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Parith Wongkittichote
*Washington University School of Medicine in St. Louis*

Gary Cunningham
*Children's National Health System*

Marshall L Summar
*Children's National Health System*

Elena Pumbo
*Children's National Health System*

Patrick Forny
*University of Zurich*

*See next page for additional authors*

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Tricarboxylic acid cycle enzyme activities in a mouse model of methylmalonic aciduria

Parith Wongkittichote\textsuperscript{a,b,c}, Gary Cunningham\textsuperscript{b}, Marshall L. Summar\textsuperscript{b}, Elena Pumbo\textsuperscript{a}, Patrick Forny\textsuperscript{d,e}, Matthias R. Baumgartner\textsuperscript{d,e}, Kimberly A. Chapman\textsuperscript{a},\textsuperscript{*}

\textsuperscript{a} Children's National Rare Disease Institute, Children's National Health System, Washington DC 20010, United States
\textsuperscript{b} Department of Pediatrics, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand
\textsuperscript{c} Department of Pediatrics, St. Louis Children's Hospital, Washington University School of Medicine, St. Louis, MO, USA
\textsuperscript{d} Division of Metabolism, the Children's Research Center, The Swiss Newborn Screening Laboratory, University Children's Hospital Zurich, 8032 Zurich, Switzerland
\textsuperscript{e} The radiz–Rare Disease Initiative Zurich, Clinical Research Priority Program for Rare Diseases, the Center for Integrative Human Physiology, University of Zurich, 8006 Zurich, Switzerland

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\textbf{ABSTRACT}

Methylmalonic acidemia (MMA) is a propionate pathway disorder caused by dysfunction of the mitochondrial enzyme methylmalonyl-CoA mutase (MMUT). MMUT catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA, an anaplerotic reaction which feeds into the tricarboxylic acid (TCA) cycle. As part of the pathological mechanisms of MMA, previous studies have suggested there is decreased TCA activity due to a “toxic inhibition” of TCA cycle enzymes by MMA related metabolites, in addition to reduced anaplerosis. Here, we have utilized mitochondria isolated from livers of a mouse model of MMA (\textit{Mut}\textsuperscript{ko/ki}) and their littermate controls (\textit{Ki/wt}) to examine the amounts and enzyme functions of most of the TCA cycle enzymes. We have performed mRNA quantification, protein semi-quantitation, and enzyme activity quantification for TCA cycle enzymes in these samples. Expression profiling showed increased mRNA levels of fumarate hydratase in the \textit{Mut}\textsuperscript{ko/ki} samples, which by contrast had reduced protein levels as detected by immunoblot, while all other mRNA levels were unaltered. Immunoblotting also revealed decreased protein levels of 2-oxoglutarate dehydrogenase and malate dehydrogenase 2. Interestingly, the decreased protein amount of 2-oxoglutarate dehydrogenase was reflected in decreased activity for this enzyme while there is a trend towards decreased activity of fumarate hydratase and malate dehydrogenase 2. Citrate synthase, isocitrate dehydrogenase 2/3, succinyl-CoA synthase, and succinate dehydrogenase are not statistically different in terms of quantity of enzyme or activity. Finally, we found decreased activity when examining the function of methylmalonyl-CoA mutase in series with succinate synthase and succinate dehydrogenase in the \textit{Mut}\textsuperscript{ko/ki} mice compared to their littermate controls, as expected. This study demonstrates decreased activity of certain TCA cycle enzymes and by corollary decreased TCA cycle function, but it supports decreased protein quantity rather than “toxic inhibition” as the underlying mechanism of action.

\textbf{Summary:}
Methylmalonic acidemia (MMA) is an inborn metabolic disorder of propionate catabolism. In this disorder, toxic metabolites are considered to be the major pathogenic mechanism for acute and long-term complications. However, despite optimized therapies aimed at reducing metabolite levels, patients continue to suffer from late complications, including metabolic stroke and renal insufficiency. Since the propionate pathway feeds into the tricarboxylic acid (TCA) cycle, we investigated TCA cycle function in a constitutive MMA mouse model. We demonstrated decreased amounts of the TCA enzymes, Mdh2 and Ogdh as semi-quantified by immunoblot. Enzymatic activity of Ogdh is also decreased in the MMA mouse model compared to controls. Thus, when the enzyme amounts are decreased, we see the enzymatic activity also decreased to a similar extent for Ogdh. Further studies to elucidate the structural and/or functional links between the TCA cycle and propionate pathways might lead to new treatment approaches for MMA patients.
1. Introduction

