Catalytic and FAD-binding Residues of Mitochondrial Very Long Chain Acyl-Coenzyme A Dehydrogenase*

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Masayoshi Souri‡§, Toshifumi Aoyama‡, Gerald F. Cox¶ and Takashi Hashimoto‡

From the ‡Department of Biochemistry, Shinshu University School of Medicine, Matsumoto, Nagano 390, Japan and the ¶Division of Genetics, Department of Medicine, Children’s Hospital, Boston, Massachusetts 02115

Very long-chain acyl-CoA dehydrogenase (VLCAD) is one of four flavoproteins which catalyze the initial step of the mitochondrial β-oxidation spiral. By sequence comparison with other acyl-CoA dehydrogenases, Glu-422 of VLCAD has been presumed to be the catalytic residue that abstracts the α-proton in the αβ-dehydrogenation reaction. Replacing Glu-422 with glutamine (E422Q) caused a loss of enzyme activity by preventing the formation of a charge transfer complex between VLCAD and palmitoyl-CoA. This result provides further evidence for Glu-422 being part of the active site of VLCAD.

F418L is a disease-causing mutation in human VLCAD deficiency. Unlike wild-type VLCAD, F418L and F418V contained no bound FAD when expressed at extremely high levels in the baculovirus expression system. Although F418T and F418Y bound FAD at a level similar to that of wild-type VLCAD, both showed reduced Vmax values toward palmitoyl-CoA, most likely due to a decrease in the rate of enzyme-bound FAD reduction. These data suggest that Phe-418 is involved in the binding and subsequent reduction of FAD. FAD-deficient VLCADs (F418L, F418V, and apo-VLCAD) showed increased sensitivity to trypsinization. Loss of FAD may change the folding of VLCAD subunits.

Mitochondrial fatty acid β-oxidation is one of the main energy-yielding metabolic pathways in eukaryotes. The initial step in the mitochondrial fatty acid β-oxidation spiral is catalyzed by four acyl-CoA dehydrogenases which have different, but overlapping, substrate chain length specificities. Very long-chain acyl-CoA dehydrogenase (VLCAD; EC 1.3.99.13), a novel mitochondrial inner membrane-associated acyl-CoA dehydrogenase, shows activity toward CoA esters of long- and very long-chain fatty acids (1). In human tissues, VLCAD accounts for the majority (≥80%) of palmitoyl-CoA dehydrogenation activity (2). VLCAD is thought to play an important role in the initial β-oxidation cycles of long-chain fatty acids, along with enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein, which catalyzes the succeeding three reactions (3, 4).

Short- (SCAD), medium- (MCAD), and long- (LCAD) chain acyl-CoA dehydrogenases are homotetramers of approximately 40-kDa polypeptides which contain 4 mol of FAD/mol of enzyme (5, 6), whereas VLCAD is a homodimer of a 71-kDa polypeptide which contains 2 mol of FAD/mol of enzyme (1). SCAD, MCAD, and LCAD share a high degree of sequence similarity throughout their entire sequences (7). Although VLCAD is highly homologous to other acyl-CoA dehydrogenases at its amino terminus, its carboxyl terminus contains a long tail of approximately 180 amino acid residues not shared by other acyl-CoA dehydrogenases (8, 9).

In the αβ-dehydrogenation reaction, the abstraction of the α-proton from the acyl-CoA substrate is catalyzed by an acidic residue in acyl-CoA dehydrogenase and is followed by the transfer of the β-hydride to the N-5 position of the enzyme-bound FAD (10, 11). In MCAD, Glu-376 has been determined to be the catalytic residue that abstracts the α-proton by mutational (12) and x-ray crystallographic analyses (13). The region surrounding Glu-376 of MCAD shows significant sequence similarity to other acyl-CoA dehydrogenases (Fig. 1). This glutamate residue is conserved in both VLCAD and SCAD. Recently, Glu-368 of SCAD was shown to be a catalytic residue by mutational analysis (14). On the other hand, in LCAD, glutamate is not conserved at the position corresponding to Glu-376 of MCAD, and instead Glu-261 has been identified as a catalytic residue (15). The presence of a glycine residue in VLCAD at the position corresponding to Glu-261 of LCAD makes it more likely that Glu-422 of VLCAD is a catalytic residue.

