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Propionyl-CoA and Adenosylcobalamin Metabolism in *C. elegans* : Evidence for a Role of Methylmalonyl-CoA Epimerase in Intermediary Metabolism

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Abstract

We have utilized *Caenorhabditis elegans* to study human methylmalonic acidemia. Using bioinformatics, a full complement of mammalian homologues for the conversion of propionyl-CoA to succinyl-CoA in the genome of *C. elegans*, including propionyl-CoA carboxylase subunits A and B (*pcca-1*, *pccb-1*), methylmalonic acidemia cobalamin A complementation group (*mmaa-1*), co(I) balamin adenosyltransferase (*mmab-1*), MMACHC (*cblc-1*), methylmalonyl-CoA epimerase (*mce-1*) and methylmalonyl-CoA mutase (*mmcm-1*) were identified. To verify predictions that the entire intracellular adenosylcobalamin metabolic pathway existed and was functional, the kinetic properties of the *C. elegans mmcm-1* were examined. RNA interference against *mmcm-1*, *mmab-1*, *mmaa-1* in the presence of propionic acid revealed a chemical phenotype of increased methylmalonic acid; deletion mutants of *mmcm-1*, *mmab-1* and *mce-1* displayed reduced 1-[¹⁴C]-propionate incorporation into macromolecules. The mutants produced increased amounts of methylmalonic acid in the culture medium, proving that a functional block in the pathway caused metabolite accumulation. Lentiviral delivery of the *C. elegans mmcm-1* into fibroblasts derived from a patient with *mut^o* class methylmalonic acidemia could partially restore propionate flux. The *C. elegans mce-1* deletion mutant demonstrates for the first time that a lesion at the racemase step of methylmalonyl-CoA metabolism can functionally impair flux through the methylmalonyl-CoA mutase pathway and suggests that malfunction of MCEE may cause methylmalonic acidemia in humans. The *C. elegans* system we describe represents the first lower metazoan model organism of mammalian propionate spectrum disorders and demonstrates that mass spectrometry can be employed to study a small molecule chemical phenotype in *C. elegans* RNAi and deletion mutants.

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Keywords

propionate metabolism; methylmalonic acidemia; methylmalonyl-CoA epimerase; methylmalonyl-CoA mutase; cobalamin; *C. elegans*

Introduction

The metabolism of propionyl-CoA to succinyl-CoA via the formation and isomerization of methylmalonyl-CoA is a critical metabolic pathway in humans (Figure 1). Defective conversion of L-methylmalonyl-CoA to succinyl-CoA in the mitochondrial matrix underlies the etiology of the hereditary methylmalonic acidemias (MMAemia, MIM 251000 & MIM 251100), a heterogeneous group of inborn errors of metabolism [1]. Patients deficient in methylmalonyl-CoA mutase (MUT) or adenosylcobalamin, the enzymatic cofactor, accumulate methylmalonic acid in their tissues and body fluids, and display secondary metabolic perturbations such as hyperglycemia, hyperammonemia and intermittent hypoglycemia [1]. Despite meticulous dietary control, affected individuals exhibit extreme metabolic instability and suffer from severe complications, such as developmental delay, renal disease, pancreatitis and metabolic infarction of the basal ganglia [2–5]. The pathophysiology of these processes and disease complications remain poorly defined.

A common feature of cell lines derived from *MUT*, *MMAA*, *MMAB* and *cblD* variant 2 deficient patients is diminished 1-[¹⁴C] propionate incorporation into macromolecules, an indirect assay of propionate flux through the pathway depicted in Figure 1 [11,12].

The function of the methylmalonyl-CoA epimerase or racemase (MCEE) *in vivo* is uncertain. One older report proposed that patients who harbor mutations at this locus are protected from developing methylmalonic acidemia because a free MMA shunt bypassed the reaction [13]. The striking conservation of the gene throughout the phyla suggests that the gene product may play a critical role in intermediary metabolism [14].

The methylmalonyl-CoA epimerase (*mce-1*) of *C. elegans* has been studied [15]. A deletion mutant has no phenotype and exhibits relative resistance to oxidative stress. While the gene product has been demonstrated to possess methylmalonyl-CoA epimerase activity *in vitro*, the biochemical phenotype of the mutant has not been evaluated. Specifically, whether it displays a functional impairment of 1-[¹⁴C]-propionate incorporation into macromolecules, suggesting an active role in propionyl- and methylmalonyl-CoA metabolism, remains unknown. Because there are no definitive reports of MMA patients with MCEE gene mutations, and no other animal models of MCEE deficiency exist, the role of the MCEE gene in methylmalonyl-CoA metabolism remains mysterious.

