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A Rare Disease-associated Mutation in the Medium-Chain Acyl-CoA Dehydrogenase (MCAD) Gene Changes a Conserved Arginine, Previously Shown to Be Functionally Essential in Short-Chain Acyl-CoA Dehydrogenase (SCAD)

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Summary

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is a serious and potentially fatal inherited defect in the β-oxidation of fatty acids. Approximately 80% of patients with MCAD deficiency are homozygous for a single disease-causing mutation (G985). The remaining patients (except for a few cases worldwide) are compound heterozygous with G985 in one allele. By sequencing of cloned PCR-amplified MCAD cDNA from a G985 compound heterozygous patient, we identified a C-to-T transition at position 157 as the only change in the entire coding sequence of the non-G985 allele. The presence of the T157 mutation was verified in genomic DNA from the patient and her mother by a PCR-based assay. The mutation changes a conserved arginine at position 28 (R28C) of the mature MCAD protein. The effect of the T157 mutation on MCAD protein was investigated by expression of mutant MCAD cDNA in *COS-7* cells. On the basis of knowledge about the three-dimensional structure of the MCAD protein, we suggest that the mutation destroys a salt bridge between arginine[28] and glutamate[86], thereby affecting the formation of enzymatically active protein. Twenty-two additional families with compound heterozygous patients were tested in the PCR-based assay. The T157 mutation was identified in one of these families, which had an MCAD-deficient child who died unexpectedly in infancy. Our results indicate that the mutation is rare. It is, however, noteworthy that a homologous mutation has previously been identified in the short-chain acyl-CoA dehydrogenase (SCAD) gene of a patient with SCAD deficiency, suggesting that the conserved arginine is crucial for formation of active enzyme in the straight-chain acyl-CoA dehydrogenases.

Introduction

Medium-chain acyl-CoA dehydrogenase (MCAD) (E.C.1.3.99.3) is one of four straight-chain acyl-CoA dehydrogenases which catalyze the first dehydrogenation step in the β-oxidation of fatty acyl-CoA esters in mitochondria (Beinert 1963; Izai et al. 1992). Human MCAD is encoded by a nuclear gene as a precursor protein of 421 amino acids with a 25-amino-acid leader peptide (Kelly et al. 1987). On import into mitochondria, the leader peptide is cleaved, producing the mature MCAD monomer (42.5 kD). In the mitochondria, the monomers are assembled to the functional homotetrameric MCAD enzyme, with one molecule of flavin adenine dinucleotide attached per subunit (Thorpe et al. 1979). MCAD and short-chain acyl-CoA dehydrogenase (SCAD), long-chain acyl-CoA dehydrogenase (LCAD), isovaleryl-CoA dehydrogenase (IVD), and 2-

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methyl-branched-chain-acyl-CoA dehydrogenase (2-meBCAD) constitute a gene family (Tanaka et al. 1990).

MCAD deficiency is a serious autosomal recessive inherited disorder (Roe and Coates 1989). The disease was first described in 1976 (Gregersen et al. 1976) and was enzymatically characterized in 1982–83 (Kølvraa et al. 1982; Rhead et al. 1983; Stanley et al. 1983). The disease manifests itself in periods of metabolic stress to the β-oxidation system and may be fatal (Roe and Coates 1989). The clinical picture of MCAD deficiency is diverse, ranging from severe episodes of hypoglycemia, lethargy, and excretion of C_6-C_{10}-dicarboxylic acids to patients who remain symptom free for years (Duran et al. 1986).

A breakthrough in the elucidation of the disease came with the identification of a prevalent mutation (G985) leading to a shift from lysine to glutamic acid at position 304 (K304E) in the mature MCAD (Kelly et al. 1990; Matsubara et al. 1990; Yokota et al. 1990; Gregersen et al. 1991*a*). The disease-causing nature of the mutation was strongly indicated by expression of K304E mutant MCAD in *Escherichia coli* (Bross et al. 1990*b;* Gregersen et al. 1991*a*) and in *COS-7* cells (Jensen et al. 1992). It was shown that the mutation leads to a drastic decrease in enzyme activity, probably caused by instability due to a defective folding of the MCAD monomer and/or a defect in assembly of the functional tetrameric enzyme (Bross et al. 1990*b;* Jensen et al. 1992; Yokota et al. 1992). In fact, recent experiments have suggested that, when the mutant tetrameric enzyme is formed, it is fully active (Yokota et al. 1992; P. Bross, unpublished data). The G985 mutation is present in more than 90% of disease-causing MCAD alleles, and approximately 80% of the patients are homozygous for this mutation (Gregersen et al. 1991*b;* Yokota et al. 1991). The disease can, in the great majority of cases, be diagnosed by a simple PCR-based assay for this mutation. But from a diagnostic point of view it is still important to identify other disease-causing mutations in the remaining 20% of the patients and to determine whether any of these mutations are prevalent. Furthermore, identification of new mutations and characterization of their biochemical consequences should contribute to a better understanding of structure-function relationships in the MCAD enzyme and thereby also shed some light on the observed variations in the clinical symptoms experienced by patients. Until now only six mutations, which are all very rare, have been reported in compound heterozygous patients with MCAD deficiency (Yokota et al. 1991; Ding et al. 1992; Kelly et al. 1992).

