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The Enzymatic Basis for the Dehydrogenation of 3-Phenylpropionic Acid: *In Vitro* Reaction of 3-Phenylpropionyl-CoA with Various Acyl-CoA Dehydrogenases

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ABSTRACT. 3-Phenylpropionic acid is an end-product of the bacterial degradation of unabsorbed phenylalanine in the intestinal lumen. As CoA ester, this metabolite has been considered to be a specific substrate for medium chain acyl-CoA dehydrogenase (MCAD). Its glycine-conjugate, 3-phenylpropionylglycine, has now been established as a pathognomonic marker in urine from patients affected with MCAD deficiency. However, no systematic studies to evaluate the reactivity of 3-phenylpropionyl-CoA with other known acyl-CoA dehydrogenases have so far been carried out to establish the specificity of this substrate for MCAD. We studied the *in vitro* reactivity of 3-phenylpropionyl-CoA with five rat and human liver acyl-CoA dehydrogenases using purified preparations. We demonstrated that MCAD effectively dehydrogenated 3-phenylpropionyl-CoA, and that no other acyl-CoA dehydrogenase exhibited any significant activity with this substrate. In the steady state condition, the Km of 3-phenylpropionyl-CoA for human MCAD was 50 μM. Gas chromatography/mass spectrometry analysis of the assay mixture identified *trans*-cinnamoyl-CoA as the product of the reaction. Furthermore, we showed by determination of the reaction products using gas chromatography/mass spectrometry selected ion monitoring that, in absence of the primary electron acceptor, 3-phenylpropionyl-CoA was slowly but significantly dehydrogenated by MCAD under aerobic conditions. These data suggest that MCAD may oxidize 3-phenylpropionyl-CoA *in vivo* using an alternative electron acceptor, to produce *trans*-cinnamoyl-CoA. This mechanism provides an explanation for the normal 3-phenylpropionylglycine excretion observed in urine from patients affected with glutaric aciduria type II and ethylmalonic/adipic aciduria. (*Pediatr Res* 27: 501–507, 1990)

Abbreviations

CI, chemical ionization
CoA, coenzyme A
DCIP, 2,6-dichlorophenol-indophenol
EI, electron impact ionization
ETF, electron transfer flavoprotein
FAD, flavin adenine dinucleotide
GC/MS, gas chromatography/mass spectrometry
IVD, isovaleryl-CoA dehydrogenase
LCAD, long chain acyl-CoA dehydrogenase
MCAD, medium chain acyl-CoA dehydrogenase
2-meBCAD, 2-methyl branched chain acyl-CoA dehydrogenase
m/z, mass to charge ratio
PMS, phenazine methosulfate
PPA, 3-phenylpropionic acid
PP-CoA, 3-phenylpropionyl-CoA
PPG, 3-phenylpropionylglycine
SCAD, short chain acyl-CoA dehydrogenase
SIM, selected ion monitoring
TMS, trimethylsilyl (derivative)

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Phenylalanine metabolites have been investigated by gas chromatography and GC/MS over the span of more than 20 y (1–3) because of their significance in the biochemical characterization of phenylketonuria and other types of inherited disorders of phenylalanine hydroxylation (4). Among them, PPA is the end-product of a minor alternative pathway: the bacterial degradation of unabsorbed phenylalanine in the intestinal lumen (5–7). *Lactobacillus pastorianus* var. *quinicus* (8), *Clostridium difficile* (9), and *Peptostreptococcus anaerobius* (10) represent some of the anaerobic bacterial strains that showed large amounts of PPA in ether-extracts from broth cultures analyzed by GC/MS (11) or liquid chromatography analysis (12). As summarized in Figure 1, several enzymatic steps are involved in this secondary pathway, where the mechanism of phenyllactic acid transformation to *trans*-cinnamic acid still requires direct experimental confirmation.

Unlike other phenylalanine metabolites (5–7), PPA is not ordinarily detected as a free acid (13) in either phenylketonuric patients (1–3) or normal subjects, even after a phenylpropionate loading test (14). However, it has been shown that homologous *p*-hydroxy intermediates were produced in tyrosine-loaded rats and rabbits along a parallel pathway (15), suggesting a different endogenous metabolism in other mammalian species. Under normal circumstances, PPA absorbed from the human intestine circulates through the portal system into the liver and diffuses into the hepatocytes, where it is activated to the CoA ester. PP-CoA is transformed to benzoyl-CoA (16), presumably via a β-oxidation of its aliphatic chain that is initiated by an acyl-CoA dehydrogenase. PP-CoA is then converted to its glycine-conjugate (hippuric acid) by a mitochondrial enzyme, benzoyl-CoA:glycine N-acyltransferase (17), and finally excreted in urine (7).

