Human Acyl-CoA Dehydrogenase-9 Plays a Novel Role in the Mitochondrial β-Oxidation of Unsaturated Fatty Acids*5

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Unsaturated fatty acids play an important role in the prevention of human diseases such as diabetes, obesity, cancer, and neurodegeneration. However, their oxidation in vivo by acyl-CoA dehydrogenases (ACADs) that catalyze the first step of each cycle of mitochondrial fatty acid β-oxidation is not entirely understood. Recently, a novel ACAD (ACAD-9) of unknown function that is highly homologous to human very-long-chain acyl-CoA dehydrogenase was identified by large-scale random sequencing. To characterize its enzymatic role, we have expressed ACAD-9 in Escherichia coli, purified it, and determined its pattern of substrate utilization. The N terminus of the mature form of the enzyme was identified by in vitro mitochondrial import studies of precursor protein. A 37-amino acid leader peptide was cleaved sequentially by two mitochondrial peptidases to yield a predicted molecular mass of 65 kDa for the mature subunit. Submitochondrial fractionation studies found native ACAD-9 to be associated with the mitochondrial membrane. Gel filtration analysis indicated that, like very-long-chain acyl-CoA dehydrogenase, ACAD-9 is a dimer, in contrast to the other known ACADs, which are tetramers. Purified mature ACAD-9 had maximal activity with long-chain unsaturated acyl-CoAs as substrates (C16:1-, C18:1-, C18:2-, C22:6-CoA). These results suggest a previously unrecognized role for ACAD-9 in the mitochondrial β-oxidation of long-chain unsaturated fatty acids. Because of the substrate specificity and abundance of ACAD-9 in brain, we speculate that it may play a role in the turnover of lipid membrane unsaturated fatty acids that are essential for membrane integrity and structure.

Unsaturated fatty acids are the most abundant form of stored fat in the human body and are vital for all living organisms. In addition to their role as an energy source, they are integral constituents of cell membranes, playing a role in membrane fluidity, cell signaling, and membrane integrity (1). Numerous beneficial physiologic effects have been attributed to unsaturated fatty acids, including protection from obesity, diabetes, cancer, and atherosclerosis (2, 3). Utilization of unsaturated fatty acids requires mitochondrial β-oxidation. Unsaturated fatty acids are more efficiently oxidized than long-chain saturated fatty acids in humans (4); however, the enzymes responsible for their catabolism have not been elucidated in their entirety. The observation that the initial cycles of β-oxidation of long-chain unsaturated fatty acids (oleic acid (cis-9-C18:1)5 and linoleic acid (cis-9,cis-12-C18:2)) occur in the absence of activity of very-long-chain acyl-CoA dehydrogenase (VLCAD) suggested the presence of an additional enzyme or alternate pathway (5). In contrast, the enzymatic β-oxidation pathway for the saturated acyl-CoA esters is well described (6). The first step of fatty acid β-oxidation is catalyzed by the acyl-CoA dehydrogenases (ACADs; EC 1.3.99.3), a family of mitochondrial enzymes with distinct substrate specificities (7). VLDL and long-chain (LCAD), medium-chain (MCAD), and short-chain (SCAD) acyl-CoA dehydrogenases have optimal activity toward acyl-CoAs of 16-, 14-, 8-, and 4-carbon chains, respectively (8–10). Deficiencies of the ACADs are important causes of human disease (8, 11, 12).

Recently, a new ACAD (ACAD-9) that is highly homologous to human VLDL was identified by large-scale random sequencing (13). The objective of this study was to investigate the function of ACAD-9. In this work, we describe the import and processing of the ACAD-9 precursor into mitochondria in vitro and in eukaryotic cells. We characterize the enzymatic properties of purified ACAD-9 following expression in Escherichia coli and purification. Surprisingly, purified ACAD-9 shows maximal activity toward long-chain unsaturated substrates, suggesting a novel role in mitochondrial β-oxidation.