Methylmalonic acidemia has been difficult to study in model organisms. Earlier efforts have used nutritional [16] and cobalamin-analog treatments [17] to induce methylmalonic aciduria in rats. Both of these treatments are artificial and produce an incomplete block, especially in the case of nutritional deficiency, causing combined methionine synthase and methylmalonyl-CoA mutase functional impairment. Establishing tractable model organism systems to study methylmalonic acidemia will be required to better understand the symptoms and complications associated with this group of disorders, develop new therapies and further define genes that may play a role in methylmalonyl-CoA and cobalamin metabolism. To date, knock-out murine models of methylmalonyl-CoA mutase have displayed a severe neonatal phenotype with uniform mortality by 24–36 hours of life [18,19]. Organisms more amenable to genetic manipulations such as yeast and *Drosophila* do not possess cobalamin-dependent metabolic

enzymes or utilize alternative pathways for propionyl-CoA metabolism, such as the methylcitrate cycle [20].

In the present report, we employed a combination of informatic, genomic, biochemical, and metabolic analyses to identify and characterize *C. elegans* genes predicted to participate in methylmalonyl-CoA metabolism. In addition to providing direct biochemical evidence for methylmalonyl-CoA mutase activity and adenosylcobalamin synthetic capacity, we show that *C. elegans* can be used to define the function of gene products previously suspected to participate in methylmalonyl-CoA metabolism in man, particularly methylmalonyl-CoA epimerase. We propose that *C. elegans* may be used in genetic and genomic efforts to identify genes that influence cobalamin and methylmalonyl-CoA metabolism and demonstrate the utility of mass spectrometry to study chemical phenotypes in *C. elegans*.

Materials and Methods

Strains

C. elegans Bristol N2, RB1434 *mmcm-1(ok1637)*, RB1347 *mmab-1(ok1493)*, VC974 *mmab-1(ok1484)* and RB512 *mce-1(ok243)* nematodes were obtained from the Caenorhabditis Genetics Center, University of Minnesota, Minneapolis, MN, USA. Deletion mutants RB1434, RB1347 and RB512 were generated by the Oklahoma Medical Research Foundation (OMRF) Knockout Group, Oklahoma City, OK, USA. Deletion mutant VC974 was isolated by the Reverse Genetics Core facility at the University of British Columbia, Vancouver, Canada. Nematodes were grown at 20 °C on nematode growth media (NGM) plates using *E. coli* strain OP50 as a food source [21].

Bioinformatics and Sequence Analysis

The BLASTP searches were performed against WormBase (www.wormbase.org) using protein sequences from the NCBI (www.ncbi.nih.gov) known to be involved in the conversion of propionyl-CoA to succinyl-CoA in humans. Reciprocal comparisons yielded identical results. The sequence accession numbers are displayed in Table 1.

Radioactive Incorporation Study

10–20 mg of *C. elegans* were incubated with 2 µCi of [1–¹⁴C] sodium propionate (PerkinElmer, Boston, MA) in 500 µl of PBS with 50 µg/ml of kanamycin for 24 hours. Worms were examined for viability and re-suspended in 500 µl of 5% trichloroacetic acid (TCA) solution, heated for 10 minutes at 85°C and then placed on ice for 20 minutes. Insoluble material was concentrated by centrifugation at 16,060 × g for 10 minutes. The pellet was rinsed with 500 µl of ice-cold 5% TCA, resuspended in 500 µL of 1 N NaOH and heated at 85°C for 15 minutes. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Incorporation of radioactive isotopes was measured using Scintillation System LS6500 (Beckman Instruments, Fullerton, CA).

Isolation of cDNA and *C. elegans* homologues

RNA was isolated from a mixed worm population using Rneasy Mini Kit (Qiagen Inc., Valencia, CA). Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was used to generate single strand cDNA libraries. The cDNAs for *mmcm-1*, *mmaa-1*, *mmab-1*, *pcca-1*, *pccb-1* and *mce-1* were isolated by PCR using primers designed against sequences obtained from WormBase. The forward and reverse primers were designed with restriction enzyme sites for directional cloning; the forward primer also contained a Kozak consensus sequence for expression. PCR products were cloned into pCR 2.1 TOPO vector

(Invitrogen, Carlsbad, CA) using TA cloning. Clones were screened by PCR and positive clones were sequenced.

Isolation of Break Point Sequences in *C. elegans* Mutants

DNA was isolated from *C. elegans* using DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). The genomic DNA from each mutant underwent PCR using the inner left and inner right primers designed by the *C. elegans* Knockout Project at OMRF for mutant screening. PCR products were cloned into pCR 2.1 TOPO vector (Invitrogen, Carlsbad, CA) using TA cloning. Five clones from each mutant strain were then sequenced and compared to the genomic DNA sequence to determine the site of the deletion. The sequences have been deposited in Wormbase under the respective gene entries.

RNA Interference

mmcm-1, *mmaa-1*, *mmab-1*, *pcca-1*, *pccb-1* and *mce-1* were cloned into the RNAi vector pPD129.36 and transformed into *E. coli* strain OP50 [22]. Feeding RNAi was performed as described [23]. The strains were grown overnight from individual colonies in LB with 100 µg/ml of ampicillin at 37° C. One milliliter of overnight culture was then placed in 30 ml of LB with 100 µg/ml of ampicillin and grown to OD of 0.4 absorbance units at 595 nm. The culture was induced with 0.4 mM IPTG for an additional 4 hours. Bacteria were concentrated by centrifugation and plated on 100 mm NGM agar plate with 12.5 µg/ml of tetracycline, 100 µg/ml of ampicillin and 1 mM IPTG. Strains were grown overnight at room temperature. The following day two 100 mM plates per strain were seeded with *C. elegans* eggs prepared using standard methods. Nematodes were grown at 20° C until plates were cleared.