Here we report the identification and characterization of another infrequent disease-associated mutation (T157) in a patient with verified MCAD deficiency. Furthermore, we report the identification of this mutation in an unrelated family with an infant with MCAD deficiency who died unexpectedly in infancy.

Patients and Methods

Patients

Index patient.—The case history of the index patient has been published previously (Duran et al. 1985; patient 4). The patient exhibited all the clinical symptoms of MCAD deficiency (Duran et al. 1985), and the MCAD enzyme defect was proved by assaying disrupted lymphocytes for specific MCAD activity by using octanoyl-CoA as substrate (Duran et al. 1992). The MCAD activity of the patient was in the same range as was seen for MCAD patients homozygous for the G985 mutation, and the MCAD activities of both parents were in the heterozygote range (table 1). Furthermore, intact cultured fibroblasts from the index patient were tested for β-oxidation activity with (1-^{14}C)-octanoate as substrate (Duran et al. 1985), as described elsewhere (Saudubray et al. 1982), and with ^{3}H-myristic acid as substrate (E. Christensen, personal communication), essentially as described elsewhere (Manning et al. 1990). β-Oxidation activity observed in intact fibroblasts from the index patient was higher than that in patients who are homozygous for the G985 mutation (table 1).

Patient 2.—After a normal pregnancy and delivery, this patient died unexpectedly on day 3 after birth. The diagnosis of MCAD deficiency was indicated by gas chromatography/mass spectrometry (GC/MS) after identification, in the urine, of metabolites specific for MCAD deficiency.

Preparation of DNA

Genomic DNA was isolated from blood samples and from cultured skin fibroblasts by standard methods (Gustafson et al. 1987). DNA from blood spots was liberated as described elsewhere (Gregersen et al. 1991*b*).

Northern Blot Analysis and MCAD cDNA Synthesis

Total RNA was prepared from frozen skin fibroblasts or transfected *COS-7* cells by the method of Chomczynski and Sacchi (1987), and northern blot analysis was performed essentially as described by Gregersen et al. (1991*a*). From fibroblast total RNA, first-

Table 1

Specific MCAD Activity and β-Oxidation Activity

	Specific MCAD Activity: GC/MS in Disrupted Lymphoblasts[a] (nmol/min/mg protein)	β-Oxidation Activity	
		$^{14}CO_2$ Release in Intact Fibroblasts[b] (nmol/10^6/min)	Tritium Release in Intact Fibroblasts[c] (pmol/min/mg protein)
Index patient	.3	.63	4.9
Father	1.7	Not done	Not done
Mother	1.9	Not done	Not done
G985 homozygote	.1–.3 (n = 5)	.21–.26 (n = 4)	1.7–2.4 (n = 4)
Controls	3.1–3.8 (n = 15)	.91–4.17 (n = 16)	10.0–28.2 (n = 19)

[a] Measurement was performed as described by Duran et al. (1992).
[b] Measurement was performed as described by Saudubray et al. (1982).
[c] With ^{3}H-myristic acid as substrate. Measurement was performed essentially as described by Manning et al. (1990).

strand cDNA was synthesized as described elsewhere (Gregersen et al. 1991*a*).

PCR Amplification of First-Strand MCAD cDNA, Cloning, and Sequence Analysis

PCR amplification of a 1,359-bp-long fragment covering the entire protein coding region of MCAD cDNA was performed essentially as described elsewhere (Gregersen et al. 1991*a*), by using recombinant *Taq* polymerase (Perkin Elmer Cetus, Norwalk, CT) and an automated thermal cycler (Perkin Elmer Cetus). The only difference was that, instead of the original 5′ primer (p-18), which covered 12 bp of the protein coding region, a primer (p-38), located farther upstream, was used. The p-38 primer (5′-CCGTGTATTAGGATCC-GAGTGGCC-3′) covers the nucleotides from position −38 to position −15, introducing a *Bam*HI restriction site. The amplified product of the expected length was cloned into a pGEM4z (Promega, Madison) vector and was sequenced with a Sequenase sequencing kit (USB, Cleveland).

