Intrinsic Isomerase Activity of Medium-Chain Acyl-CoA Dehydrogenase†

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ABSTRACT: Mitochondrial medium-chain acyl-CoA dehydrogenase is a key enzyme for the β oxidation of fatty acids, and the deficiency of this enzyme in patients has been previously reported. We found that the enzyme has intrinsic isomerase activity, which was confirmed using incubation followed with HPLC analysis. The isomerase activity of the enzyme was thoroughly characterized through studies of kinetics, substrate specificity, pH dependence, and enzyme inhibition. E376 mutants were constructed, and mutant enzymes were purified and characterized. It was shown that E376 is the catalytic residue for both dehydrogenase and isomerase activities of the enzyme. The isomerase activity of medium-chain acyl-CoA dehydrogenase is probably a spontaneous process driven by thermodynamic equilibrium with the formation of a conjugated structure after deprotonation of substrate α proton. The energy level of the transition state may be lowered by a stable dienolate intermediate, which gains further stabilization via charge transfer with the electron-deficient FAD cofactor of the enzyme. This raises the question as to whether the dehydrogenase might function as an isomerase *in vivo* in conditions in which the activity of the isomerase is decreased.

Numerous diseases have been reported in relation to fatty acids, such as cardiovascular disease (*1*), cancer (*2*), and diabetes (*3*). The regulation of fatty acid oxidation has been reported as a potential method of treating noninsulin-dependent diabetes mellitus (NIDDM) (*4*), and inhibitors of enzymes involved in the metabolism of fatty acids have been synthesized and studied as potential medicines by the Sandoz Research Institute (*5*). It has been observed that rates of fatty acid oxidation are greater in NIDDM, and it has been hypothesized that elevated free fatty acids are the cause of increased endogenous glucose production and the resultant hyperglycemia in NIDDM (*4*). A rational treatment would therefore be to inhibit the abnormally high rate of fatty acid oxidation. The incidence of NIDDM has been estimated at about 20 million in the U.S. and 50 million in China and India (*5*). Fatty acid oxidation in mitochondria is also an essential energy generation system for cells. During prolonged fasting and starvation, fatty acids are the precursors of ketone bodies, which are an important alternate fuel in extrahepatic tissues at times when the supply of glucose is limited.

The degradation of saturated fatty acids occurs in mitochondria in a sequence of four reactions referred to as the β-oxidation cycle (*6*). Acyl-CoA dehydrogenases (ACDs) catalyze the first and rate-limiting step reaction of the β-oxidation cycle, which involves conversion of saturated acyl-CoA substrate to unsaturated acyl-CoA (Figure 1). There are nine known members in the acyl-CoA dehydrogenase

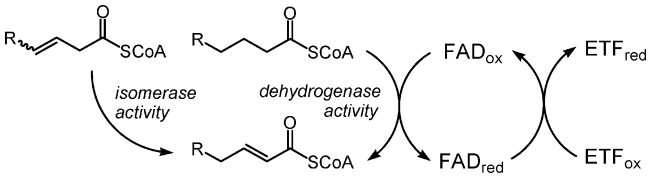

FIGURE 1: Acyl-CoA dehydrogenase-catalyzed reactions.

(ACAD) family, and five members are involved in fatty acid β oxidation; these are short-chain (SCAD), medium-chain (MCAD), long-chain (LCAD), and very long-chain (VLCAD1 and VLCAD2) acyl-CoA dehydrogenase (*6*). The four other members are involved in amino acid oxidation pathways; they are iso(3)valeryl-CoA dehydrogenase (i3VD) for leucine, iso(2)valeryl-CoA dehydrogenase (i2VD, also known as short/branched-chain acyl-CoA dehydrogenase or 2-methylbutyryl-CoA dehydrogenase) for isoleucine, isobutyryl-CoA dehydrogenase (iBD) for valine, and glutaryl-CoA dehydrogenase (GD) for lysine and tryptophan (*6*). With the exception of VLCADs, all of these are soluble homotetramers with a subunit mass of approximately 43 kDa, with each subunit containing one FAD. While each of these enzymes displays a preferential substrate specificity, they all adopt a common mechanism in which the initial step has been established to be the abstraction of the *pro-R* α proton by a conserved glutamate in the active site. This is followed by the removal of a β hydride from the nascent carbanion intermediate, and the stereochemistry of this step has been shown to be *pro-R*-specific as well (*7*, *8*). MCAD (EC 1.3.99.3) acted on C4−C16 acyl-CoAs with its peak activity toward medium-chain (C6−C12) substrates. It has been reported that between 19 and 25% of patients with undiagnosed deficiency of MCAD die during their first episode of metabolic decompensation (*9*, *10*) and that those who survive

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an initial crisis experience substantial morbidity (*9*, *10*). Several studies have also retrospectively documented deficiency of MCAD in infants who had previously received a diagnosis of sudden infant death syndrome (*11*−*13*). The MCAD purified from pig kidney has been solved at 2.0 Å resolution (*14*−*16*), and Glu376 has been confirmed as the catalytic residue. Several mechanism-based inhibitors of MCAD have been characterized in sufficient detail, which helped to understand and control the reaction catalyzed by MCAD (*17*−*19*). In our further study of MCAD, we found that the enzyme has intrinsic enoyl-CoA isomerase activity, which is probably a spontaneous process driven by thermodynamic equilibrium with formation of a conjugated structure after deprotonation of substrate α proton.