EXPERIMENTAL PROCEDURES

In Vitro Transcription and Translation of the ACAD-9 Precursor, Mitochondrial Import and Processing, and N-terminal Radiolabeling—First-strand cDNA was synthesized from fibroblast mRNA with the iScript kit (Bio-Rad) and used as template for PCR amplification of the ACAD-9 precursor sequence. The PCR product was cloned into the pGEM-T-Easy vector (Promega) using TA cloning.

The abbreviations used are: cis-9-C18:1, oleic acid/oleoyl; cis-9,cis-12-C18:2, linoleic acid/linoleoyl; VLDL, very-long-chain acyl-CoA dehydrogenase; ACAD, acyl-CoA dehydrogenase; LCAD, long-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase; MPP, mitochondrial processing peptidase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPLC, high pressure liquid chromatography; C8:0, octanoyl; C16:0, palmitoyl; cis-9-C16:1, cis-9,10-dodecanoyl; all-cis-4,7,10,13,16,19-C22:6, docosahexaenoic acid (DHA)/docosahexaenoic; ETF, electron transfer flavoprotein; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MIP, mitochondrial intermediate peptidase.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1 and Tables 1 and 2.

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ing according to the manufacturer’s instructions and then subcloned into the mammalian expression vector pCDNA3.1(+) (Invitrogen). The identity of this and all other constructs was confirmed by DNA sequencing.

ACAD-9 precursor protein was synthesized from 1 μg of pCDNA3.1(+) containing the ACAD-9 precursor cDNA by coupled in vitro transcription/translation in the presence of L-[4,5-3H]leucine (25 μCi, 160 Ci/mmol; Amersham Biosciences) or [35S]methionine (20 μCi, ~1250 Ci/mmol) as described (14, 15). For import assays, liver mitochondria were freshly prepared from young male Sprague-Dawley rats as described previously (14, 16). The final pellet was resuspended in 500 μl of 5 mM HEPES (pH 7.4), 220 mM d-mannitol, and 70 mM sucrose (HMS buffer) and diluted to a final concentration of 20 mg/ml protein (14). Thirty-five μl of translation mixture containing translated 3H- or 35S-labeled precursor were mixed with 85 μl of mitochondrial suspension and 30 μl of HMS buffer to a total volume of 150 μl and incubated at 30 °C for 75 min. The incubation mixture was then separated into supernatant and mitochondrial pellet fractions by centrifugation at 9100 × g for 2 min at 4 °C. The pellets were solubilized in 30 μl of SDS-PAGE loading buffer and boiled for 10 min. For processing with yeast mitochondrial processing peptidase (MPP, EC 3.4.24.64), 15 μl of 3H- or 35S-labeled translation mixture were incubated with 100 ng of purified recombinant yeast MPP prepared as described (15) in a total reaction volume of 60 μl of 10 mM HEPES-KOH (pH 7.4), 1 mM dithiothreitol, and 1 mM MnCl2 for 30 min at 30 °C (15).

Three μl of a 35S]methionine-labeled import reaction mixture were added to each of two 3H]leucine-labeled import reactions, and these reactions were separated by 10% SDS-PAGE utilizing piperazine diacrylamide (Bio-Rad) instead of bisacrylamide as cross-linker to prevent sequence bias underlined. The amplified fragment was subcloned into the prokaryotic expression vector pET-21a(+) (Novagen). PCR primers were designed to amplify the mature coding region of the human ACAD-9 precursor sequence previously cloned into the pGEM-T-Easy vector. The 5'-primer consisted of 94 nucleotides and included an Ndel restriction site (shown in boldface), followed by the first 81 nucleotides of the mature ACAD-9 coding sequence; the first 60 nucleotides of the coding sequence were altered to reflect Pseudomonas fragi endoproteinas ein Asp-N (Roche Diagnostics) or trypsin (Promega) and processed as described (20).

Mass spectrometry was carried out using a Bruker Daltonics Autoflex MALDI-TOF mass spectrometer with a scoutMTP ion source operating in reflector mode. Spectra were analyzed using FlexAnalysis Version 2.0 and BioTools Version 2.2 (both from Bruker Daltonics).