PCR-based Assays for the Two Mutations

The PCR-based assays for the G985 mutation and for the T157 mutation are both based on the principle of PCR-mediated mutagenesis creating new restriction-enzyme sites enabling discrimination between amplified mutation-bearing alleles and amplified normal alleles.

G985 assay.—The G985 assay was performed exactly as described elsewhere (Gregersen et al. 1991*b*).

T157 assay.—PCR amplification using two primers, p132 (sense strand) and p216 (antisense strand), produced an 84-bp-long fragment. The p132 primer covering nt 132 to nt 156, with GA instead of CT at positions 152 and 153 (5′-GCAGAAAGAATTTCAAGCTA-GAGCT-3′), creates a *Sac*I site (GAGCTC) in the amplification product from normal alleles but not in the product from alleles harboring the T157 mutation. The p216 primer covering nt 216 to nt 190 (5′-TTC-ACCAGTGAGCTCATATTCTGCAGC-3′), with GAGC instead of ATTT at positions 207 to 204, introduces a control *Sac*I site in all amplification products.

The PCR was carried out with either genomic DNA, cDNA, or boiled blood spots as DNA source in 100 μl, for 35 cycles, with 15 pmol of each primer, 2 units of recombinant *Taq* polymerase, and the following program: 95°C for 1 min, 50°C for 1 min, and 74°C for 2 min. In the last cycle the polymeration was for 10 min at 74°C. After digestion with *Sac*I, samples were electrophoresed on 12% polyacrylamide gels, together with uncleaved samples. The bands were visualized by staining with ethidium bromide.

RFLP Analysis

RFLP analysis was performed by Southern blotting of 10 μg genomic DNA digested with the restriction enzymes *Ban*II, *Pst*I, and *Taq*I. The banding pattern observed with a ^{32}P-dCTP-labeled full-length MCAD cDNA probe was denoted as described elsewhere (Kølvraa et al. 1991).

Expression of Recombinant MCAD in COS-7 Cells

A 1,341-bp-long MCAD cDNA fragment coding for the entire MCAD precursor protein but harboring the

T157 mutation was cut out, by *Bam*HI and *Hin*dIII, from one of the plasmids, which had no PCR-derived errors. After the recessed termini were filled in, the fragment was blunt-end ligated into the unique *Xho*I cloning site located between the SV40 promoter and SV40 processing signals of the expression plasmid pTPS (Jensen et al. 1992). Transfection of *COS-7* cells was performed by using a calcium phosphate coprecipitation method (Graham and van der Eb 1973). Expression of MCAD protein was assayed from hygromycin B–selected cells transfected with expression vectors harboring wild-type, G985, or T157 MCAD cDNA. Construction of the expression vectors with wild-type or G985 MCAD cDNA, as well as the conditions for growth and transfection of cells, have been described elsewhere (Jensen et al. 1992).

Determination of the concentration of protein in *COS-7* cell extracts was performed with a modified Bradford assay kit from BioRad (BioRad, Richmond, CA). To test whether expression of the MCAD cDNAs was successful, aliquots from the transfected *COS-7* cells were tested by western blot analysis, as described elsewhere (Bross et al. 1990*a;* Jensen et al. 1992). Before MCAD activity measurements were performed, the samples were lysed by sonication and were cleared from cell debris by centrifugation at 3,000 *g*. The activity measurements in the 3,000-*g* supernatant samples were performed by a method described elsewhere (Kølvraa et al. 1982). In addition, MCAD activity measurements in the 3,000-*g* supernatants from the transfected *COS-7* cells were performed with a ferricenium ion–based assay (Lehman et al. 1990).

Western Blot Analysis of Fibroblasts

Western blot analysis of protein extracts from cultured fibroblasts was performed as described elsewhere (Jensen et al. 1992).

Results

G985 Assay

The index patient and her father were heterozygous for the G985 mutation (fig. 1, lanes 1 and 2). The mother does not possess the G985 mutation (fig. 1, lane 3). The patient might therefore be a compound heterozygote, with the G985-bearing allele received from her father and with an MCAD allele, bearing another mutation, received from her mother.

