Characterization of Wild-Type and an Active-Site Mutant in *Escherichia coli* of Short-Chain Acyl-CoA Dehydrogenase from *Megasphaera elsdenii*[†,‡]

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ABSTRACT: The objective of this work is to determine the molecular mechanism and regulation of short-chain acyl-CoA dehydrogenase (SCAD) from *Megasphaera elsdenii*. To achieve this, the gene coding for SCAD from *M. elsdenii* was cloned and sequenced. Site-directed mutagenesis was then used to identify an amino acid residue that is required for the proposed mechanism. To clone the gene, the amino acid sequence of the 50 N-terminal residues of SCAD from *M. elsdenii* was determined. This sequence information was utilized to synthesize two sets of mixed oligonucleotide primers which were then used to generate a 120-bp specific probe from *M. elsdenii* DNA by the polymerase chain reaction (PCR) method. The 120-bp probe was used to screen a *M. elsdenii* genomic DNA library cloned into *Escherichia coli*. The gene encoding *M. elsdenii* SCAD was identified from this library, sequenced, and expressed. The cloned SCAD gene contained an open reading frame which revealed a high degree of sequence identity with an open reading frame protein sequence of the human SCAD and the rat medium-chain acyl-CoA dehydrogenase (MCAD) (44% and 36% identical residues in paired comparisons for human SCAD and rat MCAD, respectively). Recombinant SCAD expressed from a pUC119 vector accounted for 35% of the cytosolic protein in the *Escherichia coli* crude extract. The expressed protein had similar activity, redox potential properties, and nearly identical amino acid composition to native *M. elsdenii* SCAD. In addition, a site-directed $Glu^{367}Gln$ mutant of SCAD expressed from a pUC119 vector was shown to have minimal reductive and oxidative pathway activity with butyryl-CoA and crotonyl-CoA, respectively. Residue Glu^{367} has been proposed to initiate catalysis by abstracting the substrate α-proton. This is the first of the site-directed mutants that will enable us to characterize the mechanism and thermodynamic regulation of *M. elsdenii* SCAD.

Mammalian acyl-CoA dehydrogenases catalyze the oxidation of saturated acyl-CoA thioesters to give a 2,3-unsaturated product. This reaction is the first step in β-oxidation of these thioesters and occurs in the mitochondria. The electrons from the reduced acyl-CoA dehydrogenases are transferred to the electron transfer flavoprotein and ultimately to the electron transport chain. Most of the research on mammalian acyl-CoA dehydrogenases has focused on the medium-chain acyl-CoA dehydrogenase (MCAD),[1] which has an optimum specificity for 8-carbon chain length fatty acyl-CoAs (Thorpe et al., 1979). Electrochemical and spectroscopic studies have shown that favorable thermodynamic conditions for substrate turnover by MCAD exist only in the presence of bound substrate/product (Lenn et al., 1990). Free MCAD has a reduction potential value of −136 mV (pH 7.6), while substrate/product bound MCAD has a reduction potential value of −26 mV (pH 7.6) (Lenn et al., 1990). This positive shift in the reduction potential of MCAD upon substrate/product binding allows isopotential electron transfer to occur from substrate to enzyme. Certainly, the electrochemical observations seen with MCAD suggest it is regulated by substrate/product binding. The mechanism of this regulation, however, remains unclear.

In an attempt to resolve the structural basis of this regulation, MCAD cDNA clones from rat liver mRNA have been isolated and characterized (Matsubara et al., 1987). These data along with the 2.4-Å X-ray crystallographic data of pig liver MCAD (Kim et al., 1992) have provided insights concerning the site of FAD and substrate binding. Although human MCAD has been cloned, the yield of normal and mutant cloned MCAD expressed in *Escherichia coli* is low (about 0.9 mg/L of culture) (Bross et al., 1990) and the resultant proteins are unstable (S. Ghisla, 1990, personal communication). As an alternative approach, we have initiated the cloning and analysis of SCAD from *Megasphaera elsdenii*. The primary structural information gained from this study will provide insights into the structure/function relationships of *M. elsdenii* SCAD and the mechanism of the thermodynamic regulation observed in mammalian MCAD. The rationale for this is based on our electrochemical experiments with SCAD from *M. elsdenii*.

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[1] Abbreviations: BCoA, butyryl-CoA; CCoA, crotonyl-CoA; CD, circular dichroism; dATP, 2′-deoxyadenosine 5′-triphosphate; DCPIP, 2,6-dichlorophenolindophenol; dUTP, 2′-deoxyuridine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; HPLC, high-pressure liquid chromatography; IPTG, isopropyl thio-β-D-galactoside; MCAD, medium-chain acyl-CoA dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); PCR, polymerase chain reaction; SCAD, short-chain acyl-CoA dehydrogenase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Electrochemical studies with *M. elsdenii* SCAD have shown it behaves similarly to MCAD. Although SCAD activity *in vivo* is in the reverse direction [it shuttles reducing equivalents to crotonyl-CoA (CCoA) to form butyryl-CoA (BCoA)], *in vitro* SCAD behaves like its mammalian counterparts by catalyzing the insertion of a trans double bond between the C-2 and C-3 of its acyl-CoA thioester substrates (Brockman & Wood, 1975). *M. elsdenii* SCAD has an optimum specificity for 4-carbon length fatty acyl-CoAs (Williamson & Engel, 1984). Free SCAD has a reduction potential value of −79 mV (pH 7.0, 25 °C) (Fink et al., 1986), while substrate/product-bound SCAD has a reduction potential value of −19 mV (pH 7.0, 25 °C) (Stankovich & Soltysik, 1987). This positive shift in the reduction potential value of SCAD is similar to that of MCAD and facilitates isopotential electron transfer from substrate to enzyme. Due to this similar electrochemical behavior, structural homologies between *M. elsdenii* SCAD and mammalian MCAD are also expected, allowing the molecular analysis of SCAD to aid in the understanding of the structural basis for the regulation of MCAD redox potentials upon substrate/product binding. The three-dimensional structure of *M. elsdenii* SCAD is being determined and should shortly result in a map of the enzyme active site at 2.5-Å resolution (J.-J. P. Kim, 1993, personal communication).