Isolated methylmalonic acidemia (MMA, OMIM #251000, 251100, 251110) is a severe inborn error of metabolism caused by dysfunction of the propionate pathway, involved in the catabolism of odd-chain fatty acids, valine, isoleucine, methionine, threonine, and cholesterol to succinyl-CoA, which is subsequently metabolized in the tricarboxylic acid (TCA) cycle. Isolated MMA is characterized by a defect in conversion of (D-)methylmalonyl-CoA to succinyl-CoA, due to deficiency of the methylmalonyl-CoA mutase (MMUT) enzyme (E.C. 5.4.99.2), and may be caused by mutations in the MMUT gene, mutations in genes involved in production of its cofactor adenosyl-cobalamin, or mutations in MCEE (E.C. 5.1.99.1) or succinate synthetase (SUCLG1/SUCLA2), enzymes directly proximal and distal to MMUT [1,2] (Fig. 1). The patients who suffer from MMA can present in the neonatal period with coma and metabolic crisis (including severe metabolic acidosis and hyperammonemia), and if left untreated, die within the first few days of life [3,4].

With the establishment of newborn screening, MMA patients can be detected early and pre-symptomatic treatment can be achieved. However, despite early intervention, long-term complications are observed in these patients [5,6], including neurologic and neurodevelopmental abnormalities [2,7,8]. Renal insufficiency is also common among MMA patients and this might lead to end-stage renal disease [7,9]. The pathophysiology of these late complications is yet to be elucidated. Previous studies found abnormalities in various metabolic pathways, such as the electron transport system in mitochondria [10–12] and amino acid metabolism [13,14]. Mitochondrial dysfunction and oxidative stress were observed in patients with MMA and was thought to play important roles in the development of renal complications [15,16]. Two prominent mechanisms proposed to explain the long-term complications of MMA include 1) the concept of “toxic metabolites” derived from accumulation of methylmalonyl-CoA and propionyl-CoA excess interrupting, among other things, TCA enzyme function [17] and the urea cycle [18] and 2) shortage of TCA cycle intermediates. Propionyl-CoA itself has been shown to inhibit pyruvate dehydrogenase complex (PDHC) activity [10], while 2-methylcitrate, formed by a condensation reaction of propionyl-CoA and oxaloacetate, can inhibit various enzymes in the TCA cycle, such as, citrate synthase (CS), isocitrate dehydrogenase (IDH2/3) and the α-ketoglutarate dehydrogenase complex (OGDHc) [19]. In general, the toxic intermediate role in disease pathophysiology predicts abnormalities in dehydrogenase activity due to inhibition from toxins. On the other hand, impaired anaplerosis of the TCA cycle might be due to the decreased production of succinyl-CoA by MMUT in the propionate pathway, as well as by formation of 2-methylcitrate, which reduces availability of oxaloacetate [20,21].

Previously, TCA cycle enzymes have been proposed to work as a complex, called the “metabolon”, which allows metabolite channeling [22]. Although more evidence is needed, several studies provide support for the presence of the TCA cycle metabolon [23,24], as well as metabolons involved in other human metabolic pathways, including the initial steps of branched-chain amino acid metabolism [25]. If substrate channeling, created by the interaction of the enzymes which make up a metabolon unit, is necessary for effective metabolism, deficiency in the quantity of one enzyme might affect the function of the others. Since MMUT is a mitochondrial enzyme immediately proximal to the TCA cycle, it may function as part of the TCA cycle metabolon. By this hypothesis, pathological mutations in Mmut might affect the structure and/or function of the proposed TCA cycle metabolon and subsequently lead to decreased TCA cycle function.

We have used a mouse model of MMA (Mut-ko/ki) which has been described in Forny et al. [26] to model the human disease. These mice are genetic hybrids which carry a knock-out allele of the Mmut gene [27] in combination with the knock-in allele p.M698K, corresponding to the human p.M700K mutation [26]. Like the human disease, these mice (Mut-ko/ki) demonstrate elevated methylmalonic acid, propionylcarnitine, and 2-methylcitrate, but retain about 1% enzyme activity [26].