The *in vivo* metabolism of PPA is now receiving considerable attention in a different context in the study of inborn errors of metabolism. Its glycine conjugate, PPG, has proven to be a pathognomonic metabolite for the diagnosis of MCAD deficiency (18). The disease is an autosomal recessive defect of

mitochondrial β-oxidation of fatty acids (19). PPG was originally identified by GC/MS in urine from a case diagnosed as Reye's-like syndrome (20), and, 2 y later, was detected in urine from eight proven MCAD-deficient patients (14, 21). The specificity of PPG for the diagnosis of MCAD deficiency has been recently confirmed by us in 21 MCAD-deficient patients including 17 cases who were asymptomatic and untreated at the time of the study (18). The hypothesis that PPA is a product of bacterial metabolism has been corroborated by our observation that, among three diagnostic acylglycines, only PPG excretion was not increased in urine from a MCAD-deficient newborn, in agreement with the gradual bacteria colonization of the intestinal mucosa (Bennett MJ, Coates PM, Hale DE, Millington DS, Pollitt RJ, Rinaldo P, Roe CR, Tanaka K, unpublished data).

In view of these recent development in the study of MCAD deficiency and other inborn metabolic disorders, it is important to ascertain if PP-CoA is metabolized exclusively via MCAD to provide the biochemical basis for the use of PPG as a diagnostic marker for MCAD-deficiency. Previous *in vivo* (16) and *in vitro* (22, 23) experiments have shown that the shortening of the aliphatic chain of ω-phenyl fatty acids by two carbon atoms is catalyzed by the enzymes of mitochondrial and peroxisomal fatty acid β-oxidation (23). In an extensive study of the mechanism of the flavin reduction step in the acyl-CoA dehydrogenation pathway, Murfin (22) investigated the dehydrogenation of PP-CoA by mitochondrial octanoyl-CoA dehydrogenase, that was prepared from beef liver. However, it should be noted that there are six known acyl-CoA dehydrogenases (24, 25). Three of them, SCAD, MCAD, and LCAD, catalyze the first reaction of the β-oxidation cycle in the catabolism of fatty acids with varying chain length (26). Two others IVD (27) and 2-meBCAD (28), catalyze the third reaction in the leucine and isoleucine/valine pathways, respectively. Glutaryl-CoA dehydrogenase catalyzes the oxidative decarboxylation of glutaryl-CoA, an intermediate of L-lysine metabolism, yielding as final products crotonyl-CoA and carbon dioxide (29). The first five of these enzymes belong to the acyl-CoA dehydrogenase family (30) and share similar molecular properties and reaction mechanisms (31). All acyl-CoA dehydrogenases remove one hydrogen each from the β- and α-positions of an acyl-CoA ester. Electrons accepted by the enzyme from the substrate are transferred to coenzyme Q in the main mitochondrial electron transport chain via two obligate electron acceptors, ETF and ETF-ubiquinone oxidoreductase (29). It has been observed that the substrate specificities of acyl-CoA dehydrogenases overlap to some degree (26). So far, no evidence has ever been presented that PP-CoA is dehydrogenated exclusively by MCAD and not by other mitochondrial acyl-CoA dehydrogenases. Thus, the specificity of PPG excretion for the diagnosis of MCAD deficiency has not yet been thoroughly confirmed by excluding other enzyme defects that could impair PPA oxidation and cause PPG accumulation in body fluids.

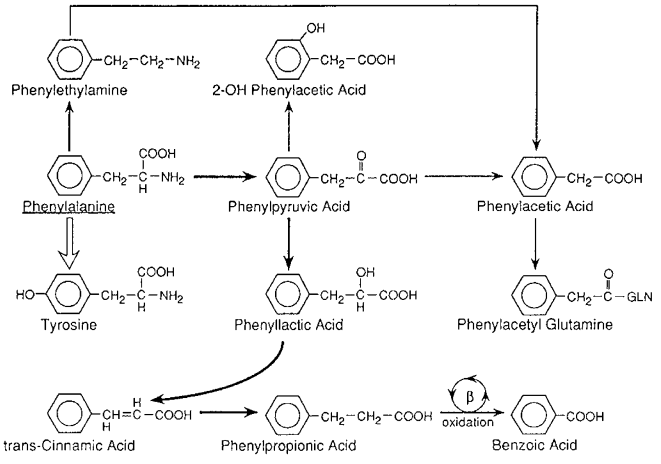

Fig. 1. Phenylalanine metabolism in the intestinal lumen. Proposed scheme for the production of PPA by bacterial degradation of unabsorbed phenylalanine in the intestinal lumen. *Wide closed arrows* indicate the metabolic pathway from phenylalanine to PPA, *narrow arrows* indicate additional mechanisms of phenylalanine catabolism that may take place in the intestinal lumen. The *wide open arrow* shows the conversion to tyrosine, which is the preferential pathway of phenylalanine catabolism. Although tyrosine is not produced from phenylalanine at this level, dietary tyrosine may be metabolized through the same pathway (*wide arrows*) producing 3-(p-hydroxyphenyl)propionic acid (phloretic acid) (15).