Besides these similarities between *M. elsdenii* SCAD and mammalian MCAD, there are also some differences which would be of interest in the molecular comparisons of these two enzymes. One difference between SCAD and MCAD is the solvent accessibility to their active centers when substrate bound. In the case of SCAD, both the substrate α and β hydrogens exchange with solvent, while in MCAD only the substrate α-hydrogen exchanges (Ghisla et al., 1984). The two enzymes also differ in their reactivity with molecular oxygen in the presence of substrate. SCAD from *M. elsdenii*, which is an anaerobic bacterium, reacts quickly with oxygen (Ellison et al., 1984), while mammalian MCAD is protected against reactivity with oxygen (Wang & Thorpe, 1991). Structural variations between the two enzymes could indicate the basis of their differences in solvent accessibility and oxygen reactivity when substrate-bound.

In this report, the cloning and sequencing of the SCAD gene from *M. elsdenii* is described. In addition, the amino acid sequence comparisons of *M. elsdenii* SCAD with human SCAD and rat MCAD are shown. The expression, purification, and selected properties of recombinant normal and $Glu^{367}Gln$ mutant *M. elsdenii* SCAD in *E. coli* are also reported. To our knowledge, this is the first report of a cloned gene from *M. elsdenii*. Previous attempts to clone *M. elsdenii* SCAD were unsuccessful (A. Westphal and P. C. Engel, 1990, personal communication), and attempts to clone *M. elsdenii* flavodoxin have also been unsuccessful (G. Voordouw, 1990, personal communication).

MATERIALS AND METHODS

Enzymes and Chemicals. T4 DNA ligase, calf intestinal alkaline phosphatase, random hexamer primer, *E. coli* DNA polymerase I Klenow fragment, and restriction endonucleases were purchased from Boehringer Mannheim Biochemical or BRL. Restriction endonucleases and DNA-modifying enzymes were used according to the recommendations of the manufacturer. Lumi-Phos 530, digoxigenin-dUTP, and the anti-digoxigenin alkaline phosphatase conjugate for the Genius nonradioactive nucleic acid labeling and detection system were obtained from Boehringer Mannheim. AmpliTaq DNA polymerase was purchased from Perkin-Elmer Cetus. DNA sequences were determined using the Sequenase version 2.0 DNA sequencing kit obtained from U.S. Biochemical Corp. [α-^{32}P]dATP was purchased from Amersham Corp. All other chemicals were obtained from Sigma Chemical Co.

Bacterial Strains and Plasmids. The *E. coli* strains Stratagene SURE cells, DH5αF′, and RS3097 and K19 (two mutants obtained from Dr. Barbara Bachmann at the *E. coli* Genetic Stock Center at Yale University) were used for the cloning of SCAD. The *E. coli* strains DH5αF′ and K19 were also used as hosts for the expression vectors. The plasmids pUC18 and pUC19 were used for the cloning of SCAD, while pUC119 was used for the expression of SCAD.

M. elsdenii Genomic DNA Preparation. The *M. elsdenii* genomic DNA was purified by a modification of the reported purification procedure (Hermann & Frischauf, 1987). Frozen *M. elsdenii* cells (0.5 g) were ground in a mortar cooled with liquid nitrogen and dispersed in a 10-mL solution containing 50 mM Tris-HCl, pH 8.5, 100 mM EDTA, and 200 mM NaCl. The cells were lysed by the addition of 0.5 mL of 10% SDS and 2.5 mg of proteinase K followed by incubation at 50 °C for several hours. The homogenate was extracted with an equal volume of 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA (Tris–EDTA buffer) saturated/equilibrated with phenol using a wrist action rocker for 15 min. The phases were separated by centrifuging the mixture at 3000 rpm for 10 min at 10 °C. This extraction was repeated once with a 1:1 phenol/Sevag (chloroform/isoamyl alcohol) mixture and then with Sevag alone.

The aqueous phase was then adjusted to 0.3 M with sodium acetate, pH 5.2. The DNA was precipitated by the addition of 2-propanol (0.8 volume). Next, the DNA pellet was removed with a plastic pipet and transferred to 70% ethanol, spun down, dried and resuspended in 0.5 mL of Tris–EDTA buffer to which RNase A was added (55 μg/mL). The yield of *M. elsdenii* genomic DNA was 1 mg of DNA/g of bacteria.