Expression in E. coli and Purification of Mature ACAD-9—The sequence of mature human ACAD-9 was inserted into the prokaryotic expression vector pET-21a(+) (Novagen). PRIMmers were designed to amplify the mature coding region of the human ACAD-9 precursor sequence previously cloned into the pGEM-T-Easy vector. The 5'-primer consisted of 94 nucleotides and included an Ndel restriction site (shown in boldface), followed by the first 81 nucleotides of the mature ACAD-9 coding sequence; the first 60 nucleotides of the coding sequence were altered to reflect E. coli codon usage bias (21, 22): 5'-A G TA C G T G C ATG CAT ATG GCT TTC GCT AAA GAA CTG TTC CTG GGC AAA ATC AAA AAA AAA AAA GAA GTT TTC CCG TTC CCG GAA GTT AGC CAA GAT GAA CTT-3’. The 3'-primer consisted of the last 26 nucleotides of the coding region (stop codon in antisense direction shown in italics), followed by an EcoRI restriction site (shown in boldface): 5’-ATT CGA GAA TTC TCA GCA GGT GCC GTC CAG AGG GTG GG-3’ (with nucleotides altered to conform to E. coli codon usage bias underlined). The amplified fragment was subcloned into the Ndel and EcoRI sites of the pET-21a(+) vector.

The pET-21a(+) vector, which expresses the mature ACAD-9 insert without a tag, was transformed into the E. coli host strain C43 (DE3) (Avidis, Saint Beauzire, France) (23), and the enzyme was then purified from a cell-free extract as described previously (16, 22). Briefly, an 8-liter culture of cells grown to mid-log phase (absorbance of 0.8–1.0 at 550 nm) at 37 °C was induced overnight at 37 °C by addition of 0.5 mM isopropyl 1-thio-D-galactopyranoside (Sigma) (22). Cells were harvested and lysed using a French press (two passes of pelleted cells at a pressure of 10,000 p.s.i. following treatment with DNase), and the lysate was subjected to centrifugation at 20,000 × g for 15 min. ACAD-9 was purified from the supernatant by fast protein liquid chromatography. Approximately 50 ml of cell-free extract were loaded onto a DEAE-Sepharose fast flow column (Amersham Biosciences), and the bound protein was eluted using a 0–300 mM NaCl gradient in 25 mM Tris (pH 8.0). Fractions with a yellow-green color were tested for enzyme activity, and peak fractions were combined and loaded onto a 20-μm ceramic hydroxylapatite column (Bio-Rad). The column was eluted with a 0–0.5 mM potassium phosphate gradient at pH 6.8. Peak samples were pooled and concentrated by ultrafiltration, and the final sample was stored in buffer containing 20% glycerol at −80 °C until used. The molecular mass of
puriﬁed recombinant ACAD-9 protein was estimated by gel ﬁltration analysis using a BioSep SEC-S3000 column in a Waters HPLC system. The column was calibrated using standards purchased from Amersham Biosciences: ribonuclease A (13.7 kDa), ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), and ferritin (440 kDa).

Production of Anti-ACAD-9 Antiserum—Rabbit antiserum to puriﬁed ACAD-9 was raised by Cocalico Biologicals, Inc. (Reamstown, PA) according to standard company protocols. Rabbits were given an initial inoculation of 200 μg of puriﬁed protein and boosted four times with 200 μg of puriﬁed protein each at 1- or 2-week intervals. Reactivity of the ﬁnal serum was conﬁrmed by Western blotting.

