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Evidence for involvement of medium chain acyl-CoA dehydrogenase in the metabolism of phenylbutyrate

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Abstract

Sodium phenylbutyrate is used for treating urea cycle disorders, providing an alternative for ammonia excretion. Following conversion to its CoA ester, phenylbutyryl-CoA is postulated to undergo one round of β -oxidation to phenylacetyl-CoA, the active metabolite. Molecular modeling suggests that medium chain acyl-CoA dehydrogenase (MCAD; EC 1.3.99.3), a key enzyme in straight chain fatty acid β -oxidation, could utilize phenylbutyryl-CoA as substrate. Moreover, phenylpropionyl-CoA has been shown to be a substrate for MCAD and its intermediates accumulate in patients with MCAD deficiency. We have examined the involvement of MCAD and other acyl-CoA dehydrogenases (ACADs) in the metabolism of phenylbutyryl-CoA. Anaerobic titration of purified recombinant human MCAD with phenylbutyryl-CoA caused changes in the MCAD spectrum that are similar to those induced by octanoyl-CoA, its *bona fide* substrate, and unique to the development of the charge transfer ternary complex. The calculated apparent dissociation constant ($K_{D\text{ app}}$) for these substrates was 2.16 μM and 0.12 μM , respectively. The MCAD reductive and oxidative half reactions were monitored using the electron transfer flavoprotein (ETF) fluorescence reduction assay. The catalytic efficiency and the K_m for phenylbutyryl-CoA were 0.2 $\text{mM}^{-1} \cdot \text{sec}^{-1}$ and 5.3 μM compared to 4.0 $\text{mM}^{-1} \cdot \text{sec}^{-1}$ and 2.8 μM for octanoyl-CoA. Extracts of wild type and MCAD-deficient lymphoblast cells were tested for the ability to reduce ETF using phenylbutyryl-CoA as substrate. While ETF reduction activity was detected in extracts of wild type cells, it was undetectable in extracts of cells deficient in MCAD. The results are consistent with MCAD playing a key role in phenylbutyrate metabolism.

Keywords

Buphenyl; Phenylbutyrate; hyperammonemia; urea-cycle disorders; medium chain acyl-CoA dehydrogenase

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1. Introduction

Impairment of urea synthesis in humans is caused by defects in the activity of enzymes in the urea cycle including carbamylphosphate synthetase, ornithine transcarbamylase, argininosuccinic acid synthetase, argininosuccinate lyase, and arginase and leads to hyperammonemia. High levels of ammonia in blood may lead to encephalopathy and death [1]. Sodium phenylbutyrate is the active ingredient in Buphenyl® (Ucyclyd Pharma) and is currently used for treating primary hyperammonemia caused by certain urea cycle defects [2]. Sodium phenylbutyrate may also be useful in treating secondary hyperammonemia that accompanies other inborn errors. In addition, phenylbutyrate has been and is being investigated in numerous clinical settings including modulation of fetal hemoglobin gene expression in sickle cell and in thalassemia, treatment of myelodysplastic syndromes and acute myeloid leukemia, cerebral and liver ischemic injury protection, among others [3–6]. As of this writing >30 trials involving sodium phenylbutyrate are listed on the www.clinicaltrials.gov website.

The mechanism proposed for ammonia clearance by phenylbutyrate administration involves its activation to phenylbutyryl-CoA, conversion to phenylacetyl-CoA, and conjugation with glutamine (Figure 1) for excretion by the kidneys [7]. The conversion of phenylbutyryl-CoA to phenylacetyl-CoA is presumed to occur through one cycle of β -oxidation in mitochondria. The first step in the β -oxidation cycle is the $\alpha\beta$ -dehydrogenation of fatty acid CoA esters catalyzed by members of the acyl-CoA dehydrogenase (ACAD) family of enzymes. Nine members of this enzyme family have been identified, each with characteristic substrate specificity profile [8–15]. Short, medium, long, saturated very long, unsaturated very long chain acyl-CoA dehydrogenases (SCAD, MCAD, LCAD, VLCAD and ACAD9) have substrate optima of C4, C8, C12, C16, and C16:1 (unsaturated very long chain among others) acyl-CoA esters, respectively, but can utilize other substrates [8,16–18]. The crystal structures of SCAD, MCAD, and VLCAD have been published, (PDB ID: 1JQI, 3MDE, and 3B96, respectively) [19–21]. The remaining four enzymes in the family are involved in amino acid metabolism and their crystal structures have been published as well, (PDB ID: 1IVH, 1SIR, 1RX0, and 2JIF) revealing a relatively restrictive active sites, rendering them highly specific for their *bona fide* substrates [22–25].