Development of a Hybridization Probe Specific for SCAD. SCAD was isolated from *M. elsdenii* as described previously (Engel, 1981). The N-terminal sequence of the first 50 amino acids of SCAD was obtained by Sandy Keilland at the Microsequencing Facility at the University of Victoria, British Columbia. The corresponding DNA sequence encoding these amino acids was then deduced. On the basis of this information, two oligonucleotides (32-mers) were designed complementary to areas near the ends of the 150-base-pair sequence; the oligonucleotides encode the polypeptides MDFNLTDIQQ and LIDELLSLGI. To aid in subsequent subcloning, *Eco*RI and *Bam*HI restriction sites were designed into the ends of the oligonucleotides. At positions of high degeneracy, deoxyinosine residues were incorporated into the two oligonucleotides as a neutral base which can pair adequately to all four conventional bases. This minimizes the effects of mixmatches between the probe and the target sequence, as the deoxyinosine residue forms a more stable hybrid with the DNA than a mixmatched base (Ohtsuka et al., 1985). These oligonucleotides were synthesized by Dr. Tom Atkinson in the University of British Columbia Biochemistry Department.

The oligonucleotides then served as primers in a polymerase chain reaction (PCR) using genomic DNA from *M. elsdenii* as the template. The PCR was performed using the Perkin-Elmer Cetus DNA thermal cycler and AmpliTaq DNA polymerase. The conditions for the PCR were as follows: denaturation, 30 s at 94 °C; annealing, 2 min at 40 °C; elongation, 1 min at 72 °C. The PCR mixture was analyzed

by agarose gel electrophoresis and a single band was obtained corresponding to the expected 120-base-pair fragment. The DNA fragment was cut with *Eco*RI and *Bam*HI and ligated into pUC19 at the *Eco*RI and *Bam*HI sites. The nucleotide sequence was determined using the dideoxy method (Sambrook et al., 1989). The sequence corresponded exactly to the amino acid sequence of SCAD except where deoxyinosine residues had been incorporated. For the screening of the *M. elsdenii* genomic libraries, a hybridization probe was made by a random primer labeling reaction (Sambrook et al., 1989) using ^{32}P-labeled dATP and the 120-bp fragment as a template. In later screenings of the subclones harboring the SCAD gene, the Genius nonradioactive nucleic acid labeling and detection system was used. The probe was labeled with digoxigenin-dUTP by the random primer labeling procedure.

Construction of the M. elsdenii Genomic Library. Due to SCAD's approximate subunit molecular weight of 43 000, the SCAD gene was expected to be about 1.2 kb in size. Thus, fragments in the 2–4-kb range were sought. In separate experiments, the genomic DNA from *M. elsdenii* was cut with a variety of restriction enzymes and in some cases with two restriction enzymes. The DNA fragments were then separated by agarose gel electrophoresis in preparation for Southern blots which were used to find the fragment size that contained the SCAD gene. On the basis of these results, larger quantities of the appropriate restriction digests were size-fractionated by using sucrose density centrifugation. Southern blot analysis was performed on all fractions. Fractions with the correct molecular weight were ligated into the relevant vector.

Appropriately sized fragments were recovered utilizing sucrose density gradient centrifugation and ligated into pUC18 plasmids. The ligations were performed according to the recommendations of Bethesda Research Laboratories (King & Blakesley, 1986) and Sambrook et al. (1989). The recombinant plasmids were used to transform Stratagene SURE cells. Transformations were accomplished by variations of the methods reported by Sambrook et al. (1989). The resulting bacterial colonies were screened by histochemical identification and colony hybridization using the 120-bp fragment as a probe. Positive colonies were grown in 2-mL cultures and the plasmid DNA was purified by the alkaline lysis method (Sambrook et al., 1989) before digestion and Southern blot analysis.

DNA Sequence Analysis. The nucleotide sequence of the entire 1.6-kb fragment contained in the pUC18 recombinant plasmid was determined by the Sanger method (Sambrook et al., 1989) utilizing the Sequenase version 2.0 DNA Sequencing Kit according to the recommendations of U.S. Biochemical Corp. Both complementary strands of the 1149-bp open reading frame were sequenced, while only one strand of the upstream 355-bp region was sequenced. The two universal primers (M13 reverse and –40) and sequence-specific synthetic primers (purchased from Oligos Etc. Inc., Wilsonville, OR) were used to attain the 1.6-kb fragment sequence.[2] The sequencing strategy was performed in a stepwise fashion as newly acquired sequence was used to build new synthetic primers to attain additional DNA sequence. Denatured double-stranded plasmid DNA was used in all sequencing reactions (Sambrook et al., 1989). Alignment of the deduced amino acid sequence of cloned SCAD with the amino acid sequences of human SCAD (Naito et al., 1989) and rat MCAD (Matsubara et al., 1987) was performed by the IntelliGenetics program in the Molecular Biology Computer Center at the University of Minnesota. The amino acid sequences of human SCAD (GenBank Accession Number A30605) and rat MCAD (GenBank Accession Number A2846) were retrieved from the GenBank (Bilofsky & Burks, 1988) data bank.