EXPERIMENTAL PROCEDURES

Materials. A HiTrap chelating metal-affinity column was purchased from Amersham Pharmacia Biotech. Octanoyl-CoA was purchased from Sigma. *Pfu* DNA polymerase, HB101 competent cells, *Escherichia coli* strain BL21(DE3) competent cells, and agarose came from Invitrogen Life Technologies. The Plasmid Mini kit and synthesized oligonucleotides were obtained from Tech Dragon Company of Hong Kong. A gel-extraction kit, T4 DNA ligase, and restriction enzymes came from MBI Fermentas of Germany. All other reagents were of research grade or better and were obtained from commercial sources. The wild-type rat mitochondrial MCAD was cloned and purified as previously described (*20*). The MCAD apoprotein without a bound FAD cofactor was prepared as described previously (*21*).

Construction of Rat Mitochondrial MCAD Mutants E376A, E376Q, and E376D. A QuikChange mutagenesis kit (Stratagene) was applied for constructing the pMCAD(E376A), pMCAD(E376Q), and pMCAD(E376D) mutant expression plasmids. The plasmid pLM1::MCAD (*20*) was used as a template for constructing expression plasmids through PCR. The following primers and their antisense primers were synthesized to introduce the mutated sequence: (1) E376A, 5′-cc aag atc tat cag att tac **gca** ggt act gca caa att c-3′, codon gaa for glutamic acid (E) was changed to codon gca for alanine (A); (2) E376Q, 5′-cc aag atc tat cag att tac **caa** ggt act gca caa att c-3′, codon gaa for glutamic acid (E) was changed to codon caa for glutamine (Q); (3) E376D, 5′-g atc tat cag att tac **gat** ggt act gca caa att cag-3′, codon gaa for glutamic acid (E) was changed to codon gat for aspartic acid (D).

PCR amplification was performed using *Pfu* DNA polymerase, and samples were subjected to 13 cycles of 0.5 min of denaturation at 95 °C, 1 min of annealing at 60−63 °C, and 12 min of elongation at 72 °C in a Mastercycler (Eppendorf). The mutant-carrying plasmid was transformed in *E. coli* HB101 competent cells (Novagen) by electroporation (Bio-Rad) for screening purposes. Positive clones were identified, and the DNA was sequenced to verify the presence of the desired mutations and the absence of any PCR-generated random mutations. Plasmids were then transformed in *E. coli* strain BL21(DE3) cells for expression purposes.

Expression and Purification of Soluble Rat Mitochondrial MCAD Wild-Type and Mutant Proteins. Established methods were again used to prepare the samples (*20*). The proteins, all observed to be apparent homogeneity by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE)[1] (Figure 2), were stored at −80 °C in 50 mM potassium phosphate buffer at pH 7.5, 0.1 mM EDTA, 5% glycerol, and 5 mM β-mercaptoethanol.

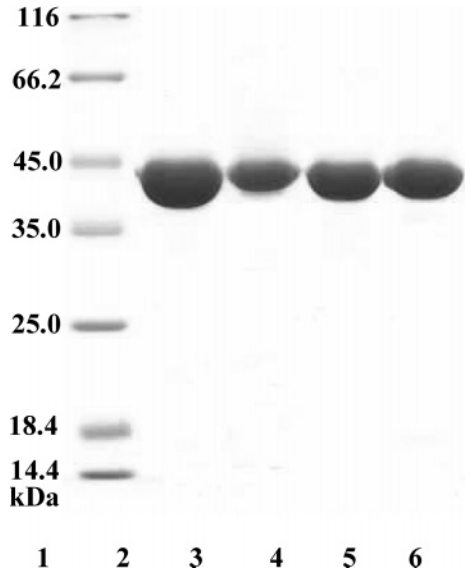

FIGURE 2: Coomassie blue-stained SDS−PAGE of the purified rat mitochondrial MCAD. Lane 1, molecular mass standards; lane 2, purified rat mitochondrial MCAD wild-type enzyme; lane 3, purified rat mitochondrial MCAD mutant E376A; lane 4, purified rat mitochondrial MCAD mutant E376D; lane 5, purified rat mitochondrial MCAD mutant E376Q.