Loading studies

Nematodes were washed from plates (two plates per strain) after feeding RNAi with 6 mL phosphate buffered saline (PBS) and purified via a sucrose gradient (30% sucrose in ultra-pure water). The animals were placed in 1.5 ml centrifuge tubes and suspended with 5 mM of sodium propionate (Sigma) in 500 µL PBS with 50 µg/mL kanamycin incubated at 20° C for 48 hours. The worms were centrifuged and the supernatant was removed for GC/MS analysis. Metabolite levels were normalized to protein concentration of the pellet or wet weight.

Gas chromatography/mass spectrometry (GC/MS)

Methylmalonic acid concentrations were determined using capillary gas chromatography-mass spectroscopy with stable isotopic internal calibration as described [24]. In some experiments, organic acids were purified after perchloric acid precipitation of macromolecules and anion exchange column chromatography [25].

Mitochondrial preparations

Isolation of intact mitochondria from *C. elegans* was performed as previously described [26], with the modifications detailed below, to obtain well-functioning mitochondria. A total of 1 to 3 g of freshly washed adult worms were washed twice with 10 ml MSM-E [150 ml MSM (40.08 g mannitol, 23.96 g sucrose, 1.047 g MOPS, 3 ml 0.1M EDTA, pH 7.4 per liter] and resuspended by centrifugation (300 × g, 10 min, 4°C). A glass Potter/Elvehjem tissue grinder with a Teflon (DuPont, Wilmington, DE) pestle (Wheaton, Millville, NJ) was then used for initial rupture: 3 slow strokes at 400 rpm. Proteinase subtilisin A (Sigma, MO) was added to the homogenate (5 mg per gram of worms) and stirred for 10 minutes on ice. Immediately afterward, the slurry was homogenized in the same apparatus as before, with 10 slow strokes at 400 rpm. An equal volume of MSM-EB (50 ml MSM-E + 0.2 g defatted bovine serum albumin) was added and the homogenate was centrifuged (300 × g, 10 min, 4°C). The supernatant was pipetted into a fresh centrifuge tube, while the pellet was re-extracted in the

same manner as described above. Centrifugation ($7000 \times g$, 10 min, 4°C) of the supernatants from both the original and re-extracted homogenizations yielded mitochondrial pellets, which were resuspended in 10 ml MSM-E, centrifuged ($7000 \times g$, 10 min, 4°C), and then resuspended in 10 ml MSM, and centrifuged as before. The final mitochondrial pellet was resuspended in approximately 100 μl of MSM (per ~ 2 g of fresh-washed adult worm pellet initially used). Total protein concentration was determined by the Lowry assay with bovine serum albumin (0–100 μg) used as standard [27]. Two microliters of protease inhibitor cocktail (P8340, Sigma, MO) were added per 100 μl mitochondrial preparation. Oxidative phosphorylation assays on the purified mitochondrial preparations verified functional activity. Isolated mitochondria were frozen at -80°C prior to enzymatic analyses.

Expression of *C. elegans* mmcm-1 in yeast

A sequence verified, full-length *C. elegans* mmcm-1 cDNA was cloned into the pYES-DEST52 Gateway vector (Invitrogen, Carlsbad, CA) and transformed into strain INVSC1. The vector carries a URA selection marker and uses the GAL4 promoter to drive expression of the insert. Expression was induced after growth in galactose containing media and confirmed by comparison to a *LacZ* expression control and enzymatic assay for the methylmalonyl-CoA mutase gene product. Lysates were prepared by homogenization with acid-washed glass beads and subsequent centrifugation and were used for enzyme kinetic assays as described [23].

Enzymatic Assays and Kinetics

Methylmalonyl-CoA mutase activity was assayed using a radioactive succinate thiokinase-linked assay as described [28]. The assay mixture contained: 95 μl of D/L-[methyl- ^{14}C] methylmalonyl-CoA (150 μCi per mmole), 1 mM in 3 mM HCl], 45 μl of Tris-HCl buffer, 0.5 M, pH 9, 45 μl MgCl_2 , 0.1 M, 45 μl GDP, 20 mM, 50 μl adenosylcobalamin (variable concentration), 20 μl of succinate-CoA ligase (succinyl thiokinase) prepared at 5 units/ml, yeast cell lysate or mitochondrial preparation extract (variable volume) and water to bring the volume to 450 μl . The tubes were incubated in a 30°C dry block for 30 minutes (in dark). The reactions were stopped by acidification and extracted with ethylacetate. An aliquot was then counted by liquid scintillography to determine activity. The wild-type murine enzyme, expressed in an identical fashion in yeast, served as a control for the kinetic studies. Assays were performed in triplicate for all concentration points and the kinetics repeated twice. Kinetic constants were determined by nonlinear regression modeling of the kinetic data and regression statistics were calculated as previously described [29].