Expression of Cloned SCAD. For the production of SCAD, transformed *E. coli* strains DH5αF′ and K19 were grown in either Luria–Bertani medium or Terrific broth. Purified SCAD originated from K19 cultures grown in Terrific broth to stationary phase at 37 °C with no induction. The purification of SCAD from the *E. coli* strain K19 followed the same order of the purification protocol reported for native SCAD with some modifications and the exclusion of the gel filtration column (Engel, 1981). After being washed with 0.8% NaCl, the pelleted *E. coli* cells were disrupted by French press. The broken cells were spun at 30 000 rpm for 30 min. The resulting supernatant was then applied to a (diethylaminoethyl)-Sepharose column equilibrated with 0.1 M potassium phosphate buffer (pH 7.0). The column was washed with 0.2 M potassium phosphate buffer (pH 7.0) followed by a linear gradient of 0–1 M KCl in 0.2 M potassium phosphate buffer (pH 7.0) to elute the cloned SCAD. For the amino acid composition analysis and N-terminal sequencing, purified SCAD was subjected to fast protein liquid chromatography using a Pharmacia Mono-Q HR 10/10 column. SCAD was eluted with a 0–1 M KCl linear gradient in 20 mM bis-Tris propane buffer (pH 7.0).

Characterization of Cloned Wild-Type and Mutant SCAD. Activity assays of the crude extract and purified SCAD were performed according to the method of Engel (1981). Normal SCAD activity from this assay is reported to be about 200 μmol of DCPIP min^{-1} (μmol of FAD)$^{-1}$ (Engel, 1981). For the activity comparisons of mutant with wild-type SCAD, phenazine methosulfate was replaced with phenazine ethosulfate. The extinction coefficient determinations were performed at 450 nm using Engel's method (Williamson & Engel, 1984) and were repeated four times to give an average value and a standard deviation. The UV–CD spectra were measured at 25 °C with a Jasco J-710 spectropolarimeter using a 0.01-cm cell. The amino acid composition analysis of the cloned and native SCAD and the N-terminal amino acid sequencing of the cloned SCAD were performed by the Microchemical Processing Center at the University of Minnesota.

Reduction potential measurements of cloned normal SCAD were performed as previously described (Stankovich, 1980; Stankovich & Fox, 1983). Experimental conditions were 20 °C and pH 7.0 in 0.1 M potassium phosphate buffer. Methyl viologen (0.1 mM) was used as the mediator dye while pyocyanine (E_m = –30 mV, pH 7.0) (5 μM) and indigo disulfonate (E_m = –96 mV, pH 7.0) (1–2 μM) were used as the indicator dyes. SCAD concentration for the spectroelectrochemistry experiments ranged from 12 to 16 μM.

In the incubations of normal and Glu^{367}Gln mutant cloned SCAD with either BCoA or CCoA, 8–12 μM protein was placed with 0.1 mM methyl viologen in a visible spectroelectrochemistry cell and degassed for 1 h. Prior to the addition of BCoA to oxidized normal or mutant cloned SCAD, the residual oxygen was removed by reducing the enzyme with 2–3 mC. The BCoA was then added by tipping the substrate into the anaerobic solution from the side arm. Prior to the addition of CCoA to either reduced normal or mutant cloned SCAD, complete reduction of the enzyme was noted by the appearance of reduced methyl viologen at 395 nm. Subse-

[2] The 1.6 kb sequence was submitted to GenBank (Bilofsky & Burks, 1988) sequence data bank and assigned the accession number L04528.

quently, CCoA was then added to the solution using a syringe.

HPLC was used as an additional method to check the Glu^{367}-Gln mutant SCAD activity with BCoA. Aliquots of both normal and Glu^{367}Gln mutant SCAD incubated (25 °C) aerobically with an 18-fold molar excess of BCoA were analyzed at various time intervals on an HPLC C-18 reverse-phase column. A 15-min gradient of 20–35% methanol in 50 mM potassium phosphate (pH 5.3) was used to separate BCoA and CCoA.

RESULTS

Isolation of the SCAD Gene from M. elsdenii. The *M. elsdenii* genomic DNA was digested with the restriction endonucleases *Bam*HI and *Pst*I. This digestion produced a 3.2-kb fragment which subsequently hybridized with the SCAD probe in a Southern blot. The 3.2-kb fragments were recovered from an agarose gel and ligated into pUC18 at the *Bam*HI and *Pst*I sites. Next, Stratagene SURE cells were transformed with the recombinant pUC18 plasmids. The Stratagene SURE cells are free of known restriction systems.

A 2.2-kb *Bam*HI–*Bam*HI fragment was inadvertently ligated along with the 3.2-kb fragment into the pUC18 plasmid. Thus, the recombinant plasmid contained 5.4 kb of foreign DNA. A *Hin*dIII site was found in the middle of the 3.2-kb fragment. This *Hin*dIII site was used to subclone a 1.6-kb *Hin*dIII–*Hin*dIII fragment out of the 5.4-kb piece and into a pUC18 vector. The N-terminus of the SCAD gene was shown to be in the subcloned 1.6-kb fragment by a Southern blot. Consequently, the nucleotide sequence of the 1.6-kb piece was determined.

Sequencing. An open reading frame of 1149 bp was found approximately 355 bp downstream from the *Hin*dIII site. The nucleotide sequence of the N-terminal region of this open reading frame matched the sequence of the 120-bp probe except where the deoxyinosine residues and the *Bam*HI and *Eco*RI linker sequences had been incorporated. In addition, the open reading frame could be translated into a protein of 383 amino acids which had an N-terminal amino acid sequence corresponding exactly to the sequence of the first 50 amino acids of native SCAD from *M. elsdenii*. As a result, it was highly probable that this open reading frame represented the cloned SCAD gene from *M. elsdenii*.