Enzyme Assays and Kinetic Analysis—Hexanoyl (C6:0)-CoA, octanoyl (C8:0)-CoA, nonanoyl (C9:0)-CoA, decanoyl (C10:0)-CoA, undecanoyl (C11:0)-CoA, lauroyl (C12:0)-CoA, myristoyl (C14:0)-CoA, myristoleoyl (cis-9-C14:1)-CoA, pentadecanoyl (C15:0)-CoA, palmitoyl (C16:0)-CoA, palmitoleoyl (cis-9-C16:1)-CoA, heptadecanoyl (C17:0)-CoA, stearoyl (C18:0)-CoA, oleoyl (cis-9-C18:1)-CoA, elaidoyl (trans-9-C18:1)-CoA, linoleoyl (cis-9,cis-12-C18:2)-CoA, and arachidoyl (C20:0)-CoA were purchased from Sigma. Arachidonoyl (all-cis-5,8,11,14-C20:4)-CoA, icosapentaenoyl (all-cis-5,8,11,14,17-C20:5)-CoA, docosahexaenoyl (all-cis-4,7,10,13,16,19-C22:6)-CoA, and docosanoyl (C22:0)-CoA were purchased from Avanti Polar Lipids (Alabaster, AL). The branched-chain substrates 2,6-dimethylheptanoyl (C7:0)-CoA and 4,8,12-trimethyltridecanoyl (C13:0)-CoA were kindly provided by Dr. R. J. A. Wanders (Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands) and Dr. P. P. van Veldhoven (Katholieke Universiteit Leuven, Leuven, Belgium), respectively. ACAD-9 activity was measured with the anaerobic electron transfer flavoprotein (ETF) reduction assay using an LS50B ﬂuorescence spectrophotometer (PerkinElmer Life Sciences) with a heated cuvette block set to 32 °C as described (24, 25). The ﬁnal substrate concentration in the assay mixtures was 50 μm. For kinetic analysis, the ETF concentration was 1 μm, and the substrate concentration was varied from 0.1 to 45 μm. K_{m} and V_{max} values were calculated using SigmaPlot software (Systat Software, Inc., Point Richmond, CA), with ﬁtting to the Michaelis-Menten equation by nonlinear regression analysis.

Mitochondrial Fractionation—Human muscle (100 mg) in 600 μl of 200 mM Tris (pH 8.0) containing a protease inhibitor mixture (Roche Diagnostics) was homogenized with a Polytron homogenizer for 10–15 s. The resulting supernatant was sonicated on ice three times for 10 s each and centrifuged at 20,000 × g for 20 min, and the supernatant containing mitochondrial matrix proteins was saved for analysis. The pellet was resuspended in 300 μl of 200 mM Tris (pH 8.0) by brief sonication, and then 30 μl of 2.5% Lubrol (polyethylene glycol ether W-1, Sigma) were added. After incubation for 15 min on ice, the sample was subjected to centrifugation at 20,000 × g for 20 min. The resulting supernatant contained mitochondrial integral and membrane-associated proteins. Fifty μg each of muscle mitochondrial membrane and matrix fractions were separated on a 12% Bistris/SDS-PAGE gel (Bio-
Rad) along with 10 ng of purified ACAD-9, VLCAD, or MCAD and then transferred to a nitrocellulose membrane. The membranes were subjected to Western blotting using rabbit polyclonal antibody against ACAD-9, VLCAD (kindly provided by Dr. A. W. Strauss, Vanderbilt University, Nashville, TN), or MCAD, respectively.

**Molecular Modeling** — A prediction of the three-dimensional structure of ACAD-9 (minus the C-terminal region that is not found in the published structures of ACADs) was obtained using Insight II 2000 software with the Homology package (Accelrys, San Diego, CA) on a Silicon Graphics O2 workstation. Modeling based on the published structure of porcine MCAD with bound C8:0-CoA substrate (Protein Data Bank code 3MDE) (26) was carried out using the Homology and Modeller modules as described previously (25). The final working model, essentially void of violations, had a tertiary fold similar to that of MCAD and other published ACAD crystal structures (26–31). An ETF-ACAD-9 complex was also modeled using the published ETF-MCAD complex atomic coordinates (Protein Data Bank code 1T9G) (32) to further confirm the validity of our ACAD-9 model and to examine the structure of the ACAD-9 ETF-docking site.