The results of the G985 assay performed on full-length amplified MCAD cDNA from the patient (fig. 1, lane 4) and her parents (fig. 1, lanes 5 and 6) indicated

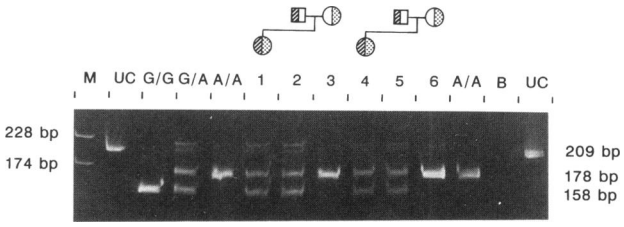

Figure 1 PCR-based assay for the G985 mutation in the index patient and her parents: ethidium bromide–stained polyacrylamide gel after electrophoresis of *Sty*I-digested and undigested PCR products. Lane M, Marker. Lane UC, Uncleaved PCR product. Lane G/G, G985-homozygous control. Lane G/A, G985-heterozygous control. Lane A/A, Normal control. Lane 1, Index patient's genomic DNA. Lane 2, Father's genomic DNA. Lane 3, Mother's genomic DNA. Lane 4, Index patient's amplified full-length MCAD cDNA. Lane 5, Father's amplified full-length MCAD cDNA. Lane 6, Mother's amplified full-length MCAD cDNA. Lane A/A, Normal control. Lane B, Blank amplification. Uncleaved PCR product is 209 bp. Cleaved G985 mutation–bearing fragments are 158 bp, and cleaved fragments with normal sequence are 178 bp.

that both MCAD alleles were transcribed at approximately the same level. Taken together with the fact that northern blot analysis of fibroblast samples from the patient and family members revealed no abnormal bands or degradation (results not shown), this indicates that transcription of both of the patient's MCAD alleles results in the production of an MCAD mRNA of normal length.

The father of patient 2 was heterozygous for the G985 mutation (results not shown), indicating that patient 2 may also be heterozygous for this mutation. Neither the mother nor an older healthy brother possessed the G985 mutation.

Western Blot

Although western blot analysis for MCAD protein was performed repeatedly on fibroblasts from the index patient, from patients homozygous for the G985 mutation, and from controls, the results were not consistent. A band of the correct size (42.5 kD) was always observed in all persons. The intensity of the band, however, varied, but it was always weaker than normal in fibroblasts from patients homozygous for the G985 mutation, as well as in the fibroblasts from the index patient. We and others have previously shown that the mutant K304E MCAD protein from G985-homozygous patients is unstable (Coates et al. 1992; Jensen et al. 1992) and therefore sometimes escapes detection by western blot analysis. Therefore we believe that, if the non-K304E mutant protein is produced, it is indistin-

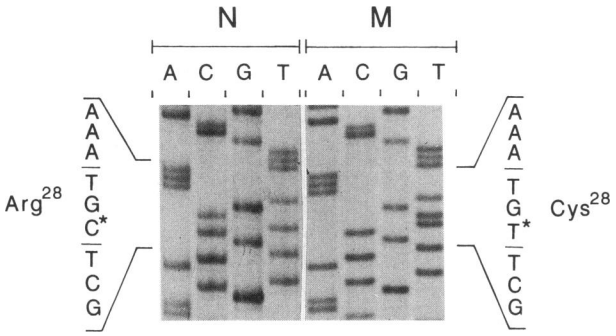

Figure 2 Sequence analysis of the cloned amplified MCAD cDNA from the index patient (M) and a normal control (N). The C-to-T mutation at position 157 is marked with an asterisk.

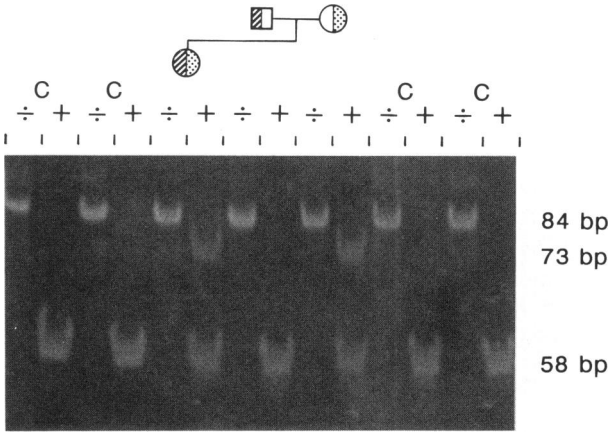

Figure 3 PCR-based assay for the T157 mutation: ethidium bromide–stained polyacrylamide gel after electrophoresis of *Sac*I-digested and undigested PCR products from amplification of genomic DNA. The PCR products were either loaded directly (lanes ÷) or digested with *Sac*I before being loaded (lanes +). The index patient and her parents are illustrated by a pedigree, and the normal controls are indicated by "C." Uncleaved PCR product is 84 bp, cleaved T157 mutation–bearing fragments are 73 bp, and cleaved fragments with normal sequence are 58 bp.

guishable, in size, from K304E MCAD, and its stability may also be drastically reduced.