Here we demonstrate that the protein levels of OGDH, the TCA cycle enzyme is decreased in Mut-ko/ki livers compared to littermate controls, despite a lack of difference in mRNA levels. Several other TCA cycle enzymes protein levels are also decreased and these contribute to decreased specific enzyme and whole pathway activity, as measured in isolated mitochondria. These data support the finding of decreased TCA cycle activity in MMA, but suggest this effect may be due to decreased metabolon function rather than an inhibitory effect of toxic intermediates.

2. Material and methods

2.1. Tissue

Flash frozen livers were taken from methylmalonyl-CoA mutase knock-out/knock-in (Mut-ko/ki) mice that have been described in Forny et al. [26]. They are referred to as MMA or Mut-ko/ki, here. Littermates, which carry one knock-in and one wild-type allele (and have 50% Mmut expression and normal metabolite levels), were used as controls (Ctl or ki/wt). Animal experiments were performed in accordance with policies of the Veterinary Office of the State of Zurich and Swiss law on animal protection. Animal studies were approved by the Cantonal Veterinary Office Zurich under license number 202/2014. All mice were sacrificed between 30 and 40 days of life and were predominately females.
2.2. Mitochondrial isolation

Mitochondria from flash frozen livers of Ctl and Mut-ko/ki mice were isolated. Briefly, each mouse liver was ground, weighed on ice and placed in 15 mL mitochondrial isolation buffer (5 mM Tris-HCl pH 7.2, 250 mM sucrose and 1 mM EDTA, pH 7.2) and homogenized. The sample was then centrifuged at 484 g for 10 min at 4°C and the supernatant collected. The supernatant then underwent centrifugation at 9800 g for 10 min at 4°C and the pellet was collected. The pellet was resuspended in 4 mL and transferred to 2 new tubes. The samples were again centrifuged at 484 g for 10 min at 4°C and supernatant collected. The supernatant then underwent centrifugation again at 9800 g for 10 min at 4°C. The pellet was collected and resuspended in mitochondrial isolation buffer and centrifuged at 18,000 g for 5 min at 4°C. The pellet is mitochondria and can be stored at -80°C. The mitochondrial pellet was resuspended in extraction buffer (which opens mitochondria) (5 mM Tris-HCl pH 7.2, 250 mM sucrose, 1 mM EDTA, 1% IDEPAL, 1 tablet Mini protease inhibitor (Roche) per 10 mL, pH to 7.5 with glacial acetic acid) prior to use in every assay. The protein quantification of mitochondria was then performed using Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific).

2.3. Enzymes assay

All TCA enzymes were measured spectrophotometrically (xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad)) using isolated mitochondria. All measurements were done in Kinetic mode at 25°C, at the wavelengths described below for each specific assay, with measurements taken every 20 s for at least 30 cycles unless otherwise specified. Each individual enzyme assay was performed in triplicate and the resulting enzyme rates averaged. The rate was determined according to the linear portion of each pattern. Each condition (i.e. Mut-ko/ki versus Ctl/WT conditions) was assayed using three separate samples from three separate animals. Distilled Water (dW) was used as a control substituting for the enzyme substrate for each reaction in the assays shown in Fig. 2. Reaction rates were not corrected for dW rates.

2.4. Citrate synthase (Cs) (EC. 2.3.3.8)

Cs activity was measured by detecting thiobis(2-nitrobenzoic acid) (TNB), a by-product of the reaction between coenzyme A (CoA-SH) and 5,5′dithiobis(2-nitrobenzoic acid) (DTNB) since CoA-SH is released by the reaction (Suppl. Fig. S1. A). The assay is started when 1 mM oxaloacetate is added to 5 μg of mitochondrial samples mixed with reaction buffer (10 mM tris-HCl pH 8.0, 0.1 mM DTNB, 0.2 mM acetyl Co-A, 0.18% Triton-X). The production of TNB was followed spectrophotometrically at 412 nm as similarly described in Shephard et al. [28] measuring every 20 s for 30 cycles. The results were graphed and slope (which correlates to enzyme rate) was determined. The enzyme activity was then calculated.

2.5. Isocitrate dehydrogenase 2/3 (Idh2/3) (EC. 1.1.1.41 and 1.1.1.42)

The activity of Idh2/3 was determined by measuring production of NADPH at 340 nm (Suppl. Fig. S2 A). The reaction was started by adding 200 µL of reaction buffer (10 mM potassium phosphate pH 7.4, 2 mM MgCl₂, 1 mM NADP and 5 mM isocitrate) to 30 µg of mitochondria [29] and measurements taken every 20 s for 30 cycles. Each analysis was done in triplicate and the resulting slopes averaged and used to determine enzyme activity.