Lentiviral Correction

The *C. elegans* mmcm-1 cDNA was cloned into pLenti6/V5-DEST (Invitrogen, Carlsbad, CA) using Gateway LR Clonase enzyme mix (Invitrogen, Carlsbad, CA). A replication-incompetent, HIV-based lentivirus was generated by using the ViraPower Lentiviral Expression System (Invitrogen, Carlsbad, CA). The viral construct has the mmcm-1 gene driven by the CMV promoter; the backbone also has a blasticidin cassette driven by the E7 promoter. A control virus expressing EGFP was also prepared and used in parallel for correction experiments. Human *mut*⁰ fibroblast cell lines were transduced with virus containing either the *C. elegans* mmcm-1 or EGFP. The transduced cells were selected and expanded in DMEM with 5% fetal bovine serum containing 2.5 $\mu\text{g/ml}$ blasticidin prior to 1-[^{14}C]-propionate incorporation assays.

Results

Protein Blast searches of human proteins involved in propionate metabolism against Wormbase identified unique worm homologues for each gene product (Table1). The homologues have been named in accordance with *C. elegans* nomenclature

(<http://biosci.umn.edu/CGC/Nomenclature/nomenguid.htm>). The high degree of similarity suggests that these proteins are functionally related. MUT, PCCB, PCCA, MMAA, MCEE, MMAB and MMAHC have similarities of 97.8, 95.3, 94.6, 82.5, 79.6 53.7% and 52% with the worm homologues (Table1). The functional domains of the predicted protein products were also highly conserved between species. This would be expected for MUT, PCCA and PCCB because of the high degree of similarity seen at the amino acid level. MMAA, MCEE and MMAB, which had a lower degree of similarity, also had conservation of all known domains between species. The *C. elegans mmab-1* gene product only has a similarity of 53.7%, but the adenosylcobalamin transferase domain of this protein displays 97.6% similarity with the same domain in the human enzyme. Such protein domain conservation may be more indicative of proteins with similar function than a simple residue alignment. The high degree of similarity and domain conservation between the human enzymes involved in propionate metabolism and *C. elegans* proteins would seem to be unlikely, unless enzymatic function was conserved. As a secondary confirmation that cobalamin metabolic pathways might be intact in *C. elegans*, we performed similar searches to identify genes that might participate in methylcobalamin metabolism. Single homologues of methionine synthase (5-methyltetrahydrofolate--homocysteine methyltransferase) and (vitamin-B12 dependent methionine synthase, MS), methionine synthase reductase (MSR) appear to be present with a similar degree of protein and domain conservation; WP: CE01609 is 80% similar to human methionine synthase (Blast e-value=0.0) and WP: CE00868 is 50% similar to methionine synthase reductase (Blast e-value=1.5e-75). However, homologues of transcobalamin I, transcobalamin II, intrinsic factor, and the *E. coli* periplasmic binding protein btuF were not detected. Queries with cubilin and megalin produced a non-specific alignment to a number of *C. elegans* genes in the EGF-like domains of these genes.

We first attempted to verify the existence of functional *C. elegans* homologues of propionate metabolism genes, which were identified using bioinformatics, using an RNAi approach. RNAi directed against *mmcm-1*, *mmaa-1* and *mmab-1* cause the accumulation of methylmalonic acid in the supernatant at 2–3 times the levels seen in the wild-type nematodes (Figure 2). Interference against *pccb-1* was used as a negative control and produced no increase in MMA versus the wild-type strain. Methylmalonic acid levels did not differ from controls, probably because *pccb-1* RNAi blocks the conversion of propionyl-CoA to D-methylmalonyl-CoA. Bacterial RNAi feeding strains were loaded with propionate in the same manner as nematodes and did not exhibit levels of methylmalonic acid above wild-type nematodes (data not shown) demonstrating that the bacteria were not producing methylmalonic acid in our experiment. Attempts were made to clone *pcca-1* and *mce-1* into RNAi vectors, but in bacteria, these vectors were unstable and toxic, respectively.

Three *C. elegans* deletion mutants suspected to remove portions of genes identified in our bioinformatic analysis, *mmcm-1* and *mmab-1*, were studied and the deletion breakpoints delineated when not known. The *C. elegans mmcm-1* deletion mutant, strain RB1434, has a portion of exon 2 and all of exon 3 deleted. Exon 3 appears to encode a portion of the highly conserved coenzyme A binding pocket and would be predicted to produce a non-functional protein. RT-PCR performed on *mmcm-1* deletion mutants did not detect mRNA (data not shown). Based on the RT-PCR results, which showed no *mmcm-1* RNA transcript, and the neonatal lethal phenotype observed in mice with a similar deletion, the *C. elegans mmcm-1* mutant would be predicted to have no enzymatic activity. Strain RB 1347 harbors a deletion of the first exon of the putative *mmab-1* gene. The second exon of *mmab-1* begins with a start codon and expression studies to characterize the RNA made by this mutant were not pursued. The deletion of *mmab-1* harbored in strain VC974 has been described by the generating laboratory and appears to remove the C-terminal exon that has high homology with the adenosyltransferase domain. Strain RB512 has previously been shown to harbor a deletion of

the *mce-1* gene [15]. Propionate incorporation and metabolic analysis of the *mce-1* mutant have not been previously described.