Cloning and Sequence Analysis of PCR-amplified MCAD cDNA

A total of 10 clones harboring the sequence coding for the MCAD precursor protein from the index patient were analyzed by sequencing. Only two consistent mutations were observed in the clones: the A-to-G transition at position 985 (i.e., the G985 mutation) and a C-to-T transition at position 157 (i.e., the T157 mutation; see fig. 2). It is surprising that the clones seemed to represent four different "alleles": one of the clones contained both mutations, four had only the T157 mutation, three contained only the G985 mutation, and two had the wild-type sequence. Because the PCR-based assays for the G985 and T157 (see below) mutations showed that the two mutations are present individually in each of the parents (table 2), it is obvious that they are situated on separate alleles. Therefore, the clones carrying both or neither of the two mutations must have been generated by PCR-mediated recombination, probably caused by nicked templates acting as primers in the PCR (Meyerhans et al. 1990; Marton et al. 1991). This observation emphasizes the importance of analyzing genomic DNA from family members for the respective mutations, to distinguish between alleles with double mutations and compound heterozygosity.

Table 2

Mutation Status in Index Patient and Her Parents

	Father	Mother	Patient
G985 assay	G/A	A/A	G/A
T157 assay	C/C	C/T	C/T
Haplotype:			
*Ban*II	1/2	1/1	1/1
*Pst*I	1/1	1/1	1/1
*Taq*I	2/1	2/1	2/1

Haplotyping of the Family

RFLP analysis of the patient and her parents by *Ban*II, *Pst*I, and *Taq*I did not reveal any gross rearrangements in the MCAD gene of either the patient or her parents. The haplotypes of the two MCAD alleles in the patient are 1-1-2 and 1-1-1 (table 2). We have previously reported that the G985 mutation exclusively occurs on MCAD alleles with the 1-1-2 haplotype (Kølvraa et al. 1991). The data suggest that the T157 mutation has occurred on the MCAD allele with the 1-1-1 haplotype. The haplotypes of the parents are consistent with this argument. The results of the haplotype analysis and the PCR-based assays for the G985 and T157 mutations (see below) are summarized in table 2.

PCR-based Assay for the T157 Mutation

The presence of the T157 mutation in genomic DNA was confirmed in the index patient. As expected, the mutation was also present in genomic DNA from her mother but not in that from her father (fig. 3). The assay

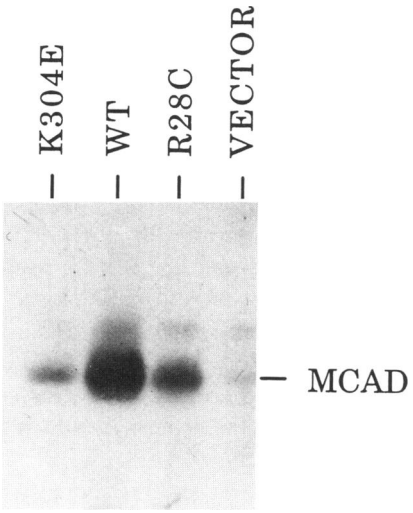

Figure 4 Expression of wild-type and mutant MCAD in *COS-7* cells: western blot of *COS-7* cells harboring expression vectors encoding G985 mutant (K304E), wild-type (WT), and T157 mutant (R28C) MCAD protein. The control is *COS-7* cells harboring the expression vector pTPS with *no* MCAD cDNA insert (VECTOR). Material corresponding to approximately 2.5×10^5 cells was loaded in each lane.

was also performed on full-length amplified MCAD cDNA from all family members (results not shown), and, as observed with the G985 assays, the results indicated that there are no significant differences in the level of MCAD mRNA transcribed from the wild-type allele, the G985-bearing allele, and the T157-bearing allele. Genomic DNA from 31 control persons (62 alleles) with different MCAD haplotypes (two of them were homozygous for the 1-1-1 haplotype, which is the haplotype of the chromosome harboring the T157 mutation in the index patient) was also tested, and it did not possess the T157 mutation.