For these reasons, we synthesized the phenylpropionyl-CoA ester and tested it *in vitro* as a substrate for five mitochondrial acyl-CoA dehydrogenases purified from rat liver (26–28) and two from human liver (32). Also, a study of the reaction mechanism was carried out by GC/MS analysis of the reaction products. Octanoyl-CoA, the optimal substrate of MCAD, and PP-CoA were allowed to react with human MCAD under various assay conditions. These experiments were carried out to confirm the identity of the trans-2-enoyl-CoA products and to explore the mechanism(s) for the normal PPG urinary excretion in patients affected with ETF or ETF-ubiquinone oxidoreductase deficiencies, commonly referred to as glutaric aciduria type II or ethylmalonic/adipic aciduria (29).

MATERIALS AND METHODS

Materials. CoA lithium salt was obtained from Pharmacia (Milwaukee, WI), FAD and DCIP were purchased from Sigma Chemical Co. (St. Louis, MO), PMS was from Eastman (Rochester, NY). Hydrocinnamic, octanoic, *trans*-2-octenoic, *trans*-cinnamic, and 4-phenylbutyric acids were purchased from Aldrich (Milwaukee, WI). D_{15}-Octanoic acid was obtained from Merck, Sharp and Dohme (Montreal, Canada). N,O-bis (trimethylsilyl)-acetamide in pyridine and 1.0 mL Reactivials were from Pierce Chemical Co. (Rockford, IL). Diazomethane was generated by the method of Schlenk and Gellerman (33) with minor modifications (34).

Synthesis of phenylpropionyl-CoA. PP-CoA was prepared from free CoA and hydrocinnamic acid via the mixed anhydride synthesis (35, 36). PP-CoA was purified by ascending paper chromatography using ethanol/0.1 M potassium acetate (1:1), pH 4.5, as the developing solvent. The CoA ester band was visualized under UV-light, cut out, and eluted using distilled water. To remove residual acid, the crude product was acidified to pH 1.5 with 0.1 N HCl and extracted six times with equal volumes of ether. The solvent phases were discarded, and the water layer was frozen and lyophilized under vacuum. Yield was checked by hydroxyamate assay (37) and by GC/MS analysis of the acyl moiety as TMS-derivative. All other acyl-CoA substrates were purchased from P-L Biochemicals (Milwaukee, WI) and used without any further purification.

Preparation of enzymes. LCAD, MCAD, SCAD (26), IVD (27), and 2-meBCAD 28 were previously purified to homogeneity from rat liver mitochondria in this laboratory (30, 33). Human SCAD and MCAD were purified also to homogeneity from homogenate of autopsy livers (32). The purified enzyme preparations were stored in 50% glycerol at −20°C.

Assay of acyl-CoA dehydrogenase activity. The *in vitro* assay of acyl-CoA dehydrogenases was carried out spectrophotometrically under aerobic conditions according to the method previously described (26), using PMS and DCIP as the primary electron acceptor and secondary electron acceptor/indicator dye, respectively. The reaction mixture contained 100 mM potassium phosphate buffer (pH 8.0), 3 mM PMS, 0.1 mM FAD, 0.048 mM DCIP, and 0.1 mM substrate (0.2 mM in the case of PP-CoA). In each control, the optimal substrate for each enzyme (butyryl-CoA for SCAD; octanoyl-CoA for MCAD; palmitoyl-CoA for LCAD; isovaleryl-CoA for IVD; and isobutyryl-CoA for 2-meBCAD) was used. The final volume of the reaction mixture

was 0.5 mL. The amount of enzymes added were: SCAD (rat), 0.05 nmol; MCAD (rat), 0.03 nmol; LCAD, 0.40 nmol; IVD, 0.15 nmol; 2-meBCAD, 0.23 nmol; SCAD (human), 0.05 nmol, and MCAD (human), 0.08 nmol. The enzyme, buffer, and FAD were preincubated at 30°C for 5 min before adding the substrate, DCIP and PMS, the latter as starter of the reaction. The initial rate of DCIP bleaching at 600 nm wavelength was determined for 30 s at 30°C using a Beckman DU-7 spectrophotometer with a microprocessor (Beckman Instruments, Fullerton, CA).