In order to verify the phenotypes observed in our RNAi treated animals, we conducted 1- ^{14}C -propionate incorporation studies on the *C. elegans* deletion mutants. 1- ^{14}C -propionate incorporation studies, routinely used to study human mutant cell lines [11,12], were adapted to detect blocks in propionate metabolism of *C. elegans* as described above. Radioactive incorporation studies with the N2 strain showed that *C. elegans* incorporates 1- ^{14}C -propionate from propionate into protein in amounts similar to those seen in human cells [11 $\pm$ 4 nmols/mg/18 hrs] [30] (Figure 3). Furthermore, a robust stimulation of incorporation was observed when hydroxycobalamin (OH-Cbl) was added to the medium in excess. *C. elegans mmcm-1*, *mmab-1* and *mce-1* deletion mutants all exhibited a diminished ability to incorporate 1- ^{14}C -propionate into protein as compared to wild-type nematodes (Figure 3). The N-terminal deletion mutant *mmab-1(ok1493)* displayed a less severe block than the C-terminal mutant *mmab-1(ok1484)*, possibly because the C-terminal deletion removes a domain critical to *mmab-1* function as the sequence analysis would suggest. The *mce-1*, *mmcm-1*, and *mmab-1(ok1484)* mutant animals exhibited a $> 80\%$ decrease in the ability to incorporate propionate relative to wild-type animal. A similar decrease in 1- ^{14}C -propionate flux can be seen in human *mut⁰* fibroblasts when compared to wild-type controls.

To assess the biochemical response to precursor loading, we incubated the N2, *mmcm-1*, *mmab-1(ok1484, ok1493)* and *mce-1* mutant strains with propionic acid and measured methylmalonic acid in the supernatant (Figure 4). Preliminary studies suggested methylmalonic acid was easily detected in the supernatant, which ranged in concentration from 0.5–10 μM and was present at higher concentrations than in whole cell worm extracts. While both the wild-type and *mmcm-1* animals produce an increased amount of methylmalonic acid after loading, the *mmcm-1* mutant accumulated 17 times more methylmalonic acid than the wild-type strain (Figure 4). The *mmab-1(ok1484, ok1493)* and *mce-1* mutant strains show approximately a two-fold increase in the accumulation of methylmalonic acid in comparison to wild-type animals. Kanamycin treatment provided a bactericidal effect on the residual feeding strain to assure bacteria had no effect on metabolite measurements; growth assays of the bacteria that were incubated for 24 hours in kanamycin showed uniform and complete sensitivity and failed to incorporate C^{14} propionate or produce metabolites. Furthermore, metabolic measurements of the supernatant from the wash steps of purification, before and after propionic acid loading indicating that the bacteria were not producing MMA (data not shown).

Attempts to demonstrate methylmalonyl-CoA mutase enzyme activity in crude worm extracts were unsuccessful. We therefore sought to examine isolated mitochondrial preparations to determine whether detectable activity might be present. We found that purified mitochondria harbored significant methylmalonyl-CoA mutase activity and the activity was destroyed by heat inactivation (Table 2 A). A yeast over-expression strain was created using a full-length sequence verified *C. elegans* methylmalonyl-CoA mutase and was employed in comparison with wild-type *C. elegans* mitochondrial preparations to examine apparent kinetic constants (Table 2B). The initial reaction velocities of the enzyme-catalyzed reactions were graphed against substrate concentrations in Michaelis-Menton plots. The kinetic parameters and Lineweaver-Burke plots were determined from these data by curve fitting using non-linear regression [29].

The apparent K_m for adenosylcobalamin for the mouse enzyme, the *C. elegans* enzyme from isolated mitochondria, and the *C. elegans* enzyme over-expressed in the yeast system, were all within the 10^{-8} M range and were similar to that reported for human enzyme [31]. The *C. elegans* mutase enzyme in the mitochondrial preparations appears to exist predominantly as

an apoenzyme and, in the presence of adenosylcobalamin, exhibits a $V_{\max}$ of 6.42 $\mu\text{mol}/\text{min}/\text{mg}$. This is comparable to the $V_{\max}$ observed for *Ascaris lumbricoides* of (4.73 $\mu\text{mol}/\text{min}/\text{mg}$) [32] but much less than the mouse enzyme.