Activity Assay for Dehydrogenase Activity of MCAD. The activity of MCAD was assayed spectrophotometrically following the decrease in absorbance at 600 nm using phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCPIP) as the intermediate and terminal electron acceptor, respectively ($\epsilon_{600nm} = 21\ mM^{-1}\ cm^{-1}$), as described previously (*22*). The reaction progress curves were recorded for 1 min on a Hitachi U-2000 UV−visible spectrophotometer. A standard assay mixture contained 33 μM octanoyl-CoA, 1.5 mM PMS, 48 μM DCPIP, 20 mM phosphate buffer at pH 7.4, and 30 μM EDTA, and the final volume was 0.7 mL. The reaction was started by adding 5 μL of appropriate diluted enzyme in the reaction mixture. A unit of enzyme activity was defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product per minute. Determination of the K_M and the V_{max} was performed using the same assay buffer with varying substrate concentrations.

Activity Assay for Isomerase Activity of MCAD. 3-Enoyl-CoA thiolester was prepared from the corresponding free acid and coenzyme A by the mixed anhydride method (*17*) and subsequently purified by reverse-phase HPLC. *cis*-3-Enoyl-CoAs were made from corresponding *cis*-3-enoic acids, which showed different retention time on HPLC profiles from those of corresponding *trans*-3-enoyl-CoAs. A previously described method was used for assaying the activity of enoyl-CoA isomerase (*23*, *24*). The assay mixture contained 50 mM of potassium phosphate (pH 7.5), 100 μM EDTA, 50 μM 3-enoyl-CoA, and an appropriate amount of

[1] Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMS, phenazine methosulfate; SDS, sodium dodecyl sulfate; UV−vis, ultraviolet−visible spectroscopy.

enzyme in a final volume of 1.0 mL. The reaction mixtures were preincubated for 1 min at 25 °C and were initiated by the addition of the enzyme. The increase in absorbance at 263 nm was monitored, which is characteristic for the conjugated α,β-unsaturated thioester group ($\epsilon = 6700\ M^{-1}\ cm^{-1}$). One enzyme unit (U) was defined as the activity that converts 1 μmol of 3-enoyl-CoA to 2-enoyl-CoA per minute. Determination of the K_M and the V_{max} was performed using the same assay buffer with varying substrate concentrations.

HPLC Analysis of Enzymatic Incubation Mixture. Enzymatic incubations were carried out at 37 °C, and the mixtures were analyzed with HPLC. Incubation reactions were terminated by filtering through a Microcon YM-10 filter (Millipore) with centrifugation. The filtrate was applied to a HPLC C_{18} reverse-phase column (3.9 × 150 mm) attached to a Waters-gradient HPLC system. The absorbance of the effluent was monitored at 260 nm. Separation was achieved by linearly increasing the methanol/H_2O (85%, v/v) content of 25 mM potassium phosphate elution buffer (pH 5.9) from 20 to 80% in 35 min at a flow rate of 0.8 mL/min.

RESULTS

Construction of Mutant Plasmids and Expression and Purification of Proteins in E. coli. We incorporated six histidine amino acids on the C terminal of the enzyme to facilitate the purification process, and no additional residue was added to minimize the effect of the His tag. The specific activity of our wild-type enzyme was 4.0 units/mg, which is within the range of pure MCAD from different sources (*25*−*27*). It should be noted that the MCAD isolated from pig kidney has been most widely used for various enzymatic studies in the past. The specific activity of pure MCAD isolated from pig kidney in our previous study is 3.5 units/mg measured with same enzyme assay method, which is close to 2.6 units/mg reported by another lab isolated from same natural source (*27*).

It has been reported that the His tag on the N terminal affected the activity of the MCAD from *Mycobacterium tuberculosis* (*28*). On the basis of the crystal structure of the MCAD from pig mitochondria (*14*), the enzyme forms a tetramer, which is arranged as a dimer of dimers. Both the N- and C-terminal ends of one dimer extend into the other dimer and lie on the surface of the molecule; however, they are not part of the active site of the MCAD. Therefore, the His tag might have hindrance in monomer interaction to form the active tetramer without affecting the active site of the enzyme. Considering that it is much easier to be purified to apparent homogeneity without possible contamination of authentic enoyl-CoA isomerase, we choose to use His-tagged MCAD for studying isomerase activity of the enzyme, which can give reproducible and reliable results.

A QuikChange mutagenesis kit (Stratagene) was applied to construct the pMCAD(E376A), pMCAD(E376Q), and pMCAD(E376D) mutant expression plasmids. The expressions of rat mitochondrial MCAD and its mutant proteins in *E. coli* BL21(DE3) were carried out at different temperatures, and IPTG concentrations and proteins were obtained for all tested conditions. It was found that the protein expressed very well even without the addition of IPTG and that it resulted in a more soluble protein at room temperature than at 37 °C. Nickel metal-affinity resin columns were used for single-step purifications of His-tagged rat mitochondrial MCAD wild-type and mutant proteins. The wild type and mutants E376A, E376Q, and E376D were obtained as soluble proteins that can be stored in a −80 °C freezer and were stable for at least 6 months, tested on the basis of their activity.