**RESULTS**

**Identification of the Mature N Terminus of ACAD-9** — Radiolabeled ACAD-9 precursor protein was synthesized by coupled transcription/translation. Analysis by SDS-PAGE revealed a band for the translated ACAD-9 precursor with an apparent molecular mass of ~70 kDa (Fig. 1A, lane 1). It was processed to a smaller mature intramitochondrial form when incubated with freshly isolated rat liver mitochondria (Fig. 1A, lane 3). When incubated with purified yeast MPP, however, the precursor was cleaved to mature product that appeared to be slightly larger (Fig. 1A, lane 2). Cleavage of the N-terminal amino acid of ACAD-9 by MPP was deduced by radiosequencing to be between pre-

**Intramitochondrial Localization, Purification, and Peptide Mass Mapping of ACAD-9 in Mammalian Cells** — To confirm the localization of ACAD-9 to the mitochondria in intact eukaryotic cells, a plasmid expressing an ACAD-9-Myc-His fusion protein was used to transfect eukaryotic Chang cells. A discrete pattern of fluorescence co-localizing with the mitochondrion-specific MitoTracker dye was observed after anti-Myc staining and was similar to that obtained for human SCAD (Fig. 2) (19). This indicates that the fusion protein was localized inside the mitochondria. This was confirmed by direct immunofluorescence of native ACAD-9 in fibroblast monolayers with anti-ACAD-9 antibody (data not shown).

To identify the mature N terminus of the ACAD-9-Myc-His fusion protein, the tagged protein was affinity-purified from extracts of transfected human embryonic kidney cells and separated by SDS-PAGE. The ACAD-9-Myc-His band was excised from the gel and in-gel digested with trypsin (cleavage of peptide bonds C-terminal to Arg and Lys) or endoproteinase Asp-N (cleavage of peptide bonds N-terminal to Asp), the resulting peptides were analyzed by MALDI-TOF mass spectrometry. A total of 42 tryptic peptides were unambiguously assigned to the ACAD-9 sequence, corresponding to a sequence coverage of 62.6%, with the most N-terminal cleavage site predicted to be between Lys51 and Gln52 (supplemental Table 1). After partial digestion with trypsin, masses matching both peptides 53-AFAKELFLGX-59 and 53-AFAKELFLGKIKK-59 were identified (data not shown). Mass analysis of peptides

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Cellular localization of ACAD-9 in transfected Chang cells using a c-Myc tag and a mitochondrion-selective dye. ACAD-9 was overexpressed as a chimera with the c-Myc tag in transfected Chang cells. A, ACAD-9 visualized by mouse anti-c-Myc and fluorescein-conjugated Alexa Fluor 488-labeled goat anti-mouse antibodies (green); B, mitochondria from the same section shown in A stained with rhodamine-labeled MitoTracker (red); C, images from A and B superimposed, showing co-localization of ACAD-9 and mitochondria (yellow-orange). Nuclear DNA was counterstained with Hoechst 33258.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Purification and size exclusion HPLC of ACAD-9. Purified ACAD-9 (50 μg) was subjected to gel filtration and compared with molecular mass standards. ACAD-9 eluted at 8.45 ml for a calculated molecular mass of 119 kDa. The inset shows 7 μg of purified ACAD-9 run on a 7.5% SDS-polyacrylamide gel and stained with Coomassie Blue. The standards for SDS-PAGE are myosin (198.1 kDa), β-galactosidase (113.6 kDa), bovine serum albumin (96.4 kDa), ovalbumin (52.9 kDa), and carbonic anhydrase (35.9 kDa).
resulting from endoproteasome Asp-N digestion of ACAD-9 identified the first Asp-N cleavage site in ACAD-9 to be localized between Gln61 and Asp62 (supplemental Table 2). Eleven peptide masses were assigned to the human ACAD-9 sequence (supplemental Table 2). In addition, a mass was detected matching sequence AFKELFLGKIKKEVFPF-PVSOQ (observed mass of 2780.52 Da; calculated mass of 2780.58 Da) that did not correspond to any of the 11 peptides, identifying Ala38 as the N-terminal amino acid of the mature ACAD-9 protein within the mitochondria (supplemental Fig. 1).