It has been observed that patients with MCAD deficiency characteristically accumulate both the glycine and carnitine conjugates of phenylpropionate, a bacterial metabolite from bowel flora that is absorbed into the blood stream [26]. Mass spectrometry based enzymatic assay of MCAD deficient patient fibroblast cells using phenylpropionyl-CoA as substrate showed lack of conversion to its α,β -unsaturated product [27]. While the crystal structure of SCAD and VLCAD and homology 3D modeling of ACAD9 show that the active site would not accommodate the phenylbutyryl acyl moiety, the active site of MCAD would. These findings and the structural similarities between phenylpropionate and phenylbutyrate implicate MCAD in the metabolism of phenylbutyryl-CoA.

In this study we tested the ability of purified human recombinant ACADs to bind and use phenylbutyryl-CoA as a substrate. We demonstrate that MCAD indeed uniquely utilizes phenylbutyryl-CoA as a substrate. In addition, we show the inability of extracts prepared from MCAD-deficient fibroblast to act upon this substrate.

2. Materials and Methods

2.1. Purification of recombinant human MCAD

Expression and purification of recombinant human MCAD was performed as previously described for isovaleryl-CoA dehydrogenase with minor modifications [28]. *E. coli*

JM105 cells (Amersham Biosciences Corp; Piscataway, NJ) containing the human MCAD high expression vector pK⁺MCAD [29] and a GroEL/GroES expression plasmid were grown overnight in a 200-ml LB broth pre-culture that was used to inoculate 4 × 2-L cultures in 2-YT broth. The cells were left to grow overnight at 37°C with shaking and MCAD expression was induced the next morning using IPTG at a final concentration of 0.5 mM for 3 hrs. Cells were harvested by centrifugation and resuspended in 2:1 weight to volume of 100 mM potassium phosphate pH 8.0, 150 mM EDTA. Cells were then lysed by sonication on ice. Including high amounts of EDTA in the cell lysis buffer is for protecting residues with groups, e.g., cysteine thiols and methionine sulfide groups, vulnerable to modification by oxygen reactive species generated during sonication cell suspension. This was effective in improving enzyme preparations resulting higher specific activity and consistent kinetic behavior [30]. Cellular debris was removed by centrifugation at 250,000 × g for 60 minutes. The final supernatant was dialyzed for 4 hours with vigorous stirring in 50 mM potassium phosphate pH 8.0, at 4°C. The sample was then loaded on a 16 × 40 mm DEAE Sepharose FF column pre-equilibrated in 50 mM potassium phosphate pH 8.0, using an ÄKTA UPC-900 pump FPLC system (Amersham Biosciences Corp; Piscataway, NJ). After washing with 300 ml of 50 mM potassium phosphate pH 8.0, MCAD was eluted with a 300 ml linear gradient from 50 to 500 mM potassium phosphate pH 8.0. Green fractions with a 270/447 nm ratio <12 containing MCAD were pooled, concentrated, and dialyzed against 25 mM potassium phosphate, pH 8.0. Pooled fractions of essentially pure MCAD (270/447 nm ratio = 5.5), were concentrated and stored at –80°C.

Other recombinant human ACADs were similarly purified except that the protocol was terminated after the DEAE-Sepharose column for LCAD as the enzyme was unstable. LCAD protein purity ~70% at this stage.

2.2. The electron transfer flavoprotein (ETF) purification

Porcine ETF was purified as previously published [31], except that the dialysis buffer used after both the 40–60% ammonium sulfate fractionation and DE-52 cellulose anion-exchange chromatography steps consisted of unbuffered 15 mM dibasic potassium phosphate and 5% glycerol.

2.3. Fibroblast cell culture and extract preparation

Wild type and MCAD deficient cells (homozygous for the K304E mutation) with the designation GM085401 and GM07844, respectively, were obtained from Coriell Institute for Medical Research, Camden, NJ. Cells were cultured in DMEM medium supplemented with glutamine and ampicillin and streptomycin, and 20% fetal bovine serum. Cells were harvested from a T175 flask by sonication with a buffer consisting of 50 mM Tris buffer and 10 mM EDTA, pH 8.0. The cell debris was removed by centrifugation and the cell free extract was assayed for protein and enzyme activity as described below.