The *C. elegans* methylmalonyl-CoA mutase cDNA was introduced into a human *mut*⁰ cell line by lentiviral delivery. Blasticidin was used to select for transduced cells; an EGFP control was confirmed by fluorescence and Western analysis using antibodies against EGFP (data not presented). Cell lines that had undergone transduction plus selection were studied by C14 macromolecule incorporation. The expression of *C. elegans* mutase significantly increased the mutase-dependent label movement into macromolecules in mutant cell lines to 30% of wild-type but failed to reach 100% normalized flux as assessed by this assay (Figure 5). The *C. elegans mmcm-1* corrected cell line also exhibited increased methylmalonyl-CoA mutase dependent label movement after supplementation with hydroxycobalamin, a precursor of the active co-factor, adenosylcobalamin.

Discussion

In these studies, we have used genetic, genomic and biochemical methods to establish the existence of an adenosylcobalamin-dependent methylmalonyl-CoA mutase in *C. elegans*. The conservation and extreme similarity between the vertebrate and predicted worm enzymes suggests that the entire propionyl-CoA to succinyl-CoA pathway depicted in Figure 1 is functionally intact in *C. elegans*. Our findings demonstrate that native *C. elegans* mitochondria do harbor an active mutase that is predominantly apoenzyme in constitution. The observed kinetic constants of the nematode methylmalonyl-CoA mutase show high affinity adenosylcobalamin binding, as seen in vertebrate mutases and a $V_{\max}$ that approximates what has been seen in other organisms. The significant fraction of apoenzyme versus holoenzyme observed in our mitochondrial preparations suggests a modulation of or dependence upon cobalamin in the environment or the food source. The accentuation of activity in whole worm incorporation experiments after incubation in the presence of hydroxycobalamin further demonstrates the existence of adenosylcobalamin synthetic capacity *in vivo* and suggests that propionate metabolism can be greatly increased when the environment is cobalamin-replete. The mechanisms for extra-cellular cobalamin uptake and transport are unclear from a bioinformatics analysis as the extracellular proteins known to participate in these processes do not appear to have *C. elegans* homologues. However, the synthesis of methylcobalamin seems likely, given the presence of gene products that are highly similar to human vitamin B12 – dependent methionine synthase and methionine synthase reductase. Future studies, perhaps similar to the ones described herein, will be required to examine the functionality and nature of these enzymatic pathways.

A number of other lines of experimental evidence indicate the presence of an adenosylcobalamin metabolic pathway in *C. elegans* when grown under laboratory conditions. The *mmcm-1* and *mmab-1* deletion mutants have diminished 1- ^{14}C -propionate incorporation compared to wild-type animals, indicating that downstream movement of the label from 1- ^{14}C -propionate into protein is impaired, presumably at the step of conversion of methylmalonyl-CoA to succinyl-CoA. These metabolic blocks are consistent with a functional defect in adenosylcobalamin synthesis in the case of the *mmab-1* mutants, and impairment in apoenzyme synthesis in the case of the *mmcm-1* deletion mutant. The deletion in the *mmab-1* (*ok1493*) mutant removes the first exon, which may contain an inverted repeat. The possibility of an internal translation product from the deleted allele with residual activity is feasible given the fact that the second exon begins with a start codon. The other mutants all display severe blocks and appear to have similar activity in this assay, consistent with the proposed roles for the gene products in methylmalonyl-CoA metabolism.

The *mmcm-1* mutant exhibits increased methylmalonic acid production, which is accentuated in the face of precursor loading. The mutant consistently produced greater than tenfold more methylmalonic acid than the N2 strain (P value = 0.02), but exhibits variability between experiments, which explains the larger standard deviation observed in this strain versus N2. The *mce-1* and *mmab-1* deletion mutant strains appear to have decreased viability in propionate-containing medium but consistently produce more methylmalonic acid than the N2 strain. Preliminary observations indicate diminished survival by these strains under propionate loading, which likely explains the milder increases in metabolite production by these mutants, especially since the mutants show display a severe impairment in the C14 incorporation assays. The results show that the *C. elegans* system might be used to examine inducers and regulators of the methylmalonyl-CoA mutase reaction by virtue of metabolite production.

A surprising finding was that the *mce-1* mutant had a severe block in C14 propionate incorporation. Although mutations in *MUT*, *MMAA* and *MMAB* have been shown to cause methylmalonic acidemia in humans, MCEE deficiency has not yet been proven to cause methylmalonic acidemia [33]. Montgomery *et al.* [13] administered a number of methylmalonic acid isotopomers to vitamin B12 deficient rats and analyzed methylmalonic acid output by mass spectrometry. They noted a rearrangement of label in the urine after peritoneal injection of methylmalonic acid into the cobalamin-deficient rats, which was not observed in control animals. To explain the label distribution observed, they posited the existence of a “free” methylmalonic acid shunt, which was able to bypass the epimerase reaction and proposed that this mechanism protected patients with MCEE lesions from developing methylmalonic acidemia. The results we have observed are not entirely consistent with the existence of a bypass in *C. elegans* because the *mce-1* deletion mutant exhibits a block as severe as the *C. elegans* MCM mutant and produces increased methylmalonic acid. If a free-shunt was functionally operational over the physiological range of methylmalonyl-CoA concentrations, selective pressure on the MCEE gene might be lessened or removed and conservation between species would not be predicted. Mutations of MCEE may result in either a mild or an embryonic lethal phenotype in humans, which could go undetected. If a free MMA metabolic shunt exists in *C. elegans*, it appears to be incapable of handling the normal flux through this pathway as assessed in the assays we have performed. Isotopomer studies using the mutant strains may be useful to further examine this issue. The results observed with the *C. elegans mce-1* deletion mutant suggest that humans with functional methylmalonyl-CoA epimerase lesions will have methylmalonic acidemia and cell lines from these patients will exhibit a block in 1-[¹⁴C]-propionate incorporation. The identification and careful clinical characterization of patients harboring suspected MCEE mutations will be required to resolve this issue.