Table 1: Comparison of Kinetic Parameters for Dehydrogenase Activity of Rat Mitochondrial MCAD Wild-Type and Mutant Proteins Using Octanoyl-CoA as the Substrate

	K_M (μM)	V_{max} (μmol mg^{-1} min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} μM^{-1})
wild type	2.3 ± 0.4	4.4 ± 0.2	3.2 ± 0.2	1.4
mutant E376D	5.6 ± 0.2	1.1 ± 0.1	0.79 ± 0.07	0.14

Kinetic Studies of Dehydrogenase Activity for Rat Mitochondrial MCAD Wild-Type and Mutant Proteins. For the kinetic characterization of rat mitochondrial MCAD wild-type and mutant proteins, rates were measured at five substrate concentrations and averages of two assays were used for each point. K_M of 2.3 μM for octanoyl-CoA and V_{max} of 4.4 μmol min^{-1} mg^{-1} were obtained for rat mitochondrial MCAD wild-type enzyme by nonlinear curve fitting using the SigmaPlot 8.0 program (Table 1). Glu376 is the catalytic residue responsible for deprotonation of the α proton. In this study, we mutated Glu376 into alanine, glutamine, and aspartic acid. The k_{cat}/K_M value of the mutant E376D is 10 times lower than that of the wild-type enzyme, which might be caused by a different position of the terminal carboxylate in the active site for the mutant. All other mutants are completely inactive, which confirmed that Glu376 is the catalytic residue for deprotonation of the α proton. It should be noted that mutation of acidic Glu376 into other nonacidic residues could affect the redox properties of the enzyme (*29*), which also affected the activity of the MCAD.

Kinetic Studies and Substrate Specificity of Isomerase Activity for Rat Mitochondrial MCAD Wild-Type and Mutant E376D. For the kinetic characterization of isomerase activity of rat mitochondrial MCAD wild-type and mutant proteins, rates were also measured at five substrate concentrations and averages of two assays were used for each point. Kinetic parameters of several substrates for wild-type enzyme were determined, as shown in Table 2. The best substrate for the isomerase activity of MCAD is *cis*-3-hexenoyl-CoA, which showed K_M of 20 ± 3 and V_{max} of 150 ± 10 nmol min^{-1} mg^{-1}. Mutants E376A and E376Q did not show any isomerase activity, which might indicate that Glu376 is also the catalytic residue for the isomerase activity of MCAD. Kinetic parameters of substrates for mutant E376D were also determined for comparison. Although the dehydrogenase activity of this mutant is decreased, the isomerase activity of this mutant for *cis*-3-hexenoyl-CoA is even higher than that of the wild-type enzyme, which showed K_M of 14 ± 2 and V_{max} of 660 ± 30 nmol min^{-1} mg^{-1}. This may be because *cis*-3-hexenoyl-CoA is better positioned in the active site of MCAD mutant E376D for deprotonation. It should be noted that different substrates were used for the measurement of dehydrogenase and isomerase activities. While saturated acyl-CoA was used as the substrate for the measurement of dehydrogenase activity, 3-enoyl-CoA was used as the substrate for the measurement of isomerase activity. Specially, the conformation of *cis*-3-enoyl-CoA can be signifi-

Table 2: Comparison of Substrate Specificity for Isomerase Activity of Rat Mitochondrial MCAD Wild-Type Enzyme and Mutant E376D

enzyme	substrate	K_M (μM)	V_{max} (nmol mg^{-1} min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} μM^{-1})
WT	*cis*-3-hexenoyl-CoA	20 ± 3	150 ± 10	0.11 ± 0.01	5.5×10^{-3}
WT	*cis*-3-octenoyl-CoA	30 ± 9	22 ± 3	$(1.6 \pm 0.2) \times 10^{-2}$	5.3×10^{-4}
WT	*cis*-3-decenoyl-CoA	62 ± 8	14 ± 1	$(1.0 \pm 0.1) \times 10^{-2}$	1.6×10^{-4}
WT	*trans*-3-hexenoyl-CoA	15 ± 3	45 ± 3	$(3.2 \pm 0.2) \times 10^{-2}$	2.1×10^{-3}
WT	*trans*-3-octenoyl-CoA	22 ± 6	12 ± 1	$(8.6 \pm 0.7) \times 10^{-3}$	3.9×10^{-4}
WT	*trans*-3-decenoyl-CoA	53 ± 7	6.2 ± 0.4	$(4.4 \pm 0.3) \times 10^{-3}$	8.3×10^{-5}
E376D	*cis*-3-hexenoyl-CoA	14 ± 2	660 ± 30	0.47 ± 0.02	3.4×10^{-2}
E376D	*cis*-3-octenoyl-CoA	18 ± 1	140 ± 3	0.10 ± 0.01	5.6×10^{-3}
E376D	*cis*-3-decenoyl-CoA	23 ± 3	125 ± 6	0.090 ± 0.004	3.9×10^{-3}
E376D	*trans*-3-hexenoyl-CoA	23 ± 2	18 ± 1	$(1.3 \pm 0.1) \times 10^{-2}$	5.7×10^{-4}
E376D	*trans*-3-octenoyl-CoA	31 ± 4	11 ± 1	$(7.9 \pm 0.7) \times 10^{-3}$	2.5×10^{-4}
E376D	*trans*-3-decenoyl-CoA		<1		