E. coli Expression, Purification, and Molecular Mass of ACAD-9—Following cloning into a prokaryotic expression vector, mature ACAD-9 was expressed in E. coli and purified. The specific activity of the crude extract was 65 milliunits/mg of protein, whereas that of the final purified sample was 780 milliunits/mg of protein. Greater than 5 mg of ACAD-9 were generally obtained per liter of induced cells. The recombinant ACAD-9 protein was ~95% pure as judged by SDS-PAGE (Fig. 3). The calculated molecular mass of mature ACAD-9 was 64.8 kDa, in agreement with the apparent subunit size of the purified protein.

Purified native ACAD-9 eluted at a volume corresponding to an apparent native mass of 119 kDa on a BioSep SEC-S3000 HPLC gel filtration column, indicating that native ACAD-9 is dimeric (Fig. 3).

Substrate Specificity and Kinetic Parameters of ACAD-9—The activity of purified ACAD-9 was characterized with a variety of short-, medium-, and long-chain saturated and unsaturated acyl-CoA substrates as well as long-chain polyunsaturated and branched-chain substrates using the ETF fluorescence reduction assay. ACAD-9 proved to have activity toward a broad range of substrates, with greater specificity for the long-chain unsaturated acyl-CoAs than for the saturated ones (Fig. 4). Maximal activity was seen with cis-9-C16:1 CoA as substrate and decreased significantly with primary chain lengths of 7 and 13 carbons. High activity was found, however, with all-cis-4,7,10,13,16,19-C22:6 CoA (DHA). There was also activity toward odd-chain acyl-CoA substrates. There was minimal activity with the two branched-chain substrates tested (primary chain lengths of 7 and 13 carbons). Similar results were obtained using the ferricenium dye reduction assay (data not shown) (37). Kinetic parameters of purified ACAD-9 were determined with C16:0-CoA, cis-9-C16:1-CoA, and cis-9,cis-12-C18:2-CoA as substrates using the ETF reduction assay. The $K_m$ values were 2.8, 0.7, and 2.1 mM for C16:0-CoA, cis-9-C16:1-CoA, and cis-9,cis-12-C18:2-CoA, and the catalytic efficiencies were $3.2 \times 10^5$, $18 \times 10^5$, and $6.2 \times 10^7$ M$^{-1}$ s$^{-1}$, respectively. The results are consistent with the finding that purified ACAD-9 had optimal activity with cis-9-C16:1-CoA compared with other long-chain substrates, with a lower $K_m$ and higher catalytic efficiency with cis-9-C16:1-CoA.
proteins. This may also imply that they have similar tertiary folding at their C termini. The hypothetical acyl moiety-binding pocket in our ACAD-9 model is long, measuring \( \sim 23 \) Å from C-1 of the substrate to the surface of the monomer. The pocket is lined with hydrophobic residues as would be expected to optimize binding to substrates with long primary carbon chains (Fig. 6B). Glu389 is in a position similar to MCAD Glu376, the active-site catalytic residue, and likely performs this function in ACAD-9.

In a modeled ETF-ACAD-9 quaternary complex, the side chain of ETF βLeu195, the key residue that is part of the MCAD recognition loop, nestles into a hydrophobic pocket surrounded by the side chains of ACAD-9 Phe85, Pro96, Leu67, Gly68, Leu69, Phe83, Met87, and Leu95 (data not shown). These are identical in position to the homologous residues in the published ETF-MCAD structure (32). Modeling of the electron transfer Domain II of ETF in the three-dimensional structure of the ETF-MCAD ternary complex has suggested that ionic interaction between MCAD Glu212 and ETF βArg249 plays an important role in stabilizing productive conformations for efficient electron transfer (32). The homologous residue in ACAD-9 is Glu223, similar to all ACADs except LCAD and isovaleryl-CoA dehydrogenase, in which it is Leu.

DISCUSSION

The role of unsaturated fatty acids in human disease is of general interest for the understanding of common disorders such as diabetes, obesity, cardiovascular disease, and cancer. Long-chain unsaturated fatty acids are the primary fatty acids found in human diets and the main components of stored body fat. They are highly abundant in brain. The enzymes involved in their catabolism have not been completely described, however.