Identification of the reaction products after incubation of PP-CoA and octanoyl-CoA with human MCAD. Reaction mixtures were prepared in triplicate as described above, containing 0.08 nmol of human MCAD and 0.2 mM PP-CoA, and were allowed to react at 37°C under aerobic conditions unless otherwise mentioned. One-tenth mM octanoyl-CoA, the optimal substrate, was separately incubated with MCAD under the same conditions to compare the efficiency of the PP-CoA dehydrogenation reaction. Anaerobic reactions were carried out after repeated cycles of evacuating and purging with argon gas the reaction mixture in a sealed cuvette, followed by a continuous argon flow throughout the incubation period. At the end of the reaction time, the mixtures were transferred to a test tube containing 50 μL of 3.5 M KOH and further incubated at 37°C for 30 min to release the free acid from each acyl-CoA ester. After cooling the mixtures at room temperature, 10 μg of 4-phenylbutyric acid (100 μL of 0.1 mg/mL solution in methanol) or 50 μg of D_{15}-octanoic acid (100 μL of 0.5 mg/mL solution in methanol) were added to the mixture containing the starting substrate PP-CoA or octanoyl-CoA, respectively. PMS was first removed by extracting the reaction mixture four times with 1.0 mL of diethyl ether at the basic pH. The samples were then acidified to pH 1.0 with 6 N HCl and extracted five times with 1.0 mL of diethyl ether. The solvent fractions were pooled, dried 1 h over anhydrous Na_2SO_4 and evaporated in a water bath at 37°C. PPA and *trans*-cinnamic acids were analyzed as TMS derivatives. They were prepared by heating the evaporated samples in 100 μL of TRI-SIL/BSA for 30 min at 60°C. The evaporated residues containing octanoate and *trans*-2-octenoate were redissolved in 100 μL of methanol, and methylated by dropwise addition of ethereal diazomethane. After 1 h at 4°C, the solvent was evaporated to a final vol of 20–30 μL on an ice-bath under a gentle stream of nitrogen.

A Dani (Finnigan MAT, San Jose, CA) gas chromatograph model 9610, coupled with a Finnigan model 4510 quadrupole mass spectrometer and a Nova 4c computer, were used in the SIM mode for the identification and quantitation of the reaction product. Samples were injected in the split mode (ratio 1:20 for total ion current analyses, 1:10 for SIM) into a high-performance fused silica capillary column (Hewlett Packard, Palo Alto, CA): 25 m length, 0.2 mm internal diameter, cross-linked 5% phenyl methyl silicone gum phase, 0.33-μ film thickness. Helium was used as carrier gas at a column flow of 1.0 mL/min. The injection port and transfer line temperatures were kept at 250 and 275°C, respectively. The gas chromatographic parameters for the analysis of PPA and *trans*-cinnamic acid TMS-derivatives, were as follows: starting temperature, 160°C; initial time, 1.0 min; ramp rate, 8.0°C/min; and final temperature, 210°C maintained for 0.8 min. The gas chromatographic method for octanoate and *trans*-2-octenoate methyl-esters used an initial oven temperature of 100°C, an initial time 0.8 min, a ramp rate of 8.0°C/min and a final temperature of 150°C. In this case, methylation was preferred over silylation to avoid significant product loss due to the heating of samples during the derivatization procedure.

Calibration curves for GC/MS selected ion monitoring analysis of the substrates and reaction products were prepared by adding increasing amounts of the authentic compound (5.0 to 50.0 μg) to a constant amount of the internal standard in a blank reaction mixture without the enzyme. In the case of PPA and *trans*-cinnamic acid, the ratio to the internal standard, 4-phenylbutyric acid, ranged from 0.5 to 2. For the quantitation of octanoic acid and *trans*-2-octenoic acid, the ratio to the internal standard, D_{15}-octanoic acid, ranged from 0.2 to 1. The calibration samples were extracted and derivatized through the aforementioned procedures. Linear regression equations gave the following coefficients: PPA, $r = 0.9992$; *trans*-cinnamic acid, $r = 0.9997$; octanoic acid, $r = 0.9999$; and *trans*-2-octenoic acid, $r = 0.9985$.

First, the identification of the reaction products was verified by the comparison of their EI ionization mass spectra with those obtained by analysis of the respective pure standards. EI mass spectrometric conditions were as used routinely in our laboratory for organic acid analysis (38). Quantitative analysis of the reaction products was then performed under CI conditions in the SIM mode. The mass spectrometer was set up as follows: ion source temperature 130°C, electron energy 70 eV and emission current 250 μA. Ammonia was used as the reagent gas at a source pressure held constant at 0.20 torr reading of the pressure gauge. CI mass spectra of the pure standards were recorded by scanning from m/z 100 to m/z 650 at a rate of 0.6 scan/s, and suitable ions were chosen for SIM analysis. The dwell time was 100 ms at each m/z. The following ions were monitored for analysis of PP-CoA reaction product TMS-derivatives: m/z 240 (ammonia adduct molecular ion, indicated as $[M + 18]^+$) for PPA, m/z 221 (protonated molecular ion, indicated as $[M + H]^+$) for *trans*-cinnamic acid, and m/z 254 ($[M + 18]^+$) for 4-phenyl butyric acid (internal standard). Methyl-esters of octanoyl-CoA reaction products were analyzed by monitoring m/z 176 ($[M + 18]^+$) for octanoic acid, m/z 174 ($[M + 18]^+$) for *trans*-2-octenoic acid and m/z 191 ($[M + 18]^+$) for D_{15}-octanoic acid (internal standard). Peak areas were calculated via computer after visual verification of baseline points by the operator.