VLCAD deficiency is a newly reported disorder of fatty acid metabolism that frequently leads to hypertrophic cardiomyopathy and sudden death in infancy (16–20). To date, 13 patients with this disease have been diagnosed and characterized at the molecular level (9, 20–22). Mutations identified in the VLCAD gene have been heterogenous with most causing rapid degradation of the mRNA, protein, or both (21).

Recently, we found a disease-causing mutation, F418L (precursor position 458), in a patient with VLCAD deficiency in whom a cross-reactive protein of normal size was detected by immunoblot analysis with anti-VLCAD antibody. We have used the baculovirus expression system and site-directed mutagenesis to investigate the roles of Phe-418 and Glu-422 in the enzymatic activity of VLCAD. Our results suggest specific catalytic functions for Phe-418 and Glu-422 in the active site of VLCAD.
EXPERIMENTAL PROCEDURES

Materials—VLCAD was purified from human liver as described (2). Human VLCAD cDNA was cloned previously (9). A Transformer™ Site-Directed Mutagenesis Kit was purchased from CLONTECH (Palo Alto, CA). MaxBac® Baculovirus Express Vector System Kit was purchased from Invitrogen (San Diego, CA). Acyl-CoAs were synthesized by the mixed anhydride method (23) and purified by DEAE-cellulose column chromatography (24). Phenazine ethosulfate (PES) and bovine spleen trypsin were purchased from Boehringer Mannheim (Germany).

Site-directed Mutagenesis and Vector Construction—VLCAD cDNA was polymerase chain reaction-amplified and subcloned into pT7Blue T-Vector (pT7B-VLCAD). HindIII and BamHI fragments from pT7B-VLCAD were independently subcloned into corresponding restriction sites of the baculovirus transfer vector, pBlueBacIII (pBIII-VLCAD).

Site-directed mutagenesis was performed by the method of Deng and Nickoloff (25) using a Transformer™ Site-Directed Mutagenesis Kit. Mutant cDNAs were synthesized according to the manufacturer’s instructions, using pT7B-VLCAD as template. BamHI fragments of the mutant pT7B-VLCAD cDNAs were inserted into the BamHI site of pBIII-VLCAD.

Preparation of Recombinant Baculoviruses—Recombinant baculovirus transfer vectors were co-transfected into Sf9 cells with wild-type Autographa californica nuclear polyhedrosis virus DNA by cationic liposome-mediated transfection. Cell transfection, plaque assays, isolation of plaque purified viruses, and preparation of high-titer stocks were performed as described in the manufacturer’s instructions.

Expression and Purification of Variant VLCAD—SP9 cells (approximately 5 x 10⁶ cells) were infected with recombinant viruses as multiplicity of infection ≥3. On day 3 after infection, the cells were collected, washed twice with phosphate-buffered saline, and resuspended in 30 ml of 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM phenylmethylsulfonil fluoride. The suspension was homogenized using a Potter homogenizer and centrifuged at 15,000 x g for 5 min. The supernatant was further centrifuged at 10,000 x g for 10 min, and the mitochondrial pellet was obtained. Expressed protein was extracted from the mitochondrial fraction and purified by phosphocellulose and DEAE-cellulose column chromatography as described previously (1). Approximately 0.5 mg of the expressed proteins were obtained and stored in 0.5 ml of 50% glycerol, 50 mM potassium phosphate (pH 7.5) at -20 °C.

FAD Content—Fifty µl of the partially purified sample (approximately 50 µg) was diluted with 350 µl of 20 mM potassium phosphate (pH 7.5), and then 25 µl of 50% trichloroacetate was added. The sample was incubated on ice for 30 min and then centrifuged at 10,000 x g for 5 min. Three hundred-fifty µl of the supernatant was transferred to a new tube, diluted with 100 µl of water, and 50 µl of NaOH (1 N) was added to adjust the pH to 2.8. The fluorescence intensity of the solution (excitation at 445 nm and emission at 520 nm) was measured using a fluorometer (Hitachi F-2000). The FAD content was calculated using standard FAD (Sigma).