2.6. α-Ketoglutarate dehydrogenase complex (Ogdcb)(EC. 1.2.4.2)

The reaction mixture containing 63 mM Tris-HCl pH 7.4, 2 mM MgCl₂, 0.63 mM potassium-EDTA, 2 mM CaCl₂, 1 mM DTT, 0.2 mM TTP, 1 mM NAD +, 0.3 mM coenzyme A, 0.5% Triton-X was added to 30 µg of mitochondria samples. The reaction started by adding 1 mM α-ketoglutaric acid. The production of NADH was measured spectrophotometrically at 340 nm [30] and measurements were taken every 20 s for 30 cycles (Suppl. Fig. S3 A). Each analysis was done in triplicate and the resulting slopes averaged and used to determine enzyme activity.

2.7. Succinyl-CoA synthetase (Scs) (EC. 6.2.1.5 and 6.2.1.4)

The Scs assay was achieved by Succinyl-CoA Synthetase Activity Colorimetric Assay Kit (BioVision Inc.). The reaction buffer was mixed according to the manufacturer’s instructions and 20 µg of mitochondrial samples were added to start the reaction and followed as instructed by the assay kit (Suppl. Fig. S4 A). Each analysis was done in triplicate and the resulting slopes averaged and used to determine enzyme activity.

2.8. Succinate dehydrogenase (Sdh) (EC. 1.3.5.1)

The activity of Sdh was determined based on its ability to reduce DCPIP, an artificial electron acceptor (Suppl. Fig. S5 A). The reaction mixture contained 50 mM potassium phosphate pH 7.4, 0.14 mM DCPIP, 0.1 mM duroquinone, 0.8 mM PMS, 8 µM rotenone, and 12 mM succinate was added to 30 µg of mitochondrial samples. The assay was measured at 600 nm [31,32] every 20 s for 30 cycles. Each analysis was done in triplicate and the resulting slopes averaged and used to determine enzyme activity.

2.9. Fumarate hydratase (Fh)

The reverse reaction of Fh starts with malate and generates fumarate which was detected at OD 250 nm (Suppl. Fig. S6 A). To start the reaction, mitochondria (30 µg) were placed in reaction buffer (50 mM Na₂HPO₄, 0.1% BSA, and 50 mM malleic acid). The reaction was followed spectrophotometrically at 250 nm and measurements are taken every 20 s for 30 cycles [33]. Each analysis was done in triplicate and the resulting slopes averaged and used to determine enzyme activity.

2.10. Malate dehydrogenase 2 (Mdh2) (EC.1.1.1.37)

The reverse function of Mdh2 was measured by following the decrease of NADH at OD 340 nm (Suppl. Fig. S7 A). The reaction mixture contained 100 mM potassium phosphate pH 7.4, 10 mM MgCl₂, 0.14 mM DCPIP, 0.1 mM duroquinone, 0.8 mM PMS, 8 µM rotenone, 0.01 mM AdoCbl, and 1 mM methylmalonyl-CoA was added to 30 µg of mitochondrial samples [34] and measured at 340 nm every 15 s for 30 cycles. Each analysis was done in triplicate and resulting slopes (correlate with enzyme activity) were averaged.

2.11. Mut-Ss-Sdh coupled assay

Mmmt, succinate synthase (Ss) and Sdh were assayed in series in a coupled assay (Suppl. Fig. S10 A). Reaction buffer containing 50 mM potassium phosphate pH 7.4, 10 mM MgCl₂, 0.14 mM DCPIP, 0.1 mM duroquinone, 0.8 mM PMS, 8 µM rotenone, 0.01 mM AdoCbl, and 1 mM methylmalonyl-CoA was added to 30 µg of mitochondrial samples. The reduction of DCPIP was followed spectrophotometrically at 600 nm [31,35]). Measurements were taken every 20 s for 30 cycles. The slope of the line created correlates with the serial enzyme activity. Since the measure is from SDH there is residual activity as seen in the control with no methylmalonyl-CoA, but with all the other components of the reaction buffer.