RESULTS

The reactivity of PP-CoA was first tested with the preparations of five acyl-CoA dehydrogenases purified from rat liver mitochondria. In the paired experiments, the activity of each enzyme toward the respective optimal substrate was determined and compared to the reactivity of PP-CoA (Table 1). PP-CoA was dehydrogenated at a significant rate by rat MCAD, averaging 48% of the activity using octanoyl-CoA as substrate under the same conditions. There was no activity when rat SCAD was used. Rat LCAD, IVD, and 2meBCAD exhibited a very low activity with PP-CoA, which represented 1 to 7% of the activity of each enzyme to dehydrogenate the respective optimal substrate.

In the assays using human SCAD and MCAD, no activity was found with SCAD, but PP-CoA was very efficiently dehydrogenated by human MCAD. The average activity in four experiments was 75% of that with octanoyl-CoA under the same experimental conditions. In the steady state condition, the Km of PP-CoA for human MCAD was 50 μM.

Mass spectral identification of the reaction products was achieved by comparison of the mass spectra of the reaction

Table 1. *In vitro activities of rat and human acyl-CoA dehydrogenases toward 3-phenylpropionyl-CoA**

Enzyme	Substrate	
	PP-CoA	Optimal substrate
	(μmol DCIP reduced/min/mg protein)	
Rat enzymes		
SCAD	0.00	3.84
MCAD	7.92	15.84
LCAD	0.07	0.96
IVD	0.03	2.43
2meBCAD	0.04	0.66
Human enzymes		
SCAD	0.00	6.14
MCAD	3.50	4.69

* Data represent the mean of assays prepared in triplicate. Zero activity refers to negative values after blank subtraction.

products, as obtained under EI conditions, with those of the respective authentic standards. Chromatographic retention times and mass spectral fragmentation patterns of the products from PP-CoA and octanoyl-CoA were essentially identical to those for *trans*-cinnamic (ω-phenyl-2-propenoic) and *trans*-2-octenoic acids as TMS-derivatives and methylesters, respectively. Figure 2 shows the mass spectra of *trans*-cinnamic acid TMS-ester obtained by analysis of the reaction mixture under CI (Fig. 2*A*) and EI conditions (Fig. 2*B*). Here the advantage of using CI for SIM quantitation is apparent by the marked reduction of fragmentation of this compound under CI conditions and by the prominent intensity of the protonated molecular ion at m/z 221. These conditions are highly desirable for quantitative analysis through the monitoring of single ionic species (41).

For the quantitative analysis of each compound, ions with the highest relative intensity in the respective CI mass spectra were selected. They were either the protonated ($[M + H]^+$) or the NH_4 adduct ($[M + 18]^+$) molecular ions. Amounts of each compound were calculated by internal standard technique. Table 2 summarizes the results of the reaction product quantitation. In blank reaction mixtures without addition of the enzyme, no signal was detected at the retention time expected for *trans*-cinnamic or *trans*-2-octenoic acid under the respective analytical conditions. After incubation for 5 min in the presence of 0.08 nmol of human MCAD, the dehydrogenation products were readily detectable in the amount that is 38 and 59% of the sum (in nmol) of the substrate and the respective reaction product for *trans*-cinnamoyl-CoA and *trans*-2-octenoyl-CoA, respectively. The amounts of both products produced after a 10-min incubation were almost identical to those of the 5-min incubation (data not shown).

In the presence of DCIP and the absence of PMS, the primary electron acceptor of the dye reduction assay, no dehydrogenated product was detected from either substrate after 10 min of incubation. After 30 min of incubation, however, both products were readily detectable in the enzyme-catalyzed reactions, and the amounts of products further increased two to three times after a 90-min incubation, with the amount of *trans*-cinnamoyl-CoA and *trans*-2-octenoyl-CoA reaching 15.5 and 3.7%, respectively, of the amount of substrate added (Table 2; Fig. 3). Each product was identified by mass spectrometry as described above. The yield of *trans*-cinnamoyl-CoA was four times higher than that of *trans*-2-octenoyl-CoA under identical experimental conditions.

When the reaction was carried out for 90 min under aerobic conditions with both PMS and DCIP omitted, only a small amount of 2-octenoyl-CoA was produced from octanoyl-CoA. In contrast, 11.3% of PP-CoA was converted to 2-*trans*-cinnamoyl-CoA under the same conditions.

DISCUSSION

In 1908, in one of the pioneering studies of the β-oxidation of fatty acids, Dakin (39) demonstrated that PPA fed or injected into a dog was oxidized to benzoic acid and excreted as hippuric acid. More recently, Murfin (22) described that PP-CoA is dehydrogenated by beef liver MCAD. However, no direct evidence has ever been presented that PP-CoA is exclusively dehydrogenated by MCAD and not by other mitochondrial acyl-CoA dehydrogenases. For this reason, we have investigated the *in vitro* reactivity of PP-CoA with five purified mitochondrial acyl-CoA dehydrogenases. These enzymes are now recognized as belonging to a gene family (30), sharing major structural and catalytic characteristics, and differing only in the length and configuration of the acyl-CoA substrates. The main aim of our study is to ascertain at the enzymatic level the rationale for using PPG for the biochemical diagnosis of MCAD deficiency, by providing definitive proof that MCAD is responsible for the first step in the PP-CoA degradation.