2.4. ETF fluorescence reduction assay

The ETF reduction assay was performed using a Jasco FP-6300 spectrofluorometer (Easton, MD) with a cuvette holder heated with circulating water at 32°C. The assay was otherwise performed as described [32], at the indicated substrate concentrations. The enzyme was diluted 1200-fold into a buffer containing 50 mM Tris, pH 8.0, 5 mM EDTA and 50% glycerol, and 10 µl were used for each assay. The ETF concentration in the reaction mixture was 2 µM. Spectra Manager 2 software (Jasco Inc) was used to collect data and calculate reaction rate and Microsoft Excel was used to calculate the kinetic parameters.

2.5. Phenylbutyryl-CoA synthesis

CoASH, octanoyl-CoA, C₁₂-CoA and phenylbutyric acid were obtained from Sigma (St. Louis, MO.) 2,6-dimethylheptanoic acid was obtained from Matreya LLC (Pleasant Gap, PA). The phenylbutyryl-CoA and 2,6-dimethylheptanoyl-CoA esters were prepared by the mixed anhydride method as described previously [33] and was purified by HPLC using a Luna 5 μ m C18(2) column (25 cm $\times$ 0.46 cm) and a linear gradient (10–60%) of acetonitrile into 50 mM ammonium phosphate, pH 5.5, at a flow rate of 1.5 mL/min over 30 min.

2.6. Monitoring the interaction of MCAD with substrates

Formation of the charge transfer ternary complex was monitored by observing the increase in absorbance at the 570 nm area, concomitant with the decrease of absorbance at 447 nm area, of the purified MCAD in 120 mM potassium phosphate spectrum under anaerobic conditions using a Jasco V-650 Spectrophotometer. Phenylbutyryl-CoA solution dissolved in water to 0.53 mM was titrated into the MCAD sample one μ L at a time using a 50 μ L Hamilton syringe attached to an automatic dispenser. Ten seconds of equilibration time were allowed after mixing before the sample was scanned at 250 to 800 nm. Final substrate concentrations varied as indicated in figure legends. All data were adjusted for the dilution resulting from substrate addition. Substrates were titrated, but with different final concentrations as indicated in the figure legends. The dissociation constant ($K_{D \text{ app}}$) was calculated with the Stockell equation as described previously [34]:

$$\frac{d}{p} = K_{D \text{ app}} \frac{1}{e-p} + n$$

where **d** is the total ligand concentration, **e** is the total molar concentration of enzyme, **p** is the fraction of enzyme sites that bind ligand multiplied by **e**, and **n** is the number of binding sites. The absorbance at 447 nm when all enzyme sites are occupied with ligand was determined separately by adding large excess of octanoyl-CoA and used to calculate the fraction of enzyme with bound ligand at various readings and assuming that at large excess of added substrate would equal to **e**.

2.7. Molecular modeling

Computer modeling of MCAD was performed using a Silicon Graphics Fuel workstation (Mountain View, CA) with the *Insight II* 2005 software package and MOE software, from Chemical Computing Group, Montreal, Canada, and the atomic coordinates of pig MCAD (3MDE) in the dimer form as a molecule [20]. Carbon atoms at positions C5-C8 of the octanoyl-CoA ligand, which co-crystallized with MCAD, were replaced with a phenyl group. The phenylbutyryl-CoA ligand conformation in the active site was refined using the Discover module. Human LCAD 3D structure was modeled using MCAD atomic coordinates as template and the *Insight II* modeling software.

3. Results

3.1. Interaction of MCAD with substrates, the reductive half-reaction

Formation of the charge transfer complex, the reductive half-reaction, is evident from the spectral scans of MCAD at various phenylbutyryl-CoA concentrations (Figure 2 and 3). The progressive decrease and increase of absorbance at 447 nm and 570 nm, respectively, are similar to those induced by octanoyl-CoA. The octanoyl-CoA binding curve is sigmoidal in contrast to the phenylbutyryl-CoA binding curve, possibly reflecting differences in enzyme mechanism of interaction. The plot of **d/p** versus **1/e-p** (the Stockell plot) was nonlinear. A line drawn at the straight area of the curve where the substrate:subunit ratio was 1:1

estimates the apparent dissociation constant ($K_{D \text{ app}}$) being 0.12 μM and 2.16 μM for octanoyl-CoA and phenylbutyryl-CoA, respectively. Other mathematical derivatives of the absorbance data all indicated that the binding sites are non-equivalent.

