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The molecular basis of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in compound heterozygous patients: is there correlation between genotype and phenotype?

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Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is the most commonly recognized defect of mitochondrial β-oxidation. It is potentially fatal, but shows a wide clinical spectrum. The aim of the present study was to investigate whether any correlation exists between MCAD genotype and disease phenotype. We determined the prevalence of the 14 known and seven previously unknown non-G985 mutations in 52 families with MCAD deficiency not caused by homozygosity for the prevalent G985 mutation. This showed that none of the non-G985 mutations are prevalent, and led to the identification of both disease-causing mutations in 14 families in whom both mutations had not previously been reported. We then evaluated the severity of the mutations identified in these 14 families. Using expression of mutant MCAD in *Escherichia coli* with or without co-overexpression of the molecular chaperonins GroESL we showed that five of the missense mutations affect the folding and/or stability of the protein, and that the residual enzyme activity of some of them could be modulated to a different extent depending on the amounts of available chaperonins. Thus, some of the missense mutations may result in relatively high levels of residual enzyme activity, whereas the mutations leading to premature stop codons will result in no residual enzyme activity. By correlating the observed types of mutations identified to the clinical/biochemical data in the 14 patients in whom we identified both disease-causing mutations, we show that a genotype/phenotype correlation in MCAD deficiency is not straightforward. Different mutations may contribute with different susceptibilities for disease precipitation, when the patient is subjected to metabolic stress, but other genetic and environmental factors may play an equally important role.

INTRODUCTION

Medium-chain acyl-CoA dehydrogenase (MCAD; EC 1.3.99.3) is one of four chain length specific, straight-chain acyl-CoA dehydrogenases which initiate the mitochondrial β-oxidation of

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straight-chain acyl-CoA esters (1,2). The human MCAD gene consists of 12 exons that span more than 44 kb encoding a precursor protein of 421 amino acids (3,4). The first 25 amino acids in the human enzyme are suggested to make up the leader peptide (5). On import into mitochondria, the leader peptide is cleaved off, producing the mature MCAD monomer (42.5 kDa). In the mitochondria, folding of the MCAD monomer is assisted by the chaperonin, mitochondrial heat shock protein 60 (Hsp60) (6). Subsequently the monomers are assembled to the functional homotetrameric MCAD enzyme, with one molecule of flavin adenine dinucleotide (FAD) per subunit (7,8). The three dimensional structure of the MCAD enzyme from pig liver has been characterized by X-ray crystallography (9).

MCAD deficiency is the most common defect of mitochondrial β-oxidation in humans (10,11). It is a potentially fatal, autosomal recessively inherited defect, which most often presents in the first years of life (11–13).

The clinical manifestations of MCAD deficiency are diverse, but usually they include fasting induced non-ketotic hypoglycemia with lethargy which may develop into coma (11,13). Biochemically the disease is characterized by urinary excretion of C_6–C_{10} dicarboxylic acids, acylglycine and acylcarnitine conjugates [hexanoylglycine, phenylpropionylglycine, suberylglycine and octanoylcarnitine (11,14,15)]. Between 20 and 25% of patients die suddenly at first presentation of the disease (13,16), but affected patients, who remain without symptoms for years have also been reported (15–18).

Part of the explanation for this wide clinical spectrum is that fasting stress, often in connection with fever, is usually required to trigger the disease. Differences in MCAD genotype could also be important for the disease manifestation. However, it is well known that the entire clinical spectrum of MCAD deficiency can be observed among patients who are homozygous for the prevalent G985 mutation (13,16; unpublished results). This clearly shows that the most common MCAD genotype—80% of patients are homozygous for the G985 mutation (19,20)—cannot be correlated with a specific disease phenotype. Despite this, it could still be speculated that patients with other MCAD genotypes exhibit a more distinct correlation between MCAD genotype and clinical phenotype.

Unfortunately, little is known about the prevalence of, and the disease phenotype associated with the different non-G985 mutations in MCAD deficiency. So far 14 non-G985 mutations have been reported (15,18,21–24), but only a small number of patients have been tested for all of them.

Due to the very variable clinical spectrum of the disease, much effort has been directed towards elucidating the molecular cell pathology in MCAD deficiency. In particular the molecular defect of the mutant protein (K304E) resulting from the prevalent G985 mutation has been investigated (6,25–28), but also three other missense mutations have been studied (18,25–27,29). These experiments showed that the folding and/or assembly of these four mutant MCAD proteins is compromised, and that this to a variable extent can be modulated by increasing the amount of available chaperonins. In addition, recent experiments have shown that the K304E mutant protein resulting from the G985 mutation is temperature sensitive. Both in human lymphoblasts and in expression experiments in *Escherichia coli* the folding of the K304E mutant protein could be improved by decreasing the temperature (26).

On this basis, we have suggested that interindividual variation of endogenous factors, especially those of the mitochondrial folding machinery, may be important in determining the susceptibility to disease precipitation (10,26,27).