When PP-CoA was used as substrate, we found no significant activity after incubation with catalytic amounts of LCAD, SCAD, IVD, or 2-meBCAD. In contrast, PP-CoA was dehydrogenated by rat and human MCAD at a rate 48–75% of that with octanoyl-CoA under the identical experimental conditions. In considering the physiologic significance of these data, the quality of preparations used in this experiment need to be taken into account, because the length of storage and the rate of activity loss during storage are different from enzyme to enzyme. The rat MCAD preparation used here was the latest and best preparation we had, and its specific activity toward octanoyl-CoA had not significantly decreased. The initial specific activities of the rat SCAD, LCAD, IVD, and 2-meBCAD preparations with the respective optimal substrate, measured at the time of purification, were 10.6, 2.0, 2.7, and 2.0 μmol of DCIP reduced/min/

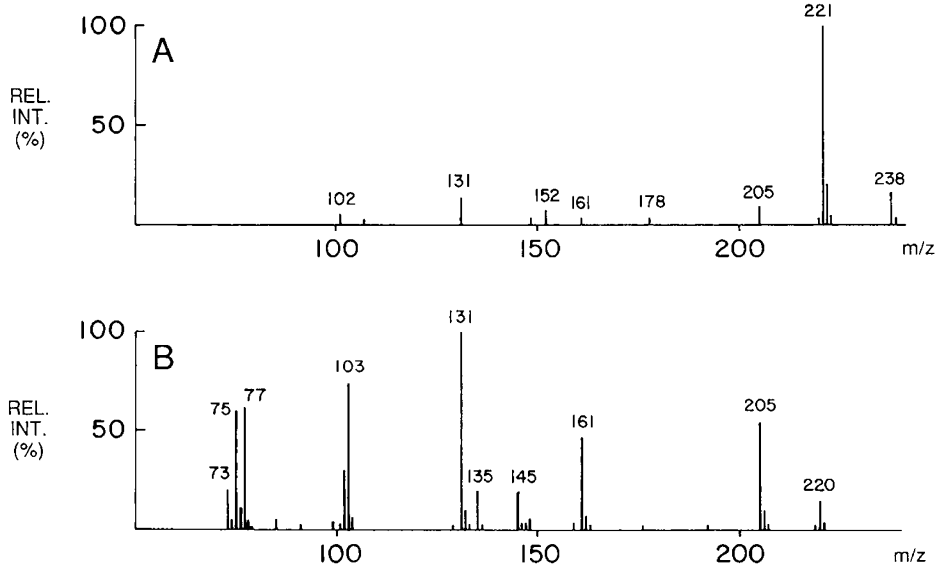

Fig. 2. Demonstration of *trans*-cinnamic acid as the reaction product. The reaction mixture containing 0.2 mM PP-CoA and a catalytic amount of human MCAD was first hydrolyzed with 3.5 M KOH for 10 min at 37°C. The hydrolyzed mixture was extracted after acidification, and the TMS derivative of the evaporated residue was analyzed by GC/MS. Instrumental conditions are described in *Materials and Methods*. *A*, ammonia (0.2 torr) CI and *B*, EI ionization mass spectra. In the CI spectrum, ions at m/z 221 and m/z 238 represent the protonated $[M + H]^+$ and ammonia-adduct $[M + 18]^+$ molecular ions, respectively.

Table 2. *GC/MS reaction product analysis after in vitro incubation of PP-CoA and octanoyl-CoA with human medium chain acyl-CoA dehydrogenase**

Enzyme added (μL)	Electron acceptors		Incubation time (min)	Amount produced	
	PMS	DCIP		*trans*-cinnamic (% of [Substrate + Product])	*trans*-2-octenoic
0	+	+	0	0.0	0.0
5	+	+	5	39.0	59.3
5	−	+	10	0.0	0.0
5	−	+	90	15.4	3.7
5	−	−	90	11.4	0.6

* The reactions producing *trans*-cinnamoyl-CoA and those producing *trans*-octanoyl-CoA were separately performed under aerobic conditions. Results from the experiments performed under identical conditions were summarized in the same lines to allow the comparison of the reactivity of the two substrates. The amount of product detected is given as percentage to the sum of the respective substrate and product, both of which were determined by SIM analysis of the respective free acid after alkaline hydrolysis and ethyl acetate extraction of the mixture.

mg as measured using the dye-reduction assay (26–28). The activities of the preparations used here were lower than these values as shown in Table 1, indicating unequal loss of activity among them. The rat IVD preparation, like the rat MCAD preparation, had lost little activity. The LCAD preparation has lost approximately 50% of the original activity, whereas those of the SCAD and 2-meBCAD preparations were 38 and 33% of the original activity (26–28). The human SCAD and MCAD activities were 84 and 69% of the respective original activity (32). Even if the loss of the activity LCAD and 2-meBCAD is taken into account, the activities of fresh LCAD and 2-meBCAD with PP-CoA would be no more than 2% of that by MCAD. Thus, the data presented in Table 1 clearly establish that MCAD is almost exclusively responsible for the dehydrogenation of PP-CoA *in vivo*, confirming the specificity and usefulness of urinary PPG determination in the differential diagnosis of MCAD deficiency from other inherited defects of fatty acid metabolism.