cantly different from that of saturated acyl-CoA. Therefore, it is not strange that MCAD mutant E376D has lower dehydrogenase activity but higher isomerase activity than wild-type enzyme. The enzyme has a higher affinity and conversion rate for the shorter chain substrate. This may be related with space or shape of the active site of the enzyme, which can better accommodate the shorter chain substrate. The MCAD can bind octanoyl-CoA better than 3-octenoyl-CoA, and the rate of isomerization catalyzed by MCAD is about 3% of that of the MCAD-catalyzed dehydrogenation reaction.

HPLC Analysis of the Enzymatic Incubation Mixture. To further confirm that MCAD and its mutant E376D have intrinsic enoyl-CoA isomerase activity, we carried out incubation studies and analyzed the metabolites of the incubation mixture with HPLC, as shown in Figure 3. *trans*-3-Octenoyl-CoA showed a single peak **a** in our HPLC profile with a retention time of 23.2 min (Figure 3A). After *trans*-3-octenoyl-CoA was incubated with MCAD, it was partially transformed to peak **b**, which may be *trans*-2-octenoyl-CoA with a retention time of 23.6 min (Figure 3B). The resulting mixture was co-injected into HPLC with *trans*-2-octenoyl-CoA, and the height of peak **b** increases as shown in Figure 3C, which indicates that peak **b** is indeed *trans*-2-octenoyl-CoA. MCAD apoprotein without the bound FAD cofactor cannot transform *trans*-3-octenoyl-CoA to *trans*-2-octenoyl-CoA as shown in Figure 3D. After *trans*-3-octenoyl-CoA was incubated with MCAD mutant E376D, it was also partially transformed to *trans*-2-octenoyl-CoA, as shown in Figure 3E. However, MCAD mutants E376A and E376Q cannot transform *trans*-3-octenoyl-CoA to *trans*-2-octenoyl-CoA as shown in parts F and G of Figure 3. When MCAD apoprotein was incubated with *trans*-3-octenoyl-CoA, followed with the addition of the FAD cofactor into the mixture (incubation experiment D followed with the addition of the FAD cofactor), MCAD showed isomerase activity again, and *trans*-3-octenoyl-CoA was transformed to *trans*-2-octenoyl-CoA as shown in Figure 3H. Peak **b** of Figure 3H was mostly converted to peak **c** of Figure 3I when incubating with enoyl-CoA hydratase, which further confirmed that peak **b** is *trans*-2-octenoyl-CoA and peak **c** is 3-hydroxyoctanoyl-CoA. This result confirmed that E376 is the catalytic residue for both dehydrogenase and isomerase activities of MCAD. The FAD cofactor is required for isomerase activity of MCAD. The lack of isomerase activity of mutants E376A and E376Q and the requirement of the FAD cofactor for the isomerase activity of MCAD indicate that the intrinsic isomerase activity of MCAD is not due to possible contaminant *E. coli* enoyl-CoA isomerase.

Inhibition Study for Rat Mitochondrial MCAD. 2-Octynoyl-CoA is a known mechanism-based inhibitor of MCAD (*30*). When we incubate MCAD with 2-octynoyl-CoA, it was found that both dehydrogenase and isomerase activities were inhibited at almost the same percentage ratio when we add different amounts of 2-octynoyl-CoA, as shown in Figure 4. This indicate that both dehydrogenase and isomerase activities are associated with the same active site of the enzyme, which further confirmed that Glu376 is the catalytic residue of the enzyme for its isomerase activity.

pH Dependence of Dehydrogenase and Isomerase Activities for Rat Mitochondrial MCAD. Both dehydrogenase and isomerase activities of MCAD were measured at different pH, and the results were shown in Figure 5. It was found that both activities have similar pH dependence curves with a maximum activity at pH 7.5. This may further indicate that both dehydrogenase and isomerase activities are associated with the same active site of the enzyme and that Glu376 is the catalytic residue of the enzyme for both dehydrogenase and isomerase activities.