3.2. Interaction of MCAD:substrate ternary complex with ETF, the oxidative half-reaction

Using ETF as the electron acceptor in the standard enzymatic assay detailed above, transfer of electrons was confirmed as evident from the reduction of ETF fluorescence in the presence of various concentrations of phenylbutyryl-CoA. The catalytic efficiency and K_m for the phenylbutyryl-CoA were 0.2 $\text{mM}^{-1} \cdot \text{sec}^{-1}$ and 5.3 μM compared to 4.0 $\text{mM}^{-1} \cdot \text{sec}^{-1}$ and 2.8 μM for octanoyl-CoA, respectively.

Molecular modeling of human LCAD shows possible accommodation of the acyl moiety of the phenylbutyryl-CoA, with the exception of residue L264, which would have one of its side chain methyl hydrogens $\sim 1.3\text{\AA}$ away from a phenyl ring hydrogen and so would hinder binding. To test if the LCAD active site has enough plasticity to accommodate this potential substrate, we measured its activity with LCAD using the ETF fluorescence reduction assay. While the partially purified recombinant human LCAD was active in the presence of various substrates including C_{12} -CoA and 2,6-dimethylheptanoyl-CoA, it was not active in the presence of phenylbutyryl-CoA. Purified SCAD, MCAD, and ACAD9 also showed no activity with phenylbutyryl-CoA as substrate.

3.3. The ETF fluorescence reduction assay of cell extract

ETF fluorescence reduction was observed using extracts from wild type fibroblast cells in the presence of 30 μM of phenylbutyryl-CoA, octanoyl-CoA, or palmitoyl-CoA. (The latter substrate was used as internal control and is a substrate of VLCAD.) Enzyme specific activity measured using these substrates was 3.41 (~ 0.53), 4.01 (~ 1.34), 9.10 (~ 2.13) $\mu\text{moles ETF reduced} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively. No activity was observed using similar amounts of extract from fibroblast cells deficient in MCAD with either phenylbutyryl-CoA or octanoyl-CoA. The measured enzyme specific activity of palmitoyl-CoA oxidation in extract from these cells was 3.91 (~ 1.34) $\mu\text{moles ETF reduced} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

4. Discussion

Following the conversion of phenylbutyrate to the CoA ester, one cycle of β -oxidation is expected to result in phenylacetyl-CoA and acetyl-CoA as the final products (Figure 4). Phenylacetyl-CoA is hydrolyzed to phenylacetate, which becomes conjugated with glutamine and is excreted in urine (Figure 1). An analysis of this first step in the β -oxidation of phenylbutyryl-CoA is important because the first step in the β -oxidation of fatty acids is postulated to be rate-limiting [35], and thus the metabolism of Buphenyl to its active form, phenylacetate, may also be modulated by similar factors that affects energy metabolism at the same step.

The effect of phenylbutyryl-CoA on the MCAD absorbance spectrum at relatively low concentrations is monitored *via* the decrease of absorbance at 447 nm and increase of absorbance at 570 nm. This confirms productive binding of phenylbutyryl-CoA to MCAD in the reductive half-reaction with lack of product release. This effect is similar to that induced by octanoyl-CoA binding to MCAD, and indicative of the transfer of a proton and a hydride to the flavin ring and formation of the charge transfer complex, which is comprised of the enzyme, reduced FAD, and enoyl product and detected by the intense absorbance band centered at 570 nm [36,37].

The inset in Figure 2 shows a sigmoidal shaped curve when octanoyl-CoA, but not phenylbutyryl-CoA, was used as substrate. This may imply positive cooperativity between

the first and second subunits with octanoyl-CoA binding that does not occur with phenylbutyryl-CoA. Although other interpretations of sigmoidal behavior in this setting are possible, including presence of various MCAD forms or other effector molecules, the argument is weakened by the fact that the only difference between the two reactions is the substrate itself. Impurities in the substrate preparation are also not likely to induce such an effect as such impurities would be present at ineffectively low concentrations at the low substrate concentrations range, between 0.25–1 and 4:1 substrate:MCAD tetramer ratio.

Reduction of ETF by the charge transfer complex in the oxidative half-reaction shows that electrons from the bound phenylbutyryl-CoA can be efficiently transferred to ETF and that the product, phenylbutenoyl-CoA, is released to complete the reaction. In contrast, none of the other ACADs are capable of catalyzing this reaction.