Enzyme Assay—VLCAD enzyme activity was measured by the dye-reduction method using PES as an electron transfer dye and dichloroindophenol as an electron acceptor. The reaction mixture contained 50 mM potassium phosphate (pH 7.5), 30 µM acyl-CoA, 35 µM dichloroindophenol, 1 mM N-ethylmaleimide, 3 mM PES, and enzyme. The reaction was initiated by adding 3 mM PES, and the reduction of dichloroindophenol was monitored by the decrease in absorbance at 600 nm. The activity was calculated using a molar extinction coefficient of 21,000 M⁻¹ cm⁻¹.

RESULTS

Expression of Wild-type VLCAD by the Baculovirus Expression System—The cDNA-directed expression of wild-type VLCAD was performed using the baculovirus expression system. A recombinant baculovirus containing full-length VLCAD cDNA with leader peptide sequence was used to infect SF9 cells. Immunoblot analysis of the cells infected with recombinant baculovirus revealed a large amount of the precursor protein along with the mature form (Fig. 2A). Subcellular fractionation showed that the expressed mature VLCAD was associated with mitochondria while the precursor protein was detected in both the mitochondrial and microsomal fractions (Fig. 2B). The precursor protein was separated from the mature form by phosphocellulose column chromatography.

The recombinant VLCAD migrated at the same position (70 kDa) as the human liver enzyme by SDS-PAGE (Fig. 2C). The native molecular mass of recombinant VLCAD was estimated to be 148 kDa by size-exclusion column chromatography, indicating that the expressed protein forms a dimer. The recombinant VLCAD showed absorption maxima near 280, 370, and 450 nm characteristic of the spectrum of FAD. The FAD content of the expressed protein was estimated to be approximately 2 mol/mol of dimer (Table I). The FAD content and catalytic properties of the recombinant VLCAD (Table II) were the same as those of the human liver enzyme.

Glut-422 Mutants—To determine whether Glu-422 is a catalytic residue in VLCAD, two mutant proteins were prepared replacing Glu-422 with Gln or Asp (Fig. 2C). The native molecular masses, absorption maxima, and FAD contents of expressed E422Q and E422D did not differ significantly from those of wild-type (Table I). The enzyme activity associated with E422Q was barely detectable with palmitoyl-CoA (Table II) or other carbon chain length substrates (data not shown). The V₅₀ value of E422Q toward palmitoyl-CoA was only 10% of that of wild-type, while the Kₘ value (Table II) and substrate chain length specificity (data not shown) did not differ significantly from wild-type. The Kₘ value of E422D for the electron transfer dye, PES, was also similar to that of wild-type (Table II).

Spectral data was used to provide evidence for the formation of a charge transfer complex (26). UV visible spectra were monitored at various concentrations of substrate added to the enzyme solution (Fig. 3). Titration of palmitoyl-CoA with VLCAD results in the quenching of absorbance at 450 nm and the appearance of a new absorption band at 580 nm (Fig. 3A), which are characteristic changes in the acyl-CoA dehydrogenase complex (27). A 15–20 nm blue shift of the 370-nm peak and a minor shift of the 450-nm peak were also observed. On the other hand, when palmitoyl-CoA was titrated with E422Q, quenching of absorbance at 450 nm and significant shifts of the absorption maxima were barely detectable (Fig. 3B). Only with 10 µM palmitoyl-CoA was slight quenching of absorbance at 450 nm detected in E422D (Figs. 3C and 4).

Phe-418 Mutants—The Phe-418 residue of VLCAD is not conserved in other acyl-CoA dehydrogenases. The corresponding residues in SCAD, MCAD, IVD, and LCAD are threonine, tyrosine, tyrosine, and glutamine, respectively (Fig. 1). To investigate the role of Phe-418 in VLCAD, four mutants of VLCAD were prepared, replacing this amino acid with leucine, threonine, tyrosine, and valine.

The final preparations of two of the four Phe-418 mutants,
F418L and F418V, were colorless despite yields that were similar to that of wild-type (Fig. 2). On the other hand, the final preparations of F418T and F418Y were yellow, the same color as wild-type. The positions of the absorbance maxima of F418T and F418Y were similar to those of wild-type, whereas F418L and F418V exhibited only 1 absorbance maximum at 280 nm (Table I). The FAD contents of F418L and F418V were barely detectable even by fluorometric measurement after liberation from the proteins. The FAD contents of F418T and F418Y were not significantly different from that of wild-type. Gel filtration analysis confirmed that all of the Phe-418 mutants formed dimers.