We also demonstrated that the *C. elegans* mutase can partially correct fibroblasts derived from *mut^o* human patients. The level of correction was significantly increased over the mutant background and GFP in all instances. The corrected human cell line displayed similar intermediate correction and could be stimulated by hydroxycobalamin addition to the media. The reason for a partial correction of the human cell line is uncertain, but may relate to the specialized mitochondrial importation sequences found at the N-terminal end of the *C. elegans* gene. Other mitochondrial enzymes have been shown to have a limited cross-species correction for this reason, even when the species barrier is relatively close, such as between human and mouse [34]. If *C. elegans* mitochondrial localized genes are to be used for cross-species correction of mutant cell lines, for example, to identify unknown cobalamin disease genes or disease gene modifiers, future studies will be required to determine optimum expression parameters.

We have documented that this model organism can be used to study the pathophysiology of human methylmalonic acidemia by demonstrating that the *mce-1* mutant has a functional deficit

in 1-[¹⁴C]-propionate incorporation and predict, based on these results, that the human homolog will cause methylmalonic acidemia in humans when mutated. The metabolic lesions generate a chemical phenotype that might be useful if RNAi or targeted deletion mutant analysis could be coupled to metabolic profiling. Such an analysis could identify mutants that produced increased amounts of methylmalonic acid and/or reduced 1-[¹⁴C]-propionate incorporation, and might identify new genes involved in intracellular cobalamin and propionyl-CoA metabolism, some of which may have human homologues that are mutated in patients with propionic acidemia, methylmalonic acidemia and/or homocysteinemia. The experiments presented here also demonstrate that sensitive mass spectrometry measurements can be performed on small numbers of animals to determine chemical phenotypes and suggest that metabolic profiling will be useful to study *C. elegans* RNAi and deletion mutants in other pathways.

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Figure 1.

Major pathway of the conversion of propionyl-CoA into succinyl-CoA in human cells. The biotin-dependent enzyme, propionyl-CoA carboxylase (PCCA, PCCB) converts propionyl-CoA into D-methylmalonyl-CoA which is then racemized into L-methylmalonyl-CoA and isomerized into succinyl-CoA, a Krebs cycle intermediate. The L-methylmalonyl-CoA mutase (MUT) reaction requires adenosylcobalamin. The class and genes associated with methylmalonic acidemia are *mut* (*MUT*), *cbIA* (*MMAA*), *cbIB* (*MMAB*), *cbID* variant 2 (unknown gene(s)), *cbIC* (*MMAHC*), *cbIF* (unknown gene(s)). Question marks indicate unknown genes or poorly defined cellular processes.

Figure 2.

Methylmalonic acid production after RNAi against putative propionate metabolic enzymes. Methylmalonic acid concentration (nM), normalized by nematode wet weight (mg), detected in supernatant after RNAi against indicated genes in the presence of 5 mM propionic acid in PBS. *mmcm-1* (P value vs WT = 0.004), *mmaa-1* (P value vs WT = 0.005), and *mmab-1* (P value vs WT = 0.33, NS) RNAi showed increases in methylmalonic acid concentrations relative to wild-type. Propionyl-CoA carboxylase subunit B (*pccb-1*) RNAi creates a block prior to the formation of methylmalonyl-CoA, therefore methylmalonic acid concentrations are not increased (P value = 0.211, NS vs wild-type). Values are from two independent experiments, averaged, with ranges indicated by error bars.

Figure 3.

1- $[^{14}\text{C}]$ -propionate incorporation in *C. elegans* deletion mutants. 1- $[^{14}\text{C}]$ -propionate incorporation of methylmalonyl-CoA mutase *mmcm-1(ok1637)*, methylmalonyl-CoA epimerase *mce-1(ok243)*, and *mmab-1* mutants *mmab-1(ok1493)* and *mmab-1(ok1484)* expressed as a percentage of the mean propionate incorporation of wild-type (WT = 6.8 nanomoles propionate/mg protein/24hrs). Values are averages from 6 (N2, *mmcm-1*), 5 (*mce-1*) and 5 (*mmab-1*) independent experiments with triplicate or quadruplicate measurements of each sample. Error bars represent mean $\pm$ SD. P-values vs wild-type: *mmcm-1(ok1637)*=2.9 E-22; *mce-1(ok243)*=2.6 E-19, *mmab-1(ok1493)* = 0.008, *mmab-1(ok1484)*=1.4 E-08, N2 plus OHcbl = 2.03 E-06.