ACAD-9, the ninth member of the family of ACADs, has been shown previously to be active with C16:0-CoA (within a limited range of substrates tested), similar to the homologous VLCAD protein (13, 40). Beyond this, however, it remained uncharacterized, and its physiologic function was unknown. To study enzyme function, we first identified the N terminus of the mature protein.

The ACADs are encoded in the nucleus as precursor proteins that are transported into the mitochondria and processed to their mature form by cleavage of a target peptide (41). With the exception of VLCAD, the ACADs are homotetrameric flavoenzymes with subunits of \( \sim 40 \) kDa (41). VLCAD assembles as a homodimer of 70-kDa subunits (10). Although most of VLCAD shares 30% sequence homology with the other
ACADs, its C-terminal 180 amino acids do not (42–44). Additionally, although the other ACADs are mitochondrial matrix proteins, mature VLCAD is associated with the inner mitochondrial membrane (10, 38).

Our cell transfection, mitochondrial import, and direct immunofluorescence studies confirmed that ACAD-9 localizes to mitochondria. In addition, we have shown that a 37-amino acid leader peptide is removed by sequential cleavage of MPP, followed by MIP. Whereas MPP is essential for global mitochondrial protein processing (36, 45), MIP is required for the maturation of only a small subset of mitochondrial proteins. Most natural human MIP substrates are proteins of oxidative phosphorylation, but a few involved in other metabolic functions have been identified (35, 46, 47). Among the family of ACADs, ACAD-9 is the first to show processing by two-step sequential cleavage.

The amino acid sequence of ACAD-9 is homologous to the other ACADs except at its C-terminal end, where it shares an additional tail of 180 amino acids only with VLCAD. Overall, it is 47% amino acid identical and 65% similar to VLCAD. In addition, the precursor proteins of both ACAD-9 and VLCAD contain leader peptides of similar lengths of 37 and 40 residues, respectively. These are longer than leader peptides of other ACADs, including SCAD, MCAD, LCAD, and isovaleryl-CoA dehydrogenase, which are 25–30 residues (48). Interestingly, this corresponds to the length of the first cleavage product of ACAD-9 by MPP.

Thus, ACAD-9 and VLCAD appear to have evolved from a more recent gene duplication than the remainder of the ACAD gene family, with subsequent divergence likely leading to the acquisition of an MIP cleavage site in ACAD-9. We have additionally demonstrated that ACAD-9 is a dimer (as is VLCAD) associated with the mitochondrial membrane. The additional 180 amino acid residues at the C termini of these two enzymes may play a role in their intramitochondrial location and may also be important in interactions with other enzymes involved in mitochondrial $\beta$-oxidation as functional complexes (49).

To begin to characterize the physiologic role of ACAD-9, we tested purified recombinant enzyme for activity with an expanded panel of substrates. Surprisingly, it showed maximal activity with long-chain unsaturated substrates. ACAD-9 was >50% more active with cis-9-C16:1-CoA (the optimal substrate tested) than with C16:0-CoA, and both cis-9-C18:1-CoA and cis-9,cis-12-C18:2-CoA gave greater activity than stearoyl-CoA. This is consistent with the hypothetical acyl moiety-binding pocket of our ACAD-9 model, which is long enough to accommodate substrates with long primary carbon chains. Activity decreased with longer and shorter chain substrates, and ACAD-9 was not active against the branched-chain substrates tested. ACAD-9 was also active with 9–11- and 15-carbon substrates (Fig. 4). This is not unexpected based on findings with other ACADs that are active against odd-chain substrates of similar length to the even-chain substrates that they utilize. Similar to unsaturated fatty acids, odd-chain fatty acids originate mainly from dietary sources (50) and are constituents of lipid membranes.