By testing 21 patients and family members from 22 families with compound heterozygous patients with diagnosed MCAD deficiency, we identified the T157 mutation in the mother of another presumed G985-compound-heterozygous patient with MCAD deficiency (patient 2). Moreover, the T157 mutation was identified in genomic DNA obtained from both sibs of the mother of patient 2. The T157 mutation was not present either in DNA from the father or in DNA from an older, healthy brother. Unfortunately, it has not yet been possible to verify the presumed compound heterozygosity for the G985 and T157 mutations in patient 2. None of the remaining compound-heterozygous patients (including one patient with the 1-1-1 haplotype) had the T157 mutation.

Expression of Recombinant MCAD in COS-7 Cells

Northern blot analysis indicated that the level of MCAD mRNA present in *COS-7* cells transfected with the expression vector with wild-type or mutant MCAD cDNA inserted did not vary significantly and that it was approximately 100-fold higher than that of the endogenous MCAD mRNA (not shown). Western blot analysis showed that immunoreactive protein with a size corresponding to that of mature MCAD protein (42.5 kD) was expressed from all three expression vectors with the different MCAD cDNA inserts and that only tiny amounts of immunoreactive protein, representing the endogenous MCAD protein, were present in cells harboring the expression vector *without* MCAD cDNA inserted (fig. 4). The amount of MCAD protein observed in cells expressing the K304E mutant protein was much lower than that observed in the cells expressing the wild-type MCAD protein. However, a substantial amount of the R28C protein was present, although it was still less than the amount seen in wild-type transfected cells. These results suggest that the two mutant proteins are labile/unstable (although not exactly to the same extent).

Measurement of the MCAD activity in *COS-7* cells revealed that the R28C mutation, like the K304E mutation, results in decreased enzyme activity when compared with wild-type protein (table 3). The residual activity observed in cells expressing the R28C mutant protein is nearly twofold higher than the residual activity observed in cells expressing the K304E mutant protein and is about half that of the wild-type protein (table 3).

Discussion

MCAD deficiency is an autosomal recessively inherited disease. The index patient must therefore be a

Table 3

MCAD Enzyme Activity in *COS-7* Cells

Cell Type	MCAD Enzyme Activity[a] (nmol/mg/h)
Vector	478
Wild type	10,574
G985 mutant	2,394
T157 mutant	5,463

[a] Mean of two readings, measured by the "product-formation-assay" (Kølvraa et al. 1982) performed on the 3,000-*g* supernatants. The results were verified by measuring the same samples with the ferricenium ion assay (Lehman et al. 1990).

compound heterozygote, since she and her father were both heterozygous for the disease-causing G985 mutation. The G985 mutation was not present in the mother. No gross rearrangement in the MCAD gene of the patient or her parents could be detected. Analysis of MCAD mRNA and MCAD cDNA from the patient and her parents established that MCAD mRNA of normal length was present and that both of the MCAD alleles were transcribed at approximately the same level. These data therefore suggest that the defect of the non-G985–bearing allele is a point mutation in the protein-coding region.

Sequence analysis of MCAD cDNA clones, together with analysis of genomic DNA, revealed that a C-to-T transition at position 157 (T157 mutation) was the only consistent change in the entire protein-coding region of the non-G985 allele from the patient. On the basis of this, and because we could show that the father and the mother were carriers of the G985 and the T157 mutations, respectively, we conclude that the index patient is a compound heterozygote with the G985 mutation in one allele and the T157 mutation in the other allele.

The T157 mutation could be expected to affect the protein, because it results in a rather drastic change—i.e., from arginine, which has a positively charged and bulky side chain, to cysteine, which has a smaller and uncharged side chain at position 28 (R28C)—in the mature MCAD protein. Our experiments with expression of mutant MCAD in *COS-7* cells showed that the T157 mutation, in fact, leads to a decrease in MCAD enzyme activity, corresponding to about half the activity of the wild type. The fact that the MCAD activity decrease resulting from the T157 mutation is not as pronounced as the decrease caused by the G985 mutation corresponds well with the MCAD activity measurements in intact patient fibroblasts. Here it was observed that the index patient had a high residual MCAD activity when compared with patients who are homozygous for the G985 mutation, suggesting that the molecular defect caused by the T157 mutation might be milder than the defect resulting from the G985 mutation (table 1). This may seem to be in conflict with the measurements of the specific MCAD activity in lymphocytes from the index patient, in which she could not be distinguished from patients homozygous for the G985 mutation. Furthermore, measurements of specific MCAD activity in lymphocytes from her parents showed that the T157 and G985 mutations may cause an identical decrease in MCAD activity in carriers (table 1).