PPG excretion has consistently been found not to be increased (18) (Rinaldo P, Tanaka K, unpublished results) in urine from several patients affected with ETF or ETF-ubiquinone oxidoreductase deficiency (29), despite the known requirement of MCAD *in vivo* for ETF and ETF-ubiquinone oxidoreductase. These findings suggest that an alternative mechanism might be responsible for the *in vivo* dehydrogenation of PP-CoA in a situation where the flow of electrons via ETF and ETF-ubiquinone oxidoreductase to the main mitochondrial electron transport chain is blocked. Our search for the alternative reaction mechanism revealed that in the presence of DCIP alone, omitting PMS, no activity was detectable after 10 min of aerobic incubation with PP-CoA or octanoyl-CoA, but a considerable amount of PP-CoA was dehydrogenated after 90 min. In contrast, the amount of octanoyl-CoA dehydrogenated was only 25% that of PP-CoA under the same condition. When PMS and DCIP were both omitted, PP-CoA was still dehydrogenated after 90 min in an amount slightly smaller (75%) than in the presence of DCIP, whereas the amount of octanoyl-CoA dehydrogenated was negligible. These results indicate that, in the absence of ETF or PMS, PP-CoA can be dehydrogenated by MCAD that is reduced by oxygen or other biologic oxidants. The dehydrogenation of PP-CoA in the absence of ETF or PMS is not nearly as efficient as in the presence of ETF or PMS, but the rate of this alternative reaction appears to be fast enough to process small amounts of phenylpropionic acid produced *in vivo*. In this regard, the catalytic behavior of PP-CoA is similar to that of β-(2-furyl)propionyl-CoA, a chromophoric pseudosubstrate with a structure very similar to PP-CoA. McFarland *et al.* (40) reported the appearance of trans-β-(2-furyl)acryloyl-CoA in a system

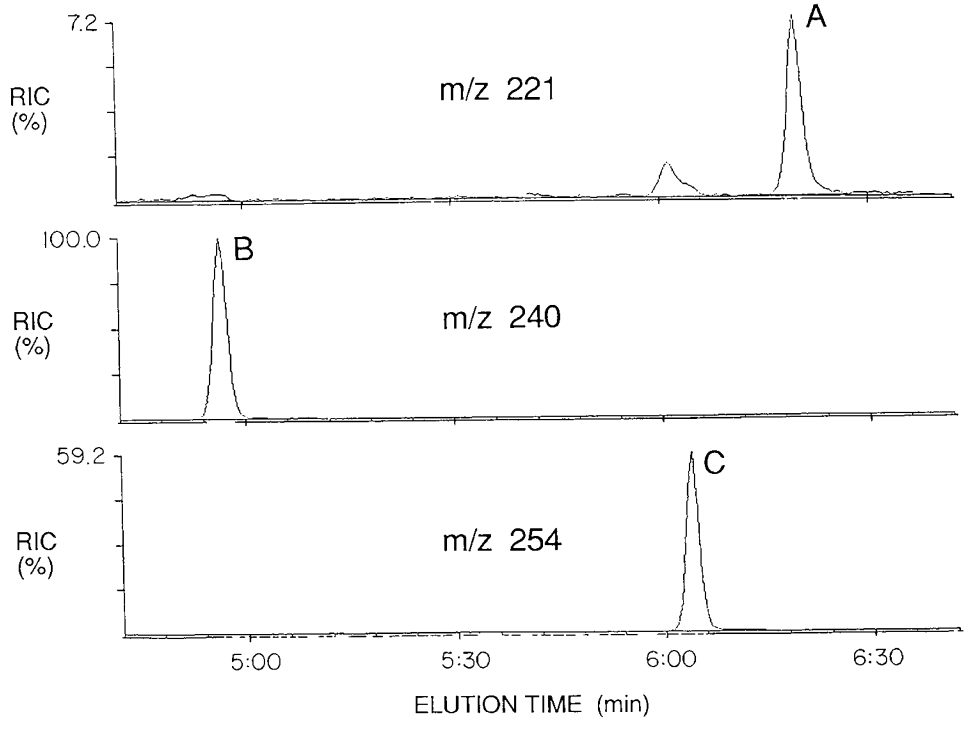

Fig. 3. CI (ammonia, 0.2 torr) $[M + H]^+$ ion chromatograms of products from PP-CoA by MCAD in the absence of the PMS. The reaction mixture containing 0.2 mM PP-CoA, a catalytic amount of human MCAD and 0.048 mM DCIP was incubated for 90 min under aerobic conditions without the addition of PMS. The reaction mixture was then hydrolyzed, extracted, and derivatized. The mol wt cited below are those of the corresponding TMS-derivatives. Peak legend: *A*, *trans*-cinnamic acid (mol wt 220, $[M + H]^+$: m/z 221); *B*, PPA (mol wt 222, $[M + 18]^+$: m/z 240); *C*, 4-phenylbutyric acid (internal standard, mol wt 236, $[M + 18]^+$: m/z 254).