2.12. Immunoblotting

For each sample, 5 µg protein (in mitochondrial lysate) was loaded into an Any kD™ Mini-PROTEAN™ (Bio-rad) acrylamide gel. The primary antibodies used for probing each enzyme were anti-Cs (ab96600,
Abcam, 1:5000), anti-Idh2 (ab94359, Abcam, 1:500), anti-Ogdh (ab137773, Abcam, 1:5000), anti-Suclg2 (ab187996, Abcam, 1:5000), anti-Sdha1 (ab139181, Abcam, 1:2000), anti-Fh (ab95947, Abcam, 1:2000), anti-Mdh2 (ab96193, Abcam, 1:1000), anti-Pcca (A304-324A, Bethyl, 1:5000), anti-Vdac1 (ab15895, Abcam, 1:2000). Anti-Pccb (1:4000) and anti-Mut (1:5000), were made by Bio-Synthesis, Lewisville, TX, USA against peptides containing amino acids \( \text{NH}_2-\text{CRLRATFAGLYSSLDVGEEGDQ}-\text{OH} \) for Pccb and \( \text{NH}_2-\text{CPEWAALAKKQLKGKNPED}-\text{OH} \) for Mut. Chicken anti-Rabbit IgG-HRP (sc-2955, Santa Cruz, 1:2000) was used as secondary antibody.

Protein quantification was performed by measuring the amount of absorbance on a ChemiDoc™ MP Imaging System (170–8280; Bio-Rad) using Image Lab Software 5.1 by BioRad.

All immunoblot normalizations were performed using Vdac1, a mitochondrial membrane voltage dependent anion channel which is independent of the propionate and TCA cycle pathways, as loading control.

**Enzyme rates calculation:**

\[
\text{Enzyme rate} = \frac{\text{Unit}}{(m)g \text{ protein}} = \frac{\mu \text{mol}}{\text{min}} \times (m) g^{-1}
\]

The slope of the measured analyte on the spectrophotometer is equal to the rate of the conversion (measured in units \( \ast \text{sec}^{-1} \)) and so slope \( (60 \ast \mu \text{mol} \ast \text{min}^{-1} \ast \text{mg}^{-1}) \) = slope \( 60 \ast \mu \text{mol} \ast \text{min}^{-1} \ast \text{(amount of mitochondrial protein or total protein used for assay = mg}^{-1}) \) is equal to the enzyme rate. All enzymatic figures use enzyme rate measured as \( \mu \text{mol} \ast \text{sec}^{-1} \) in which the amount of protein used is the amount of protein measured in mitochondrial lysate.

### 2.13. RNA isolation and expression profiling

RNA was isolated from two additional livers from each of the Mut-ko/ki and Ctl mice using the mirVANA isolation kit (Ambion, Life technologies) following manufacturer’s instructions. RNA was analyzed for quality by electrophenogram and Northern blot prior to use to confirm that the RNA was of good quality as is standard in our institution. Expression was determined using the Clarion S mouse Array (Applied Biosystems). Fold change, \( p \)-values (with Bonferroni correction) and FDR \( p \) values were determined for 29, 129 probes. Data are all loaded under GEO number: GSE121060.

### 2.14. Statistics

All enzyme assays were done in triplicates in livers isolated from three separate animals (3 from Mut-ki/ko and 3 from Ctl animals). Means and standard deviations were calculated for all assays. Student t-tests were used to determine statistical significance \( p < .05 \). Linear regression was done to evaluate the ratio of enzyme activity versus the normalized quantity of enzyme detected by immunoblot. Calculations were performed using GraphPad Prism v8.

### 3. Results

Here, we set out to define the effect of loss of Mmut on TCA cycle proteins/function. We used flash frozen liver taken from mice with a defect in methylmalonyl-CoA mutase (Mut-ko/ki, MMA) and compared them to littermate controls (ki/wt, Ctl).
Table 1
Amounts measured by Clarion S mouse Array (Applied Biosystems) of mRNA listed in the order of significance by p-value. Fold change (Log2) is also listed for each of the enzymes including Mmut, Fh, Idh2, Mdh2, Sdha, Cs, Ogdh, SucIg2, Vdac1.