SCAD Expression in E. coli and Its Characterization. The 1.6-kb fragment was subcloned into pUC119 using *Pst*I and *Hin*dIII, keeping the correct orientation of the 1149-bp open reading frame for expression by the *lac* promoter. The recombinant pUC119 vector was then used to transform the *E. coli* strain DH5αF′. SCAD activity assays of crude extracts from the transformed and nontransformed *E. coli* strain DH5αF′ grown to stationary phase were performed. These assays showed SCAD activity in the cells containing the recombinant pUC119 plasmid. Induction of the cells with IPTG for different lengths of time did not increase the amount of the cloned protein production with respect to that seen in the noninduced cells. It appears *E. coli* RNA polymerase is able to use the *M. elsdenii* promoter which was cloned in front of the 1149-bp open reading frame. Thus, the expression of the 1149-bp reading frame in the transformed *E. coli* strain DH5αF′ was not dependent upon induction with IPTG.

To develop a rapid method for the detection of *M. elsdenii* SCAD expression in *E. coli*, the *E. coli* strains K19 and RS3097 were transformed with the recombinant pUC119 plasmid. The *E. coli* strain K19 has a mutation in the *fadE* gene (Klein et al., 1971), while the strain RS3097 has a constitutive mutation in the regulatory *fadR* gene (Spratt et al., 1981).

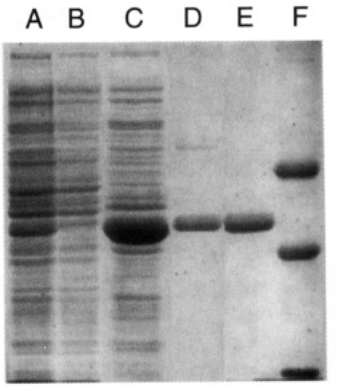

FIGURE 1: SDS–polyacrylamide gel of *E. coli* crude extracts and purified *M. elsdenii* SCAD. Lanes A and B are the crude extracts from the *E. coli* strain K19 grown less than 24 h with and without the recombinant plasmid, respectively. Lane C is the crude extract from the *E. coli* strain K19 grown over 24 h showing the amount of *M. elsdenii* SCAD present in the cytosolic fraction, and lane D is purified *M. elsdenii* SCAD from the *E. coli* strain K19. Lanes E and F are native *M. elsdenii* SCAD and the molecular weight markers (21 000, 36 500 and 55 000), respectively.

K19 and RS3097 cells with and without the recombinant pUC119 vector were plated on various agar media containing as the sole carbon source fatty acids ranging from butyric to oleic acid. It was anticipated that the mutant cells harboring the recombinant pUC119 vector would grow better on at least one of the fatty acid media than the plasmidless mutant cells. The results were inconclusive, as no real difference in the growth between the nontransformed and transformed cells was observed. Increased expression of the cloned protein, however, was detected in K19 compared to DH5αF′. Consequently, the *E. coli* strain K19 was chosen as the host for the pUC119 expression vector. As with the *E. coli* strain DH5αF′, little difference in the amount of cloned protein expression was observed between induced and noninduced K19 cultures with IPTG.

For the production of the recombinant *M. elsdenii* protein, the *E. coli* strain K19 was grown to stationary phase. Figure 1 shows an SDS–polyacrylamide gel of K19 crude extracts with and without the pUC119 vector. It can be seen that, without the recombinant pUC119 vector, the *E. coli* strain K19 does not produce a protein of similar molecular weight to SCAD. Figure 1 also shows that the cloned protein makes up 35% of the cytosolic protein determined by densitometry and runs similarly to the native SCAD from *M. elsdenii*.

Purification of the recombinant *M. elsdenii* SCAD from the *E. coli* strain K19 followed a similar protocol to that of the native SCAD from *M. elsdenii* (Figure 1) (Engel, 1981). The yellow form of SCAD was obtained in cultures grown for 24 h. The visible spectrum of the yellow form of the cloned SCAD was typical of the native SCAD yellow form from *M. elsdenii*. In cultures grown for approximately 32 h, however, a small amount of the green form of the *M. elsdenii* SCAD began appearing. A charge transfer band at 710 nm was observed in the visible spectrum of the green cloned SCAD similar to the green form of native SCAD from *M. elsdenii*. From the 710 nm/430 nm ratio, it was estimated that the green form constituted 5% of the purified cloned SCAD (Williamson et al., 1982). The slight green color was removed by dialysis of the ammonium salt fractionated SCAD pellet with 10 mM sodium dithionite in 0.1 M potassium phosphate buffer (pH 7.0) for 2 h. This was followed by dialysis with 0.1 M potassium phosphate buffer (pH 7.0, no sodium dithionite) for another 2 h, which generated the yellow form of *M. elsdenii* SCAD. Approximately 20 mg of SCAD was

Table I: Amino Acid Composition Comparisons

amino acid	recombinant SCAD	native SCAD	predicted from sequence
Asp	35.1	36.3	35
Thr	27.3	27.2	28
Ser	14.6	16.0	14
Glu	45.1	45.8	44
Pro	8.6	8.6	9
Gly	42.3	45.3	41
Ala	43.1	42.6	42
Cys	–[a]	–[a]	3
Val	23.2	22.2	23
Met	11.3	10.9	12
Ile	22.8	22.4	24
Leu	29.8	29.9	30
Tyr	13.6	13.4	14
Phe	17.4	17.4	18
His	5.5	5.3	5
Lys	30.9	30.8	31
Arg	8.6	8.6	9
Trp	–[a]	–[a]	1

[a] Analysis did not determine cystine and tryptophan composition.

obtained from a 1-L culture of the transformed *E. coli* strain K19.