An explanation for some of the variability that we have observed in MCAD activity in the different experiments/systems could be that the molecular defect caused by the T157 mutation does not manifest itself as seriously in intact cells as it does in disrupted cells. This observation may give clues to the molecular disease mechanism, and it is being investigated.

The observed very low amount of immunodetectable protein in fibroblasts from the index patient may indicate that the T157 mutation, like the G985 mutation (Coates et al. 1992; Jensen et al. 1992), results in production of a labile MCAD protein. This is also supported by our finding of decreased amounts of immunodetectable MCAD in the *COS-7* expression experiments. It could therefore be speculated that the R28C mutation affects the process by which functional MCAD enzyme protein is formed.

On the basis of the three-dimensional structure at 3-Å resolution (Kim and Wu 1988), arginine^{28} is localized in the last part of helix A in the MCAD monomer, close to helix D (fig. 5, *inset*). This is distant (30 Å) from both the active site and the sites where the cofactor and substrate bind. It is therefore not likely that the mutation directly affects the catalytic mechanism of the enzyme. Arginine^{28} is located at the surface of the tetrameric molecule, and, when this part of the three-dimensional structure is analyzed at 2.2-Å resolution (J.-J. Kim, unpublished results), it is found that its positively charged side chain is pointing toward the negatively charged side chain of glutamate^{86} in helix D, with the two side chains being only 3.1 Å apart (fig. 5). A salt bridge between arginine^{28} and glutamate^{86} is thus likely to exist. This salt bridge could be important in generating or maintaining the correct three-dimensional configuration of the MCAD monomer, probably by assisting in keeping the helices A and D close. The replacement of arginine^{28} by cysteine results in loss of the positive charge, which is necessary for the salt bridge to glutamate^{86}. In addition, the resulting change in hydrophilicity might be important energetically for the formation of functional tetrameric enzyme, as arginine^{28} is located at the surface of the molecule. This could explain the low amount of mutant MCAD present in fibroblasts from the index patient.

Comparison of the deduced amino acid sequences of the three straight-chain acyl-CoA dehydrogenases SCAD, MCAD, and LCAD from human and rat (Kelly et al. 1987; Matsubara et al. 1987, 1989; Naito et al. 1989*b*; Indo et al. 1991) reveals that both arginine^{28} and glutamate^{86} are conserved in all. It is interesting that a mutation changing the conserved arginine to tryptophan (R22W) in SCAD is associated with lack of enzyme activity in a patient with SCAD deficiency (Naito

