn. 1 (solid line) or Eqn. 2 (dashed line) using a non-linear least squares method. Initial velocities were determined from progress curve data shown in Fig. 8 and S7. **A)** Acetyl-CoA was varied as a function of glycerol at relative viscosities (η_{rel}) of 1, 1.5, 2, and 3-fold. Note that the dashed lines from fitting to Eqn. 2 are hidden due to the overlay from Eqn. 1 fitting. **B)** CoA was varied as a function of glycerol at relative viscosities (η_{rel}) of 1, 1.5, 2, and 3-fold. **C)** Acetyl-CoA was varied as a function of sucrose at relative viscosities (η_{rel}) of 1, 1.5, 2, and 3-fold. **D)** CoA was varied as a function of sucrose at relative viscosities (η_{rel}) of 1, 1.5, 2, and 3-fold.

Table. 1^aCalculated steady-state constants for the ordered bi-bi mechanism fitting

<i>Parameter</i>	Best-fit	<i>b</i> Lower bound	<i>b</i> Upper bound
$k_{cat}^f(s^{-1})$	568	389	1.01×10^3
$k_{cat}^r(s^{-1})$	376	255	548
$K_m^{Carn}(\mu M)$	81.7	0.97	298
$K_m^{AcCoA}(\mu M)$	40.7	20.6	93.9
$K_m^{CoA}(\mu M)$	6.31	2.83	13.6
$K_m^{AcCarn}(\mu M)$	109	26.1	322
$K_i^{Carn}(\mu M)$	490	389	793
$K_i^{AcCoA}(\mu M)$	164	58.1	1.73×10^3
$K_i^{CoA}(\mu M)$	102	42.2	276
$K_i^{AcCarn}(\mu M)$	1.87×10^3	1.3×10^3	2.17×10^3

^aSteady-state constants, with definitions given in eqn. S3, were calculated according to a distribution of fit rate constants as described further in Materials and Methods.

^bUpper and lower bounds were calculated from the maximum and minimum calculated parameters within a 0.83 relative χ^2 threshold described previously [20].

Table 2.

Initial rate analytical fitting as a function of double mixing time

	acetyl-CoA premixing		CoA premixing	
^a <i>Single exponential decay</i>				
$\left(y = Ae^{-k_{obs}t}\right)$	value	95% C.I.	value	95% C.I.
A	0.890	(0.694, 1.09)	0.793	(0.642, 0.945)
k_{obs} (min ⁻¹)	6.88	(0.475, 13.3)	0.965	(−0.0893, 2.02)
r^2		0.910		0.866
^a <i>Double exponential decay</i>				
$\left(y = A_1e^{-k_{obs(1)}t} + A_2e^{-k_{obs(2)}t}\right)$	value	95% C.I.	value	95% C.I.
A_1	0.442	(0.329, 0.554)	0.538	(0.326, 0.75)
A_2	0.563	(0.478, 0.649)	0.376	(0.187, 0.566)
$k_{obs(1)}$ (min ⁻¹)	61.7	(24.0, 99.4)	12.1	(1.17, 22.9)
$k_{obs(2)}$ (min ⁻¹)	0.918	(0.490, 1.35)	0.100	(−0.0151, 0.216)
r^2		0.996		0.977

^aThe left-hand side of the exponential equation (y) gives the normalized initial rate

Table 3.

Analytical fitting of initial velocity viscosity data

Condition	Parameter	value	95% C.I.
acetyl-CoA varied with glycerol as a viscogen	k_{cat} (s^{-1})	382	(348–417)
	K_{m} (μM)	21.5	(13.8–29.3)
	m	0	(–0.69 – 0.69)
	n	1.13	(0.88 – 1.37)
acetyl-CoA varied with sucrose as a viscogen	k_{cat} (s^{-1})	386	(343 – 429)
	K_{m} (μM)	23.3	(12.31 – 34.33)
	m	0.23	(–0.74–1.19)
	n	1.29	(0.87–1.71)
CoA varied with glycerol as a viscogen	k_{cat} (s^{-1})	322	(299 – 344)
	K_{m} (μM)	14.0	(8.48–19.4)
	m	0.91	(–0.47 – 2.29)
	n	1.56	(1.23 – 1.89)
CoA varied with sucrose as a viscogen	k_{cat} (s^{-1})	308	(270 – 346)
	K_{m} (μM)	12.7	(3.36 – 22.0)
	m	2.27	(–0.96 – 5.49)
	n	1.02	(0.56 – 1.47)