Activity assays of both the crude extract and purified cloned SCAD showed the cloned SCAD to have normal activity ranging from 100 to 400 μmol of DCPIP min^{-1} (μmol of $FAD)^{-1}$. The K19 cells without the SCAD gene showed little intrinsic activity in the SCAD assays, demonstrating that its contribution was insignificant to the overall SCAD activity observed in the transformed K19 cell crude extracts. All activity assays were performed with the yellow form of SCAD. An extinction coefficient of 13.7 ± 0.3 cm^{-1} mM^{-1} at 450 nm was determined for cloned SCAD (yellow form). This value compares reasonably with the two previously reported extinction coefficients for native SCAD from *M. elsdenii* of 12.5 cm^{-1} mM^{-1} (Engel & Massey, 1971) and 14.2 cm^{-1} mM^{-1} (Williamson & Engel, 1984).

Amino acid composition analysis (which has about 5% experimental error) of both the cloned SCAD and native SCAD showed the two proteins to have nearly identical compositions (Table I). In addition, the 10 amino acid N-terminal sequence was determined for cloned SCAD. The cloned SCAD N-terminal sequence matched the N-terminal sequence of native SCAD for these first 10 amino acids. From the nucleotide sequence, the predicted molecular weight of cloned SCAD is 41 400 per subunit, corresponding to 383 amino acid residues. Finally, electrochemistry experiments have shown that cloned SCAD has a reduction potential value of –76 mV, similar to the value reported for the native *M. elsdenii* SCAD (–79 mV) (Fink et al., 1986).

Mutation at the 367 Amino Acid Position of Cloned SCAD. Glu^{367} was replaced with Gln by the Kunkel method (Sambrook et al., 1989). The mutation was identified by a *Sty*I digest whose site had been incorporated into the mutant DNA strand and was then confirmed by DNA sequencing of the region containing the Glu^{367}Gln mutation. The mutant protein was expressed in the *E. coli* strain K19 by growing the cells to stationary phase (20–24 h). The Glu^{367}Gln mutant constituted about 45% (determined by densitometry) of the cytosolic protein and was purified by a protocol similar to that for normal cloned SCAD. Approximately 60 mg of mutant protein was obtained from a 1-L culture. The mutant protein had the appearance of the green form (≈8.0%), although the cultures were not grown over 24 h. The green color was removed by overnight anaerobic dialysis with 10 mM sodium dithionite in 0.1 M potassium phosphate buffer (pH 7.0)

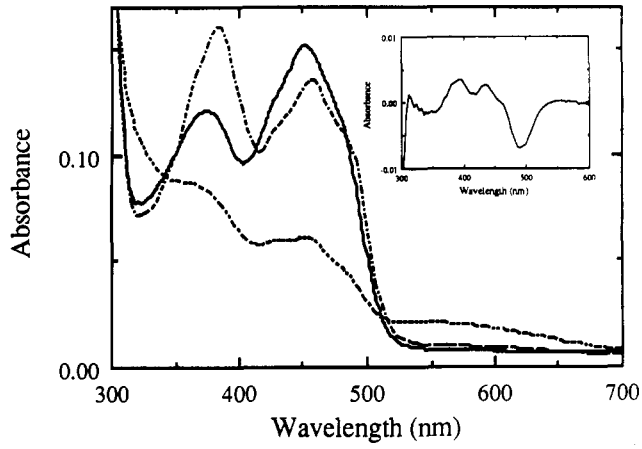

FIGURE 2: Visible spectra of Glu^{367}Gln mutant SCAD before BCoA addition (—), Glu^{367}Gln mutant SCAD after 16 h of incubation with BCoA (- - -), and normal SCAD showing the corresponding change immediately after addition of BCoA (- - -). The mutant and normal SCAD protein concentrations were normalized. The inset is a difference spectrum of the mutant and normal SCAD, showing the red shift of the mutant SCAD visible spectrum.

followed by 2 h of aerobic dialysis in 0.1 M potassium phosphate buffer (pH 7.0, no sodium dithionite) to generate the yellow form. The visible spectrum of the Glu^{367}Gln mutant (yellow form) is similar to the spectrum observed for the normal cloned SCAD except for a slightly higher resolution and red shift of the 450-nm peak (Figure 2). The UV–CD spectra of the normal and Glu^{367}Gln mutant SCAD are the same, illustrating that no global conformational changes have occurred in the mutant protein. The catalytic activity of the purified Glu^{367}Gln mutant was determined to be ≤0.03% of the activity seen in normal cloned SCAD.

The flavin spectrum of the Glu^{367}Gln mutant protein was recorded at various times during the incubation (25 °C, pH 7.0) of the Glu^{367}Gln mutant with a 30-fold molar excess of substrate (BCoA). The binding of BCoA to the mutant enzyme was evident by the immediate red shift of the 450-nm peak and the appearance of shoulders in the flavin spectrum. After 16 h of incubation (Figure 2), the amount of bleaching in the flavin spectrum was only 20% of the bleaching seen instantaneously upon substrate addition to normal cloned SCAD during an identical experiment (Figure 2). In experiments with both mutant and normal cloned SCAD, red anionic semiquinone absorption at 385 nm was evident. HPLC analysis of the Glu^{367}Gln mutant activity was also performed. Over a 24-h time period, very little CCoA was detected on the HPLC chromatograms from the Glu^{367}Gln reaction, while in the normal SCAD reaction a BCoA/CCoA ratio of 1.4:1 was detected on the chromatogram.