The existence of another dehydrogenase involved in the oxidation of long-chain unsaturated acyl-CoA substrates has been proposed previously (5). In in vitro studies with VLCAD-deficient human fibroblasts incubated with cis-9-C18:1, cis-9,cis-12-C18:2, or their trans-isomers, oxidation is effective from 18 down to 14 carbons and ceases at the level of tetradecenoyl (cis-5-C14:1)-CoA, tetradecadienoyl (cis-5,cis-8-C14:2)-CoA, or their respective trans-compounds. This suggests that VLCAD is responsible for the 2,3-dehydrogenation of substrates with cis-5- or trans-5-compounds and that another dehydrogenase or alternate pathway is responsible for the initial $\beta$-oxidation of long-chain unsaturated fatty acids. The observed substrate specificity with an optimum toward long-chain unsaturated acyl-CoAs is consistent with the hypothesis that ACAD-9 serves this function. Thus, it appears that ACAD-9 is likely to play a primary role in the $\beta$-oxidation of long-chain unsaturated substrates.

Human LCAD has been reported previously to utilize C$_{14}$ and C$_{16}$ unsaturated CoA substrates, whereas VLCAD purified from human liver is much less active with these substrates (40). Of note, in contrast to ACAD-9 and VLCAD, LCAD efficiently utilizes long branched-chain substrates (51–53). The observed overlap in substrate specificity among the three long-chain ACADs and their apparent functional redundancy require further consideration. Because patients with a genetic deficiency of VLCAD show severe derangements in the $\beta$-oxidation of long-chain fats, it is unlikely that ACAD-9 can substitute for this function in vivo in high oxidative organs such as skeletal muscle and kidney despite its ability to use long-chain saturated substrates. This may be due in part to differential tissue expression. Although LCAD is localized to the mitochondrial matrix, ACAD-9 and VLCAD are associated with the inner mitochondrial membrane. The relative roles of LCAD and ACAD-9 may thus be primarily different. It is interesting to note that human deficiencies of all the ACADs have been reported except LCAD and ACAD-9. One could hypothesize that the overlap in substrate specificity between LCAD and ACAD-9 may abrogate the effects of a deficiency of either one. However, ACAD-9 cannot compensate for the loss of branched-chain substrate utilization by LCAD. Possible functional redundancy could be studied in knockout mouse models, which are available for VLCAD and LCAD, but not yet for ACAD-9 (54, 55). These models indicate a discrepancy between the substrate specificity pattern in vitro and metabolite profiles of enzyme deficiency observed in vivo. Therefore, possible clinical and metabolic implications of a deficiency of ACAD-9 in vivo are currently difficult to predict and will require further characterization of the physiologic function of ACAD-9 relative to the other long-chain ACADs.

In addition to long-chain monounsaturated fatty acids, our study has also shown significant ACAD-9 activity toward docosahexaenoic acid (all-cis-4,7,10,13,16,19-C22:6), an essential n-3 ($\omega$-3) polyunsaturated fatty acid. This is of particular interest in view of the proposed role of all-cis-4,7,10,13,16,19-C22:6 in brain lipids and neuroprotection through cell signaling and protection of postsynaptic proteins (56). Low all-cis-4,7,10,13,16,19-C22:6 has an impact on lipid membrane peroxidation and free radical production and has been associated with synaptic loss, critical in neurodegenerative processes, particularly Alzheimer disease (56, 57). Although VLCAD, MCAD, and ACAD-9 all show high levels of mRNA in human heart, skeletal muscle, liver, and kidney (58, 59), ACAD-9 appears to be abundant in brain (13). There, it could play a role in the turnover of lipid membrane unsaturated fatty acids that are important to maintain membrane integrity and structure (1).

In conclusion, we have identified the mature N terminus of ACAD-9 and its localization to the mitochondria, produced it in an E. coli expression system, and characterized the purified recombinant protein. Our data indicate a previously unrecognized role for ACAD-9 in the oxidation of long-chain unsaturated fatty acids, complementing the function of VLCAD in long-chain fatty acid catabolism. Because of the high activity of ACAD-9 toward polyunsaturated fatty acids and its abundance in brain, we speculate that it may play an additional structural role in neural lipid membranes.

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REFERENCES

1. Benatti, P., Peluso, G., Nicolai, R., and Calvani, M. (2004) J. Am. Coll. Nutr. 23, 281–302

* J. Vockley, unpublished data.