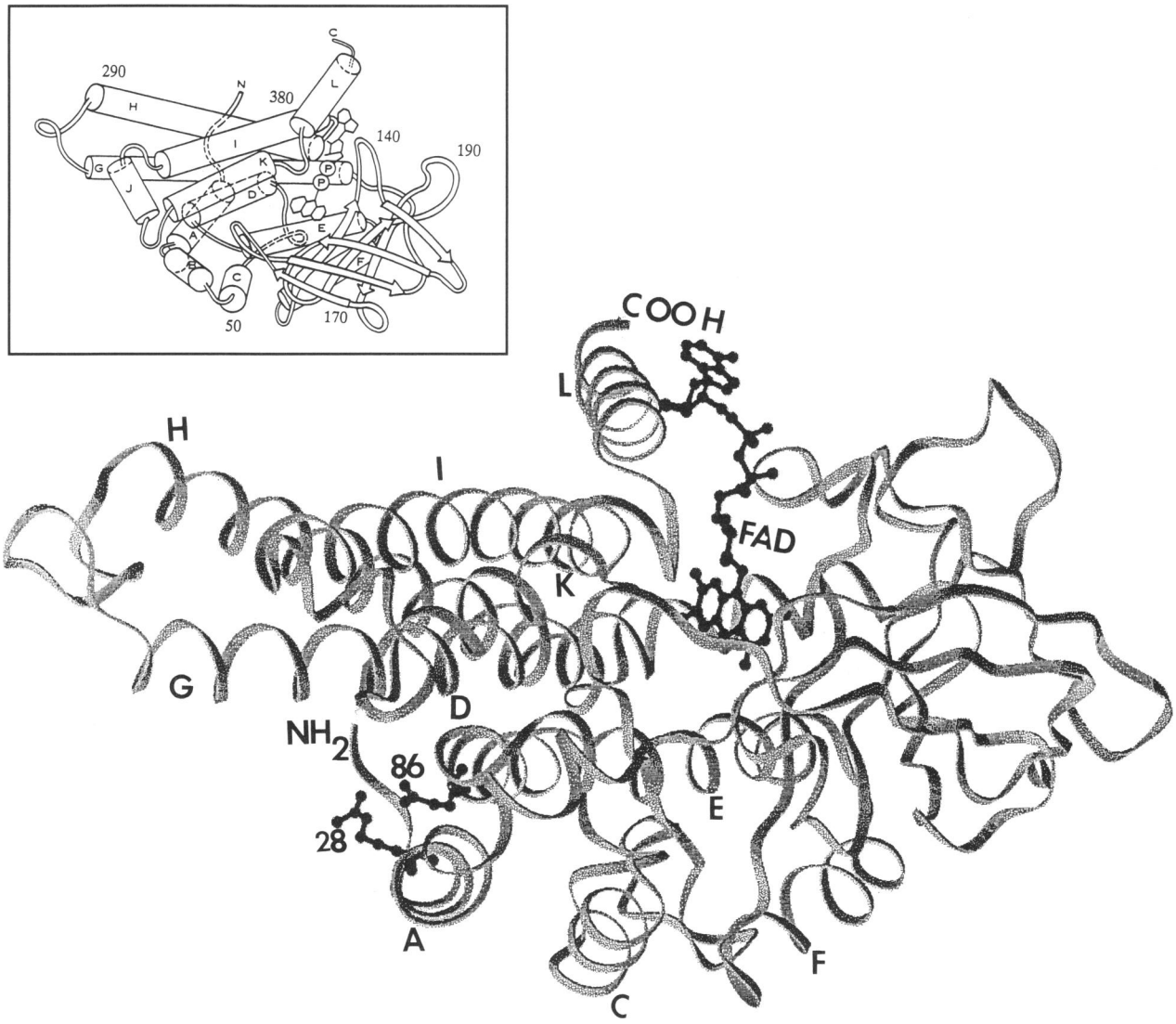

Figure 5 Structure of the MCAD monomer. The ribbon drawing of the MCAD monomer is based on the crystal structure at 2.2-Å resolution, with the flavin adenine dinucleotide and the side chains of arginine[28] and glutamate[86] drawn in. The α-helices are lettered sequentially from the NH_2 terminus, and the positions of the NH_2 and COOH termini are indicated. The inset shows a schematic backbone drawing of the MCAD monomer (Kim and Wu 1988). The cylinders in the inset represent α-helices, and the flat arrows represents β-sheets. The ribbon drawing of the MCAD monomer is slightly tilted (approximately 15%) as compared with the inset figure.

et al. 1990). Combined immunoblot analysis and pulse-chase experiments of cultured fibroblasts from this patient showed that the R22W mutant SCAD, like R28C MCAD, is unstable (Naito et al. 1989*a*, 1989*b*). This indicates that the effect of a mutation in the conserved arginine is similar in both MCAD and SCAD, regardless of the fact that the change is to cysteine in MCAD and to tryptophane in SCAD.

Thus, we believe that the conserved arginine may be important for formation of correct enzyme structure in the straight-chain acyl-CoA dehydrogenases. In this context, it is worth noting that both of our two characterized disease-associated mutations in MCAD (i.e., G985 and T157) seem primarily to affect the formation of correct enzyme structure and not directly the catalytically active regions of the enzyme.

That the T157 mutation is only present in 2 of 23 families with compound-heterozygous patients with MCAD deficiency indicates that it is rare. The PCR-based assay for this mutation could thus not be ex-

pected to contribute significantly to a better diagnostic efficiency for MCAD deficiency. Other investigators' observation of six infrequent mutations (Yokota et al. 1991; Ding et al. 1992; Kelly et al. 1992), as well as the fact that the T157 mutation also is apparently infrequent, together with our finding that the investigated compound-heterozygous patients possess several different RFLP haplotypes, indicates that the mutational spectrum in the compound-heterozygous patients with MCAD deficiency consists of many different mutations. It is important to keep these observations in mind when one is considering a better diagnostic strategy for the approximately 20% of compound-heterozygous patients. Despite the apparent rarity of T157, we find that the identification of this mutation is interesting, because it points to an important conserved arginine residue, which is crucial for formation of enzymatically active MCAD and SCAD, thereby contributing to the understanding of common characteristics of the straight-chain acyl-CoA dehydrogenases.

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