To test the oxidative pathway activity of the Glu^{367}Gln mutant, a similar experiment with product (CCoA) was performed. Immediately upon the addition of a 3-fold molar excess of CCoA to the fully reduced Glu^{367}Gln mutant, a protein to ligand charge transfer band was evident at 570 nm. After 16 h of mutant enzyme incubation with CCoA, anion radical absorption at 385 nm appeared and reoxidation was observed by an increase in the absorption at 450 nm. This reoxidation in the mutant, however, was only about 20% of the reoxidation seen immediately after the addition of CCoA to reduced normal cloned SCAD in an identical experiment.

DISCUSSION

Alignment of the deduced cloned SCAD amino acid sequence from *M. elsdenii* with the deduced amino acid sequences of both human SCAD (Naito et al., 1989) and rat

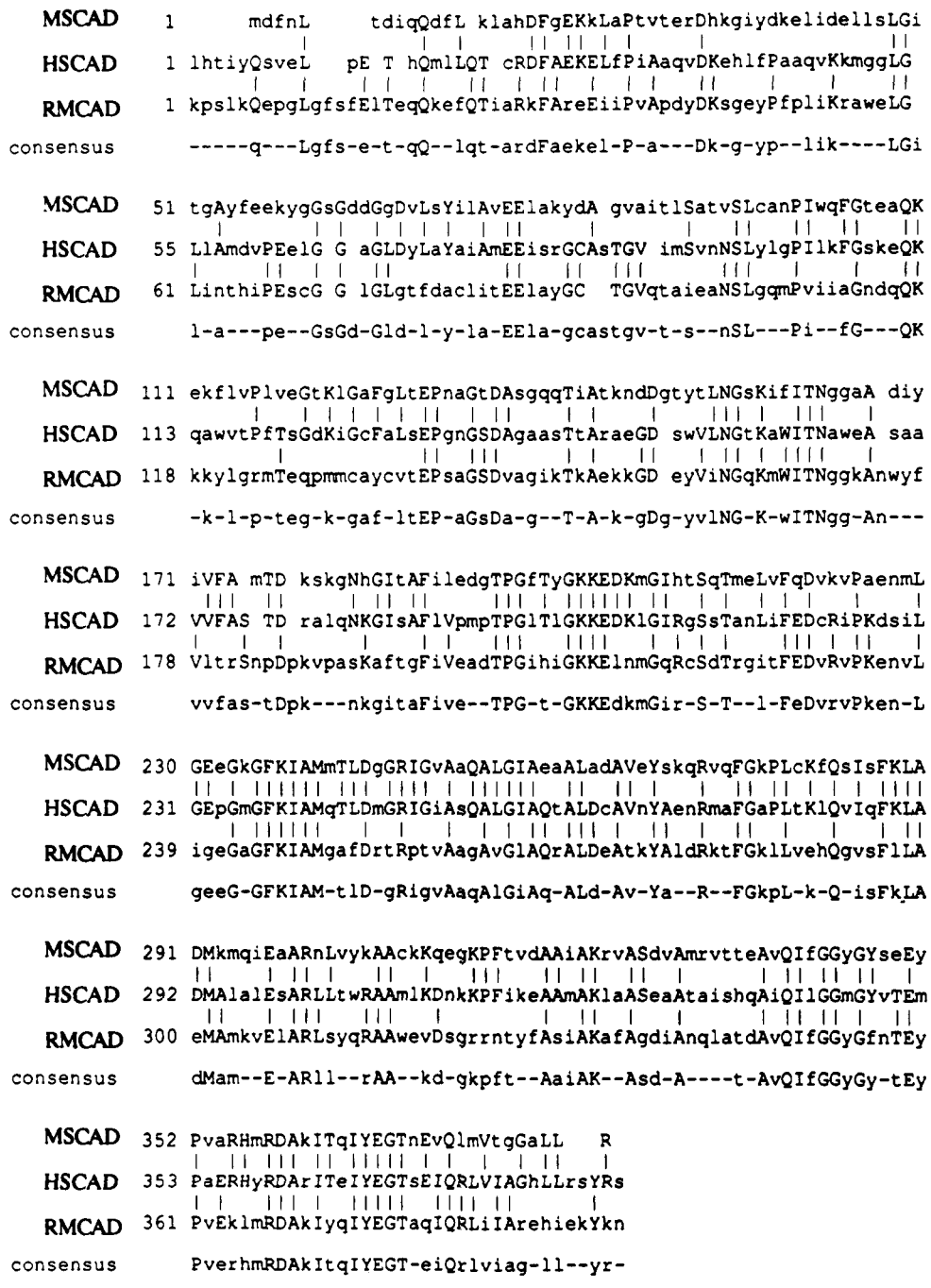

FIGURE 3: Alignment of the amino acid sequences of *M. elsdenii* SCAD (MSCAD), human SCAD (HSCAD), and rat MCAD (RMCAD). Matches in the sequences are connected by lines.