Figure 4. Methylmalonic Acid production by the deletion mutants. Wild-type (N2), *mmcm-1*, *mmab-1* (*ok1493*, *ok1484*) and *mce-1* deletion mutant were incubated in PBS containing 10 mM propionic acid and the supernatant analyzed by GC-MS. Values are averages from independent experiments shown in table below the figure and measured in nM MMA/mg protein. Error bars represent mean $\pm$ SD. P-value wild-type versus mutants were calculated using the student t-Test and are shown below the figure.

Figure 5.

Lentiviral correction of a mutant human cell line with *C. elegans* methylmalonyl-CoA mutase. A full-length *C. elegans mmcm-1* cDNA carried in pLENTI6 was used to transduce a human *mut^o* fibroblast cell line. 1-[¹⁴C]-propionate incorporation was measured in triplicate and expressed as a percentage of the mean propionate incorporation of wild-type (5.2 nanomoles 1-[¹⁴C]-propionate /mg protein/18 hrs). Error bars represent mean $\pm$ SD. P values: Wild-type vs *mut^o* = 0.001, *mut^o* vs *mut^o* plus *mmcm-1* = 0.0078; *mut^o* plus OHcbl vs *mut^o* plus *mmcm-1* plus OH-cbl = 0.0001.

Putative *C. elegans* Homologues of Genes Involved in Propionyl-CoA Metabolism
The percent similarity and e-values of the p-BLAST scores between human proteins involved in propionate metabolism and putative *C. elegans* orthologs as well as the conserved domains between human and *C. elegans* proteins is shown.

Human Protein	Protein	<i>C. elegans</i>	Gene	Protein % Similarity/e- value	Domains present in Human Protein	Domain(s) % Similarity/e- value
Propionyl-CoA carboxylase alpha subunit (PCCA) NP000273.1	WP:CE04451	F27D9.5	<i>pecca-1</i>	94.6/5.2e-215	Carbamoyl-phosphate synthase Lchain, ATP binding domain, pfam 02786	89.8/2.00e-26
Propionyl-CoA carboxylase beta subunit (PCCB) NP000523.1	WP:CE07269	F52E4.1	<i>peccb-1</i>	95.3/1.4e-212	Biotin carboxylase C-terminal domain pfam02785	100/1.00e-35
Methylmalonyl-CoA Epimerase (MCE) NP115990.2	WP:CE09082	D2030.5	<i>mce-1</i>	79.6/2.3e-45	Carbamoyl-phosphate synthase, N-terminal domain pfam00289	100/2.00e-27
Methylmalonyl-CoA Mutase (MUT) NP000246.1	WP:CE30404	ZK1058.1	<i>mmcm-1</i>	97.8/7.29e-280	Biotin-requiring enzyme pfam00364 Carboxyl transferase domain pfam01039 Glyoxalase pfam00903	100/5.00e-12 100/0.00e-00 100/7.00e-17
Methylmalonic aciduria Type A (MMAA) NP758454.1	WP:CE31822	T02G5.13	<i>mmaa-1</i>	82.5/1.2e-89	Methylmalonyl-CoA mutase B12 binding domain cd02071 Methylmalonyl-CoA mutase pfam01642	100/5.00e-43 100/0.00e-00
Methylmalonic aciduria Type B (MMAB-1) NP443077.1	WP:CE01159	C26E6.11	<i>mmab-1</i>	53.7/5.7e-35	ArgK protein pfam03308 Cobalamin adenosyltransferase pfam01923	100/3.00e-105 97.6/4.00e-50
MMACHC [35]	WP:CE34461	ZK546.17	<i>chlc-1</i>	52/5e-31	None proven	

Table 2**Properties of the Methylmalonyl-CoA Mutase Enzyme from *C. elegans* (A) Heat Lability (B) Apparent Kinetic Constants**

(A) Heat lability and specific activity of the native *C. elegans* methylmalonyl-CoA mutase in mitochondrial preparations, with and without adenosylcobalamin, added at standard assay concentrations. (B) Apparent Km for adenosylcobalamin and Vmax under standard assay conditions in *C. elegans* mitochondrial preparations and yeast extracts. The murine enzyme served as a positive control.

A.				
Sample	Adenosylcobalamin	nmol/hr/mg total protein		
Heat inactivated mitochondria	present			0.45
<i>C. elegans</i> mitochondria	absent			13.47
<i>C. elegans</i> mitochondria	present			101.17
B.				
Sample	Adenosylcobalamin Km (M)	SD	Vmax (nmol/hr/mg)	SD
<i>C. elegans mmcm-1</i> - yeast	6.97×10 ⁻⁸	1.71×10 ⁻⁸	196.35	6.32
<i>C. elegans</i> - native mitochondria	5.02×10 ⁻⁸	1.21×10 ⁻⁸	75.54	2.22
Murine - recombinant in yeast	1.23×10 ⁻⁸	3.38×10 ⁻⁹	1,794.83	78.96