Sequence Comparison between MCAD and Δ^3-Δ^2-Enoyl-CoA Isomerase. Sequence alignment was carried out for comparison between acyl-CoA dehydrogenase and Δ^3-Δ^2-enoyl-CoA isomerase; however, no significant similarity was found between these two enzymes. This might suggest that the isomerase activity of MCAD may be a spontaneous process after the initial deprotonation of the α proton by MCAD, driven by thermodynamic equilibrium with the formation of a conjugated structure.

DISCUSSION

Scientists have traditionally considered that an enzyme has only one physiological role. Although it is true in most cases, sometimes the enzyme can also have some other minor enzymatic activity. It has been reported that MCAD has intrinsic oxidase activity (*31*, *32*) and hydratase activity (*33*), too. The metabolism of unsaturated fatty acids usually requires auxiliary enzymes including Δ^3-Δ^2-enoyl-CoA isomerase, $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase, and 2,4-dienoyl-CoA reductase. The rate-limiting step of the above three enzymatic reactions is catalyzed by 2,4-dienoyl-CoA reductase, which has been studied in sufficient detail recently (*34*−*36*). Our study raises the question as to whether the dehydrogenase might function as an isomerase *in vivo* in conditions in which the activity of the isomerase is decreased.

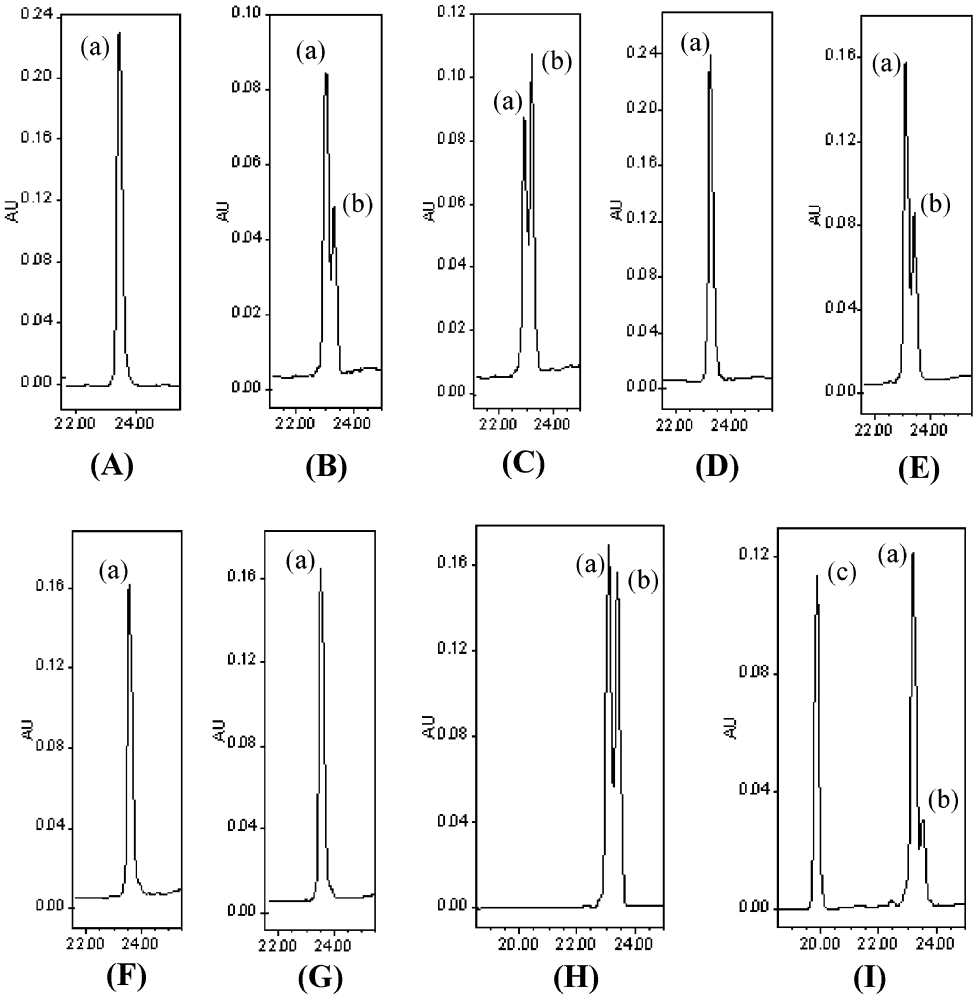

FIGURE 3: Analysis of incubation mixture using HPLC. (A) *trans*-3-Octenoyl-CoA. (B) Incubation of *trans*-3-octenoyl-CoA with MCAD. (C) Incubation of *trans*-3-octenoyl-CoA with MCAD, and the resulting mixture was co-injected with *trans*-2-octenoyl-CoA. (D) Incubation of *trans*-3-octenoyl-CoA with MCAD apoprotein (FAD was removed). (E) Incubation of *trans*-3-octenoyl-CoA with MCAD mutant E376D. (F) Incubation of *trans*-3-octenoyl-CoA with MCAD mutant E376A. (G) Incubation of *trans*-3-octenoyl-CoA with MCAD mutant E376Q. (H) Incubation of *trans*-3-octenoyl-CoA with MCAD apoprotein (FAD was removed), followed with addition of FAD cofactor (incubation experiment D followed with the addition of FAD cofactor). (I) Incubation of *trans*-3-octenoyl-CoA with MCAD and enoyl-CoA hydratase (add enoyl-CoA hydratase to mixture H). Possible identity of substrate or product peaks: (a) *trans*-3-octenoyl-CoA; (b) *trans*-2-octenoyl-CoA; and (c) 3-hydroxyoctanoyl-CoA.