where β-(2-furyl)propionyl-CoA was incubated aerobically with general acyl-CoA dehydrogenase in the absence of electron acceptors. In this condition, oxygen was acting as the electron acceptor resulting in the production of H_2O_2. They concluded that "general" acyl-CoA dehydrogenase, as MCAD was referred to in earlier studies, acts as a slow oxidase catalyst producing enoyl-CoA species and H_2O_2. Ikeda *et al.* (41) subsequently pointed out that the oxidase reaction of MCAD with β-(2-furyl)propionyl-CoA is likely due to its unusual structure. Upon dehydrogenation at C-2 and C-3, this substrate would have a C = C double bond conjugated with an aromatic system and a carboxylate oxygen. Thus, the C-3 hydrogen of this substrate is highly activated, leading to the complete reduction of the enzyme and dissociation of the reduced enzyme/product complex. In contrast, in the reaction of the natural substrates with acyl-CoA dehydrogenases, only the substrate leaves the enzyme/substrate complex (30). As noted above, PP-CoA shares a similar structure with β-(2-furyl)propionyl-CoA. This peculiar structure of PP-CoA with the activated C-3 hydrogen is likely to represent the basis for its ability to use an alternative electron acceptor in its reaction with MCAD. As McFarland *et al.* (40) suggested for β-(2-furyl)propionyl-CoA, nicotinamide adenine dinucleotide, ubiquinone, or oxygen are plausible alternatives for ETF. Based on these results, we propose that relatively small but significant amounts of PP-CoA produced *in vivo* could be dehydrogenated even in the absence of either of the natural electron acceptors. We found that octanoyl-CoA undergoes the same mechanism to a much smaller extent, but its rate seems inadequate to be of any significance in the flow of fatty substrates through the mitochondrial β-oxidation pathway.

In the disposal of PP-CoA, we must consider the possible role of the peroxisomal β-oxidation system. Using a partially purified enzyme preparation and phenyl-lauryl-CoA as a model substrate, Yamada *et al.* (23) demonstrated that approximately 60% of the β-oxidation of this substrate in the rat liver was peroxisomal, increasing to 85% in clofibrate-treated rats. However, they found only trace amounts of phenyl-butyryl-CoA by GC/MS analysis of chain-shortening products, consistent with the poor ability of the peroxisomal β-oxidation system to metabolize acyl-CoA moieties with a chain length shorter than C_6 (42). Therefore, it appears credible that PPA metabolism is mainly a mitochondrial process. This view is well supported by our previous data that PPG excretion is significantly increased in urine from patients with MCAD deficiency (18).

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Erratum

A line was omitted from the abstract of the article entitled "Deficient herpes simplex virus-induced interferon-α production by blood leukocytes of preterm and term newborn infants" by Britta Cederblad *et al.* (Pediatr Res 27:7–10, 1990). The printer regrets this error. **The following is the complete abstract:**

ABSTRACT. The ability of peripheral blood mononuclear cells (PBMC) from newborn infants, gestational age 24–42 wk, to produce interferon-α (IFN-α) on the first day after birth was studied *in vitro*. Human amnion cells (WISH) coated with herpes simplex virus type I and fixed by glutaraldehyde were used as IFN-α inducers. Individual IFN-α producing cells (IPC) among PBMC were determined by an immunoplaque assay. The frequency of IPC was low in all premature (≤36 wk) infants (median 0.3 IPC/10^4 PBMC, range 0.0–2.6), and significantly higher (median 2.0 IPC/10^4 PBMC, range 0.0–16.4) in term infants (>37 wk). The frequencies were lower in both groups of infants than in adults (7.3 IPC/10^4 PBMC, range 2.0–23.7). When a conditioned medium from cultures of herpes simplex virus type I-stimulated PBMC from adults was added to the IFN induction cultures, the frequencies of IPC increased in PBMC from both preterm and term infants, and in the latter group did not differ significantly from adult levels. The median production of IFN-α per IPC was 1.1 U (range 0.0–2.8) in premature infants, 1.0 U (range 0.0–8.8) in term infants and 3.2 U (range 1.5–8.0) in adults. When concentrations of PBMC in the cultures were decreased, a decline of IPC frequencies occurred. This decline was more marked and started at higher PBMC concentrations in infants than in adults, and was prevented by addition of conditioned medium from herpes simplex virus type I-stimulated cultures of PBMC from adults. The results suggest that PBMC of preterm infants on the first day after birth are deficient both with respect to the proportion of actual IPC and to accessory mechanisms necessary for a normal IFN-α response. In contrast, IPC frequencies in term infants approach levels of adults, but accessory functions may still be deficient. (*Pediatr Res* 27: 7–10, 1990)