The enzyme activities of F418L and F418V toward various carbon chain length acyl-CoAs were hardly detectable (Table II). No increase in enzyme activity was observed when FAD (final 20 μM) was added to the F418L and F418V preparations. On the other hand, the V_max values of F418T and F418Y toward palmitoyl-CoA were 68 and 25% of wild-type, respectively. The K_m values of F418Y for palmitoyl-CoA and PES were similar to those of wild-type, while those of F418T were somewhat higher than those of wild-type. The substrate chain length specificities

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**Table I**

| Native molecular mass (kDa) | Absorbance ratio (mol/mol of subunit) | FAD content |
|-----------------------------|--------------------------------------|-------------|
|                             | 280 | 365 | 450 |
| Wild-type                   | 148 | 0.9 | 1   | 0.95 |
| E422Q                       | 148 | 0.8 | 1   | 0.88 |
| E422D                       | 148 | 0.8 | 1   | 0.94 |
| F418L                       | 148 | 1.2 | 1   | 0.03 |
| F418Y                       | 148 | 0.8 | 1   | 0.78 |
| F418T                       | 148 | 0.9 | 1   | 0.97 |
| F418V                       | 148 | 1.1 | 1   | 0.01 |

*Estimated by gel filtration analysis.*

F418L and F418V were colorless despite yields that were similar to that of wild-type (Fig. 2C). On the other hand, the final preparations of F418T and F418Y were yellow, the same color as wild-type. The positions of the absorbance maxima of F418T and F418Y were similar to those of wild-type, whereas F418L and F418V exhibited only 1 absorbance maximum at 280 nm (Table I). The FAD contents of F418L and F418V were barely detectable even by fluorometric measurement after liberation from the proteins. The FAD contents of F418T and F418Y were not significantly different from that of wild-type. Gel filtration analysis confirmed that all of the Phe-418 mutants formed dimers.

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The UV visible spectra of F418T and F418Y were monitored at various concentrations of palmitoyl-CoA. F418T strongly quenched absorbance at 450 nm even at 1 μM palmitoyl-CoA, reaching its maximum at ∼3 μM (Figs. 3E and 4). In contrast, F418Y showed less quenching of absorbance at 450 nm compared with wild-type (Figs. 3F and 4). The estimated $K_{\text{m}}$ values (the substrate concentration causing half-maximum quenching of the absorbance at 450 nm) of wild-type, F418T, and F418Y were 3.3, 2.1, and 4.9 μM, respectively. The estimated $V_{\text{max}}$ values (the maximum decrease in absorbance at 450 nm) were 0.027, 0.038, and 0.017, respectively.

**DISCUSSION**

Two subfamilies of acyl-CoA dehydrogenases may be distinguished by the position of a catalytic glutamate residue: one subfamily corresponds to Glu-376 of MCAD, and the other corresponds to Glu-261 of LCAD (12, 13, 15). Sequence alignments suggest that SCAD and VLCAD belong to the MCAD subfamily (Glu-376), while IVD belongs to the LCAD subfamily (Glu-261) (Fig. 1). Recently, Glu-368 of SCAD, which corresponds to Glu-376 of MCAD, was confirmed to be a catalytic residue (14). In the present study, we provide mutational evidence for Glu-422 of VLCAD being a catalytic residue.

Replacing Glu-422 of VLCAD with Gln caused a complete loss of enzyme activity, while replacement with Asp decreased the $V_{\text{max}}$ value to 10% of wild-type without a significant change in the $K_{\text{m}}$ (Table II), suggesting that the carboxylate of Glu-422 is intimately involved in the catalytic function in VLCAD protein. The αβ-dehydrogenation reaction performed by acyl-CoA dehydrogenases involves the formation of a charge transfer complex, which is initiated by the abstraction of the α-proton from acyl-CoA by the active site carboxylate, followed by transfer of β-hydride to the N-5 position of enzyme-bound FAD (10, 11, 27). The charge transfer complex quenches the FAD absorbance at 450 nm and produces a new absorption band at 580 nm due to the disruption of the extended π-electron system of the FAD isoaflavoxine ring (26–29). Measuring the UV visible spectra in the absence and the presence of palmitoyl-CoA dem

![Fig. 4](image-url)  
**Fig. 4.** Palmitoyl-CoA-dependent quenching of the absorption at 450 nm. D, wild-type; A, E422Q; □, E422D; △, F418T; ■, F418Y.