| Gene symbol | Mut-ki/ko mRNA (Avg log2) | Ctl mRNA (Avg log2) | Fold change | P-val |
|-------------|--------------------------|---------------------|-------------|-------|
| Mmut        | 6.59 ± 0.37              | 9.48 ± 1.37         | −7.41       | 0.0119 |
| Fh          | 1.27 ± 0.32              | 10.22 ± 0.03        | 2.07        | 0.0138 |
| Idh2        | 10.36 ± 0.4              | 9.51 ± 0.15         | 1.79        | 0.0419 |
| Mdh2        | 7.81 ± 0.76              | 9.01 ± 0.97         | −2.3        | 0.1196 |
| Sdha        | 7.22 ± 0.82              | 7.77 ± 0.01         | −1.47       | 0.2771 |
| Cs          | 10.29 ± 0.59             | 10.54 ± 0.47        | −1.19       | 0.5829 |
| Ogdh        | 9.14 ± 0.76              | 8.91 ± 0.15         | 1.17        | 0.6206 |
| SucIg2      | 9.82 ± 0.42              | 9.91 ± 0.66         | −1.06       | 0.8477 |
| Vdac1       | 9.84 ± 0.54              | 9.88 ± 0.82         | -1.03       | 0.9402 |

* p value < .05.

To look at whether quantity of enzyme impacted activity, we measured levels of mRNA by microarray and protein amounts by immunoblot. Tables 1 and 2 summarize the mRNA expression results listing fold changes, Average log2 levels (Avg log2) and p-values for Ctl liver samples (Avg log2) and the Mut-ki/ko model (Mut-ki/ko Avg log2). Table 2 also lists the calculated amount of mRNA detected based on the standard Log2 average levels as reported standardly as mRNA quantification from microarrays seen in Table 1. Briefly, the Mut-ki/ko and Ctl had differences of expression of two-fold or more for Fh, Mdh2 and Mmut. More specifically, the Mut-ki/ko compared to the Ctl had 2.07-fold more Fh mRNA detected with a p value of 0.014. On the other hand, the liver from Ctl sample had 2.3-fold increased expression in Mdh2 than the Mut-ki/ko, but this difference does not meet the criteria for a significant p value. Mmut mRNA is significantly different in the Mut-ki/ko mouse compared to Ctl as is expected given its genotypy with a fold change of −7.41. All other enzymes do not have fold changes greater than (or less than) 2 as well as non-significant p values.

To determine whether these differences correlate with protein amounts, we used semi-quantitative immunoblotting as measured by the Chemdoc system of each TCA cycle protein. Isolated mitochondria from isolated, partially opened mitochondria. The enzymatic activity of individual TCA cycle enzymes in samples prepared from flash frozen liver from mice (Ctl and Mut-ki/ko) and the Ctl mouse liver samples. In general, protein levels appear to better correlate to enzyme activity than mRNA amounts.

Table 2
Comparison of quantitation of mRNA, protein, and enzyme activity identified for the TCA enzymes measured and Mmut in the Mut-ki/ko and the Ctl mouse liver samples. In general, protein levels appear to better correlate to enzyme activity than mRNA amounts.

| Enzyme | Mut-ki/ko mRNA (Avg log2) levels | Ctl mRNA (Avg log2) levels | Mut-ki/ko ratio with Ctl mRNA ratio | Mut-ki/ko ratio with Protein (Amt/Amt Vdac1) | Mut-ki/ko ratio with Protein (Amt/Amt Vdac1) | Mut-ki/ko ratio with Protein (Amt/Amt Vdac1) | Mut-ki/ko ratio with Protein (Amt/Amt Vdac1) | Ki/wt Protein (nmol* s−1 * mg−1) | Ki/wt Enzyme (nmol* s−1 * mg−1) | Ki/wt Enzyme (nmol* s−1 * mg−1) |
|--------|---------------------------------|---------------------------|-----------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------|----------------------------------|----------------------------------|
| CS     | 10.29 ± 0.59                    | 10.54 ± 0.47              | 0.97                              | 0.96 ± 0.18                                 | 1.0 ± 0.12                                 | 0.95                                       | 68                          | 62                              | 62                               |
| IDH2(3) (Idh2) | 10.36 ± 0.4                    | 9.51 ± 0.15               | 1.09                              | 0.75 ± 0.15                                 | 1.02 ± 0.22                               | 0.74                                       | 43                          | 50                              | 50                               |
| OGDHc  | 9.14 ± 0.76                    | 8.91 ± 0.15               | 1.03                              | 0.58                                        | 1.01                                       | 0.57                                       | 1.2                         | 1.7                             | 1.7                              |
| SCS    | 9.82 ± 0.42                    | 9.91 ± 0.66               | 0.99                              | 0.95                                        | 1.01                                       | 0.93                                       | 140                         | 130                             | 130                              |
| SDH1 (Sdha) | 7.22 ± 0.82                    | 7.77 ± 0.01               | 0.92                              | 0.97                                        | 1.04                                       | 0.67                                       | 15                          | 16                              | 16                               |
| Mdh2   | 11.27 ± 0.22                   | 10.22 ± 0.03              | 1.1                               | 0.57                                        | 1                           | 0.57                                       | 7.8                        | 12                              | 12                               |
| Mut (via SS-SDH1) | 6.59 ± 0.37                 | 9.48 ± 1.37               | 0.69                              | 0.49                                        | 1                           | 0.49                                       | 0.293                      | 1.033                           | 0.28                             |