Modeling of a phenylbutyryl moiety in the active site in place of the octanoyl moiety observed in the MCAD crystal structure shows the phenyl moiety accommodated in the acyl moiety binding site pocket with a conformation perpendicular to the aromatic ring of Y375, Figure 5. Other residues involved in binding the phenyl moiety include E99, A100, Leu103, and V259. Furthermore, modeling predicts that the phenyl ring *para* and/or *meta* positions are candidate expansion sites for adding a functional group that may improve binding, while addition at the *ortho* position would prevent the derivative from binding to MCAD.

Based on the kinetic parameters of MCAD with phenylbutyryl-CoA as substrate, individuals with MCAD deficiency are likely to experience a functionally relevant decrease in the ability to metabolize the medication, though indications for use in these patients are likely to be rare. Of note, since octanoyl-CoA has been reported to provide thermal stability to the MCAD K304E mutant [38], it is possible that phenylbutyryl-CoA would behave similarly and may be of benefit *in vivo* in patients carrying at least one copy of this mutation. This requires additional investigation in cellular or whole body systems. It is unknown if carriers for MCAD deficiency, a much more common situation, will display altered metabolism of phenylbutyrate. In other indications where the functional effects of phenylbutyrate are less well characterized, modulation of MCAD activity might be of benefit to alter drug metabolism and/or its half-life and increase its efficacy, depending on the mechanism of action of the medication in each disorder. Detailed biochemical studies to determine the drug's mode of action and its pharmacokinetics for such indications are thus crucial.

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Abbreviations

ACAD	acyl-CoA dehydrogenase
ETF	electron transfer flavoprotein
LCAD	long chain acyl-CoA dehydrogenase
MCAD	medium chain acyl-CoA dehydrogenase
SCAD	short chain acyl-CoA dehydrogenase

VLCAD very long chain acyl-CoA dehydrogenase

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Figure 1. Schematic showing a proposed overall pathway for metabolism of phenylbutyrate to its final metabolite.

Figure 2.

Monitoring the formation of the charge transfer complex with purified MCAD upon addition of increasing amounts of octanoyl-CoA. The absorbance maxima at ~370 nm and ~447 nm are reduced and a new peak centered at 570 nm appears with addition of increasing substrate. Selected scans are shown with octanoyl-CoA concentration at 0, 3.25, 7.1, 10.8, 15.6, 18.0, 21.5, and 28.2 μM . The inset shows the kinetics of these changes. Equation for the decrease at 447 nm is: $y = -1 \times 10^{-9}x^6 + 1 \times 10^{-7}x^5 - 2 \times 10^{-6}x^4 + 2 \times 10^{-5}x^3 - 0.0003x^2 - 0.008x + 0.3489$. Equation for the increase at 570 nm is: $y = 6 \times 10^{-10}x^6 - 5 \times 10^{-8}x^5 + 1 \times 10^{-6}x^4 - 2 \times 10^{-5}x^3 + 0.0003x^2 + 0.0003x + 0.0008$. Enzyme concentration was 25.2 μM .

Figure 3.

Monitoring the formation of the charge transfer complex with purified MCAD upon addition of increasing amounts of phenylbutyryl-CoA. The absorbance maxima at ~370 nm and ~447 nm are reduced and a new peak centered at ~570 nm appears with addition of increasing substrate. Selected scans are shown with phenylbutyryl-CoA concentration at 0, 4.2, 8.3, 16.3, 24.1, 31.6, 40.7, and 80.2 μM . The inset shows the kinetics of these changes. Equation for the decrease at 447 nm is: $y = 5 \times 10^{-10} x^5 - 1 \times 10^{-7} x^4 + 9 \times 10^{-6} x^3 - 0.0002 x^2 - 0.0061 x + 0.3707$. Equation for the increase at 570 nm is: $y = -1 \times 10^{-10} x^5 + 3 \times 10^{-8} x^4 - 3 \times 10^{-6} x^3 + 7 \times 10^{-5} x^2 + 0.0012 x + 0.0036$. Enzyme concentration was 25.2 μM .

Figure 4. Detailed proposed pathway of metabolism of phenylbutyrate to its active form, phenylacetyl-CoA.

Figure 5. Stick representation of MCAD active site residues and ligands with phenylbutyryl-CoA modeled in place of octanoyl-CoA. The crystal structure of pig MCAD with bound octanoyl-CoA (PDB: 3MDE, [20]) was used to create the model using MOE modeling software. The E376 carboxylate is the active site catalytic base responsible for the substrate C2 proton abstraction to initiate catalysis.