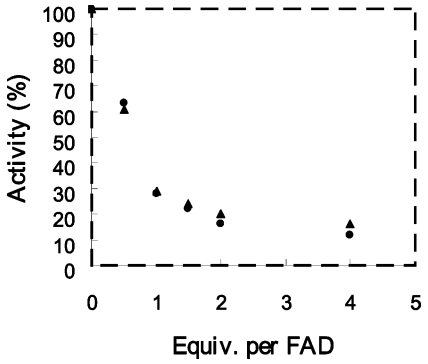

FIGURE 4: Titration of MCAD with 2-octynoyl-CoA. The indicated levels of 2-octynoyl-CoA were added to 30 μM of enzyme in 50 mM phosphate buffer at pH 7.5 and 25 °C. The dehydrogenase (▲) and isomerase (●) activities were recorded 10 min after each addition.

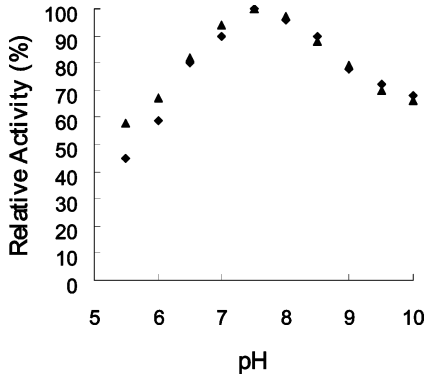

FIGURE 5: Effect of pH on dehydrogenase (▲) and isomerase (◆) activities of MCAD.

So far, five mammalian enzymes with enoyl-CoA isomerase activities have been described. They are mitochondrial enoyl-CoA isomerase (*37*, *38*), mitochondrial long-chain enoyl-CoA isomerase (*39*), mitochondrial enoyl-CoA hydratase or crotonase (*40*), peroxisomal enoyl-CoA isomerase (*41*), and multifunctional enzyme 1 (*42*, *43*). The first three enoyl-CoA isomerase activities are present in mitochondria, and the last two enzymes are associated with peroxisomes. These enzymes have different substrate specificities, which compensate each other so that the isomerase reaction can occur on all spectra of substrates with different chain length. Acyl-CoA dehydrogenase is another class of enzyme, which has

Table 3: Kinetic Properties of Enoyl-CoA Isomerase Activity of Enzymes from Various Sources

enzymes	substrate	k_{cat} (s^{-1})	K_M (μM)	reference
rat liver mitochondrial enoyl-CoA isomerase	*cis*-3-hexenoyl-CoA	200 ± 8	240 ± 16	*38*
rat mitochondrial enoyl-CoA hydratase	*trans*-3-hexenoyl-CoA	0.13 ± 0.01	66 ± 16	*40*
human liver peroxisomal enoyl-CoA isomerase	*cis*-3-octenoyl-CoA	18		*41*
Rat liver peroxisomal multifunctional enzyme 1	*trans*-3-hexenoyl-CoA	1.1		*43*
rat liver MCAD	*cis*-3-hexenoyl-CoA	0.11 ± 0.01	20 ± 3	this paper
rat liver MCAD mutant E376D	*cis*-3-hexenoyl-CoA	0.47 ± 0.02	14 ± 2	this paper

intrinsic enoyl-CoA isomerase activity. The comparison of kinetic properties for enoyl-CoA isomerase activity of enzymes from various sources is listed in Table 3. The k_{cat}/K_M value of MCAD for the isomerization reaction is about 1% of that of authentic rat mitochondrial Δ^3-Δ^2-enoyl-CoA isomerase (*37*, *38*) and is comparable to that of rat mitochondrial enoyl-CoA hydratase (*40*).

Enoyl-CoA isomerase belongs to crotonase superfamily, and enzymes in this superfamily have similar overall peptide folding (*44*). Methylenecyclopropylformyl-CoA (MCPF-CoA) is a well-studied irreversible inhibitor of crotonase (*45*−*47*), which only showed competitive inhibition against enoyl-CoA isomerase and MCAD. MCAD did not show any detectable 3-hydroxyacyl-CoA dehydrogenase (HAD) and 3-ketoacyl-CoA thiolase (KT) activities, and both HAD and KT have been purified and characterized in our lab (*48*, *49*).