* p < .05.
** p < .01.
enzyme activity between that of the Mut-ko/ki mouse and Ctl for Ogdhc which appears to be explained by amount of enzyme available. We observed no statistical differences in the quantity of most TCA enzyme (excluding Ogdh, Mdh2, and Fh) in the Mut-ko/ki and Ctl samples. In our hands, Ogdh, Fh, and Mdh2 show significant differences in quantity in the Mut-ko/ki mouse and Ogdhc also shows this in measured enzymatic rate. Increased statistical power from a larger number of samples may have allowed demonstration of a significant decrease in Fh and Mdh2 activity as well, since the difference in the amount of protein was small and the difference in activity would be expected to be small as well.

MMA’s long-term complications are hypothesized to be a result of the accumulation of toxic intermediates (e.g. propionyl-CoA, methylmalonyl-CoA, 3-hydroxypropionate and 2-methylcitrate, among others) which lead to abnormalities in enzyme functions resulting in decreased activity. The TCA dehydrogenases are a particularly concerning direct target [10,36–38]. In our study, we note some variation in enzyme function, but this variation correlates with the amount of enzyme observed (Fig. 2, Fig. 3, and Table 2). Here we see decreased OGDH activity and amounts, this could greatly impact the turnover in the TCA cycle since OGDH is a known regulator as well as would be a choke-point.

Several studies have implicated the toxic intermediates from dysfunctional Mmut causing dehydrogenase dysfunction [10,19,39]. The Mut-ko/ki mice used here have elevated markers of disease include methylmalonate and propionylcarnitine to acetylcarnitine ratios which are associated with elevated propionyl-CoA [26] and so we expect that if such an inhibition of enzyme activity could be observed, we should have been able to detect it in this model. In our samples, the dehydrogenases (Ogdh, Idh2/3, Mdh2, and Sdh) do not have greater inhibition in the Mut-ko/ki mouse samples than other enzymes in the TCA cycle when activity is corrected for enzyme availability (Suppl. Fig.S8). This provides evidence against the toxic inhibitor hypothesis. However, it does not address whether amount of enzyme available for function is impacted by these toxins or whether higher amounts of these toxins would change this result.

There are some limitations to our study. Of note, we choose to not establish the aconitase assay since its iron‑sulfur complex becomes dissociated with freezing [40] and felt that this assay was less informative at baseline. We acknowledge that these are flash frozen samples whereas fresh samples may potentially be better for assays since they are less likely to be impacted by freezing, but the goal was to demonstrate activity using this model so that other samples (e.g. human flash-frozen liver samples) which are not typically available in fresh states can be analyzed.

We recognize that fasting versus fed state can impact TCA enzyme...
activity, but here we have harvested the organisms from Ctrl and Mut-ko/k mice at the same time under the same conditions (fed state). Further studies are necessary to explore differences in quantitation under different conditions. There were no differences detected between female and male mice for enzyme activity in preliminary studies, here all are female mice.

In this paper, we have assayed TCA enzymes from flash frozen material from Ctrl and Mut-ko/k livers proving that this can be done on other flash frozen samples. We observed that TCA enzyme activity does vary for some enzymes, but this variation correlates with enzyme quantity. Finally, for Ogdl, the enzyme with the most different activity between the Mut-ko/k and Ctrl tissues, we have demonstrated that the protein quantitation differences are not reflected in the level of transcription. This suggests either changes in post-transcriptional processing or increased degradation of enzyme. Other enzymes in our study have differences at the level of transcription which reflect in differences in protein amounts.

These studies help clarify how complicated TCA cycle regulation is with differing levels of enzymes from the same amount of mitochondrial protein in the extract. This impacts the development of therapies for MMA, since the decreases in key elements of energy production may affect efforts in anaplerotic manipulation. These studies support the observation that MMA is a complex enzymatic phenotype with both upstream and downstream consequences.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jymgme.2019.10.007.

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