![Fig. 5](image-url)  
**Fig. 5.** Tryptic cleavage of wild-type, mutant, and apo-VLCADs. Three μg of protein was incubated with 0.03, 0.1, or 0.3 μg of trypsin in 20 μl of 50 mM potassium phosphate (pH 7.5) at 37 °C for 30 min. The reaction was terminated by the addition of phenylmethylsulfonyl fluoride (final concentration 1 mM) and then subjected to SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250.

![Fig. 6](image-url)  
**Fig. 6.** Phylogenetic tree of human acyl-CoA dehydrogenase family. The regions 1–443 of VLCAD (9), 1–441 of acyl-CoA oxidase (30), and entire sequences of SCAD, MCAD, LCAD, and IVD were analyzed by a MALIGN program at DDBJ homepage server. AOX, acyl-CoA oxidase.
glutamate exists at the position corresponding to Glu-376 of LCAD but not at the position corresponding to Glu-261 of MCAD (30). Catalytic residue in the ancestor protein might be located at the position corresponding to Glu-376 of MCAD.

F418L is a disease-causing mutation in human VLCAD deficiency. In the present study, a large amount of the F418L located at the position corresponding to Glu-376 of MCAD but not at the position corresponding to Glu-261 of MCAD was colorless because of the absence of enzyme-bound FAD (Table I and Fig. 3D) which led to a complete loss of catalytic activity (Table II). Replacing Phe-418 with valine also caused the loss of enzyme-bound FAD (Table I). These findings indicate that Phe-418 is important for the binding of FAD to VLCAD. We prepared two additional Phe-418 mutants, replacing phenylalanine with threonine and tyrosine, which correspond to the residues in SCAD and MCAD, respectively (Fig. 1). F418T and F418Y bound as much FAD as wild-type, but had only 68 and 25% of the $V_{\text{max}}$ value of the wild-type enzyme, respectively, without a significant change in the $K_m$ toward palmitoyl-CoA and the electron transfer dye (Tables I and II). Interestingly, the spectral data indicated that these two mutants could form a charge transfer complex but with altered $\Delta_{\text{max}}$ and $K_{\text{req}}$ values (Fig. 4). In MCAD-tyrosyl-CoA complex (13), the $\alpha$-β bond of tyrosyl-CoA is sandwiched between the carboxylate of Glu-376 and the isoalloxazine ring, likely to allow the electron transfer easily (Fig. 7). Tyr-372 of MCAD, forming between the catalytic residue Glu-422, the carboxylate of Glu-376 and the isoalloxazine ring, likely to change the position of the isoalloxazine ring in the complex formed between the catalytic residue Glu-422, the $\alpha$-β bond of acyl-CoA, and the site interacting with the electron acceptor.

Saijo and Tanaka (31) demonstrated that the isoalloxazine ring of FAD is required for the formation of the core in the folding of MCAD subunit into the tetramer assembly. Wild-type VLCAD contains a domain that is resistant to trypsinization (Fig. 5) and extends from Lys-23 to Arg-470 of mature VLCAD (which includes residues Phe-418 and Glu-422). This 48-kDa peptide corresponds to the highly homologous regions of other acyl-CoA dehydrogenases. Trypsinization of VLCAD produced a 48-kDa homodimer that contained the same amount of FAD as native VLCAD. These findings suggest that the VLCAD subunit tightly binds between amino acids 23 and 470 and that this region includes the catalytic domain. On the other hand, the FAD-deficient VLCADs (F418L, F418V, and apo-VLCAD) were capable of forming dimers, but showed increased sensitivity to trypsinization. Some degree of folding change may occur by the lack of FAD.

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**Fig. 7.** Positions of Tyr-372 and Glu-376 in three-dimensional structure of MCAD complexed with octanoyl-CoA. The data was obtained from Brookhaven Protein Data Bank with accession code 3MDE-A.