It should be noted that the isomerization of *trans*-3-octenoyl-CoA by the MCAD represents a sizable rate enhancement over the uncatalyzed reaction. In control experiments, no isomerization of *trans*-3-octenoyl-CoA was detected over several hours in the absence of the wild-type enzyme. The following evidence support that isomerase activity of MCAD was not due to contamination from *E. coli*. First, no isomerization of *trans*-3-octenoyl-CoA was detected in the presence of E376A or E376Q mutants of MCAD after incubation for several hours, and these mutant proteins were purified following the same procedure for purification of the wild-type MCAD. Second, no isomerization of *trans*-3-octenoyl-CoA was detected in the presence of MCAD apoprotein without the FAD cofactor (Figure 3D). When the FAD cofactor was added into the above incubation mixture, the MCAD showed isomerase activity again and *trans*-3-octenoyl-CoA was converted into *trans*-2-octenoyl-CoA in the HPLC profile (Figure 3H). It should also be noted that the isomerase activity of MCAD should be significantly higher than the hydratase activity of MCAD reported previously (*33*). As shown in Figure 3H, although nearly 50% of *trans*-3-octenoyl-CoA was converted into *trans*-2-octenoyl-CoA that is the substrate of hydratase, no detectable 3-hydroxyoctanoyl-CoA was generated as the product caused by hydratase activity of MCAD. The k_{cat} for hydratase activity of MCAD is estimated to be 0.5 min^{-1} when optimal substrate, crotonyl-CoA was used as the substrate (*33*), which is more than 10 times slower than the k_{cat} (~6 min^{-1}) for isomerase activity of MCAD when *cis*-hexenoyl-CoA was used as the substrate.

The available evidence strongly suggests that the isomerase activity of MCAD is associated with the active site employed for substrate dehydrogenation. Thus, isomerase activity is inhibited by known MCAD inhibitor, 2-octynoyl-CoA. It has been shown that the hydratase activity of MCAD is also inhibited by 2-octynoyl-CoA, which is used as evidence for the association of hydratase activity with the MCAD active site (*33*). Both isomerase and dehydrogenase activities show similar pH dependence curves with maximum activities at pH 7.5. It was found that the FAD cofactor is required for the isomerase activity of MCAD. Interestingly, the FAD cofactor is also required for the hydratase activity of MCAD, as reported previously (*33*). On the basis of the crystal structure determined earlier (*14*−*16*), the substrate is sandwiched between catalytic residue Glu376 and the FAD cofactor. Therefore, the orientation of the substrate in the catalytic site of MCAD could be significantly changed without the bound FAD cofactor, which makes MCAD lose both isomerase and hydratase activities. It has been demonstrated with the crystal structure that an enolate intermediate exist for the inactivation of the MCAD by 3,4-dienoyl-CoA thioesters, and the catalytic base, Glu376, has sufficient flexibility to access the C-2 position of 3,4-dienoyl-CoA thioesters (*16*). Therefore, for the isomerization reaction catalyzed by the MCAD, the carbanion at C-2, formed after initial deprotonation by Glu376, could be stabilized as a dienolate prior to protonation, as shown in Figure 6. Such a carbanion might gain further stabilization via charge transfer with the electron-deficient FAD cofactor of the MCAD (*50*, *51*). These may have significantly lowered the energy level of the transition state, which explains the observed enoyl-CoA isomerase activity of the MCAD.

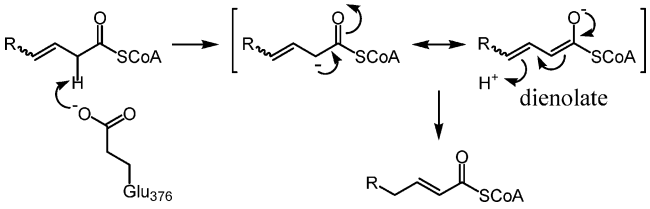

FIGURE 6: Proposed mechanism for the conversion of *trans*-3-enoyl-CoA to *trans*-2-enoyl-CoA.

It should be noted that *trans*-3-octenoyl-CoA has been previously determined as an inhibitor of pig kidney MCAD (*52*). Although a charge-transfer complex has been observed on UV spectra, no significant isomerization of *trans*-3- to *trans*-2-octenoyl has been observed by NMR (*52*). We think that the NMR instrument might not be sensitive enough to tell the difference between *trans*-3- and *trans*-2-octenoyl-CoA in an incubation mixture without separation. Our HPLC analysis clearly demonstrates that MCAD can catalyze the isomerazation reaction, and our result is also consistent with a previous report that butyryl-CoA dehydrogenase can catalyze isomerization of 3-butenoyl pantetheine to 2-butenoyl pantetheine (*53*).

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