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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 ($K_{D\ app}$) 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 mM $34^{-1}\cdot sec^{-1}$ and 5.3 μM compared to 4.0 $mM^{-1}\cdot sec^{-1}$ 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.

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, argininosucinate 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 myleodysplastic 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.clinicaltrial.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 (Fig. 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 αβ-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)

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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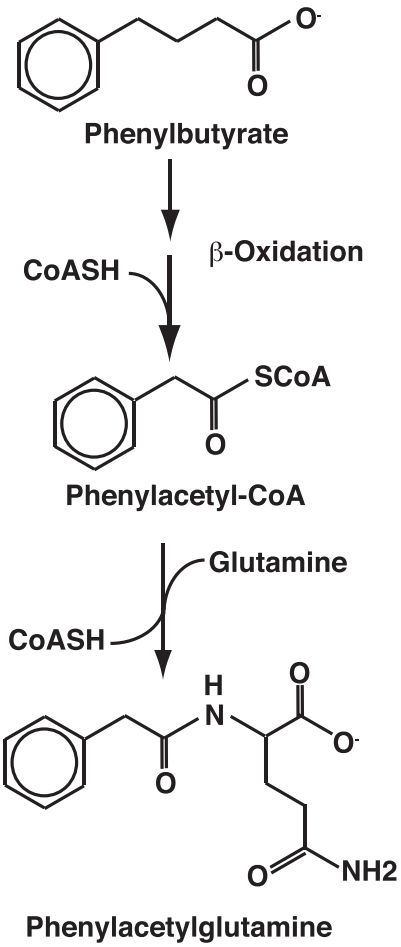

Fig. 1. Schematic showing a proposed overall pathway for metabolism of phenylbutyrate to its final metabolite.

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 p*Ke*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 min. 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.