MCAD (Matsubara et al., 1987) is shown (Figure 3). The amino acid sequence of cloned SCAD shares 44% and 36% identical residues in paired comparisons with human SCAD and rat MCAD, respectively. Some of the amino acids in the FAD binding site identified in the X-ray crystal structure of MCAD are either identical or chemically similar in the *M. elsdenii* SCAD sequence. For example, Asp^{253}, Asn^{169}, and Tyr^{375}, which have been identified in the FAD binding region of MCAD, are also present in *M. elsdenii* SCAD (Kim et al., 1992). Tyr^{375} in MCAD is suspected of providing protection to the flavin moiety from oxygen when MCAD is substrate-bound (Ghisla et al., 1992). It is interesting that this Tyr is conserved in *M. elsdenii* SCAD, as substrate-bound SCAD is readily oxidized by oxygen. In addition, Trp^{166}, also in the FAD binding region of MCAD (Kim et al., 1992), is replaced by phenylalanine in the *M. elsdenii* SCAD amino acid sequence, conserving an aromatic residue at that position.

The alignment also shows that Glu^{376} in MCAD is conserved in the *M. elsdenii* SCAD. Glu^{376} in MCAD is thought to be responsible for the initiation of catalytic activity by performing the abstraction of the α-hydrogen (Powell & Thorpe, 1988). *In vitro* substitution of the human MCAD Glu^{376} residue for a Gln produced an inactive protein (Bross et al., 1990). Data from substrate analogue inhibitions of *M. elsdenii* SCAD also suggest the initiation of the α-proton is performed by a Glu (Fendrich & Abeles, 1982). As a result, this Glu identified in the *M. elsdenii* SCAD active site is most likely the Glu^{367} residue. Similar to the variations seen in the N-terminal regions of human SCAD and rat MCAD, the amino acid sequence of *M. elsdenii* SCAD also varies considerably with the human SCAD and rat MCAD in this area. This is not surprising since the N-terminal region is believed to be responsible for substrate specificity and binding (Kim & Wu, 1988).

M. elsdenii SCAD expression in *E. coli* is not increased upon induction with IPTG. It is most likely *M. elsdenii* SCAD expression in *E. coli* depends very little on the *lac* promoter for its production. A Shine–Dalgarno sequence was found 10 bp upstream of the translational start codon of the SCAD gene.

The appearance of the green form of *M. elsdenii* SCAD from the K19 cultures grown for 32 h was surprising. The additional time (8 h) the cells spent in stationary phase apparently raised the level of CoA persulfide inside the cell to an amount sufficient for the generation of the green form of *M. elsdenii* SCAD. A possible explanation for this could be the inability of the cell to regenerate reducing cofactors (e.g., NADPH) due to the shut down of cell metabolism. Proteins such as thioredoxin would then become inactive, resulting in the increased formation of disulfide bonds. As a result, CoA persulfide could then become available to complex with *M. elsdenii* SCAD.

The activities in both the reductive and oxidative pathways of the $Glu^{367}Gln$ SCAD mutant are greatly diminished compared to normal cloned SCAD activities. These data confirm Glu^{367} as the residue responsible for initiating catalytic activity in SCAD (Fendrich & Abeles, 1982). The gradual appearance of red anionic semiquinone (385 nm) during the incubations of both mutant and normal SCAD with either BCoA or CCoA is most likely the result of a comproportionation reaction between FAD and $FADH_2$ (Jiang & Thorpe, 1983; Byron et al., 1990). Red anionic semiquinone has been reported for native SCAD during equilibration with both BCoA and CCoA in 10-fold molar excess under anaerobic conditions (Stankovich & Soltysik, 1987). In contrast, red anionic semiquinone was not observed during the 16-h incubation of the $Glu^{376}Gln$ MCAD mutant with the substrate octanoyl-CoA (Bross et al., 1990). Slow residual mutant SCAD activity in the reductive or oxidative directions would eventually generate a mixture of oxidized and reduced SCAD. Substrate/product-complexed SCAD, however, has a higher affinity for the FAD anionic semiquinone compared to FAD and $FADH_2$. This has been shown through substrate analogue studies with SCAD (C. Pace and M. T. Stankovich, 1993, personal communication) and also with MCAD (B. D. Johnson, 1992, personal communication). As a result, substrate/product-bound SCAD would facilitate the one-electron intermolecular transfer between $FADH_2$ and FAD, forming two anionic semiquinone SCAD species.

Future electrochemical experiments with the $Glu^{367}Gln$ SCAD mutant will determine whether the binding of substrate, product, or both causes the positive shift in the redox potential of SCAD. These experiments will be possible due to the slow intrinsic turnover rate shown for $Glu^{367}Gln$ SCAD. Thus, the direct effects of substrate and product binding on the $Glu^{367}Gln$ SCAD redox properties can be studied without significant contributions from substrate turnover. These experiments are not possible with the human MCAD clone because the $Glu^{376}Gln$ MCAD mutant has a $t_{1/2}$ ($\approx$ 84 min, 5 °C) which is too fast for equilibrium redox measurements (Ghisla et al., 1992).

The fundamental insights gained from the cloning, sequencing, and mutagenesis of *M. elsdenii* SCAD can be used to help generate a model system. Indeed, *M. elsdenii* SCAD is an excellent model for the studies of the redox properties and the regulation of the mammalian acyl-CoA dehydrogenases, specifically MCAD. The structural homology between *M. elsdenii* SCAD and mammalian MCAD is evident when their amino acid sequences are compared. This primary structural homology, along with their similar electrochemical behavior, will allow the molecular analysis of *M. elsdenii* SCAD to aid in the understanding of the structural basis of the regulation of MCAD redox potentials by substrate/product binding.

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