On the other hand, Brackett and co-workers (21) have stated that compound-heterozygosity with the G985 mutation and one of the non-G985 mutations, A583, gives rise to a particularly severe presentation of the disease. Based on this one might speculate whether in some instances the nature of the non-G985 mutation may in itself be sufficient to explain the phenotypic expression of MCAD deficiency in patients who are not homozygous for the G985 mutation.

In the present study we have investigated if and to what extent a correlation between MCAD genotype and clinical phenotype can be made. We have determined the prevalence of all the 14 known and seven new mutations in members from 52 unrelated families with MCAD deficiency who all harbor one or two non-G985 allele(s), using a combination of mutation-specific PCR/restriction enzyme digestion based assays and direct sequencing of PCR amplified DNA. Then we characterized the molecular consequences of the identified mutations using our *E.coli* based expression system with or without co-overexpression of the chaperonins GroEL and GroES (25–27). Finally, we related the MCAD genotype to the observed clinical phenotype in the 14 patients in whom we identified non-G985 mutations in the present study.

RESULTS

Identification of mutations

We used PCR/restriction enzyme digestion based mutation specific assays to test for five of the known non-G985 mutations located outside exon 11, namely T157, Δ343-48, A447, C730 and A799 (20,24,29,30). The A583 mutation located in exon 7 (21), and the seven known non-G985 mutations located in exon 11 (15,18,20,30,31) were tested for by direct sequencing of amplified genomic DNA.

In 50 of 52 families with non-G985 mutations the index patients were analyzed. In the remaining two families in whom materials from the index patients were not available, parents and siblings were analyzed instead. Using this approach we identified both disease-causing mutations in 14 of the families. In addition we have in our previous studies identified both mutations in seven of the 52 families (15,18,29).

In families where a non-G985 mutation was identified, we also analyzed all available family members for the mutation. Mutations identified by the mutation specific assays were verified in the index patient by direct sequencing of amplified genomic DNA (not shown). Moreover, in those patients in whom we identified non-G985 mutations we investigated whether the two mutations were the only mutations present in the two MCAD alleles. This was done by performing direct sequence analysis of amplified genomic DNA covering the coding region of all exons of the MCAD gene from these patients (see Materials and Methods). In all the index patients from the 14 families described below, and the seven families previously described (15,18,29) the presence of more than one mutation in each allele was excluded in this way. Thus, we were able to ensure that the phenotypes of these patients

were not influenced by additional unrecognized mutations in their MCAD gene.

Mutation specific assays

The T157 mutation was only detected in the two families who have previously been described (29).

In two unrelated families (Families 1 and 2) of British origin we identified the A447 mutation. In Family 1 the mutation was identified in the index patient and his asymptomatic brother (Fig. 1A), both of whom are carriers of the G985 mutation. This corresponds well with the fact that the MCAD enzyme defect had also been indicated in the brother, although he had never experienced any symptoms. We also detected the A447 mutation in an unrelated British family (Family 2). In this family the index patient and her three year younger asymptomatic sister, both of whom are carriers of the G985 mutation, are heterozygous for the A447 mutation. This patient is the same as the one described previously by Yokota and co-workers (20).

The C730 mutation, previously reported by Yokota and co-workers (20), was not observed in any of the families we examined here.

The A799 mutation, previously reported by Yokota and co-workers (20), was observed in an Australian family (Family 3). The mutation was present in heterozygous form in the index patient (Fig. 1B). She is heterozygous for the G985 mutation.

Analysis for the Δ343-48 mutation (24), by *Rsa*I digestion of a PCR product harboring exon 5 (Fig. 1C) revealed one apparently heterozygous patient who is also a carrier of the G985 mutation. However, sequence analysis of the amplified fragment showed that the lack of cleavage was not, as expected, due to removal of the diagnostic *Rsa*I site but instead caused by a 347G→A transition (A347), which abolished the *Rsa*I recognition sequence (GTAC at position 347–350). The A347 mutation changes the second nucleotide of the TGT codon encoding cysteine^{91} of the mature MCAD protein to TAT for tyrosine. The A347 mutation has not previously been reported.

Sequencing of exon 7

In order to test for the presence of the A583 mutation located in exon 7 (21), we PCR amplified and sequenced a 305 bp fragment spanning the entire exon 7 and part of the flanking introns.

The A583 mutation was identified in four unrelated families of Scottish, Irish, Northern-Irish and American origin. In all four families (Families 4–7) the index patient was heterozygous for the G985 mutation.

Analysis of the parents from Family 4 showed that the mother was heterozygous for the A583 mutation and the father was heterozygous for the G985 mutation. In Family 5 analysis of the parents showed that the mother was heterozygous for the A583 mutation and the father was heterozygous for the G985 mutation. Analysis of three healthy siblings, two of whom are heterozygous for the G985 mutation, showed that the A583 mutation was not present in any of them. In Families 6 and 7 there was no material available from family members.

In a French family (Family 8) sequence analysis of the index patient, who is heterozygous for the G985 mutation, showed that this patient was heterozygous for a 474T→G transition (G474). The G474 mutation has not previously been reported. It changes the second nucleotide of the TAT codon encoding tyrosine^{133} of the mature MCAD protein to a TAG stop codon. If translated such

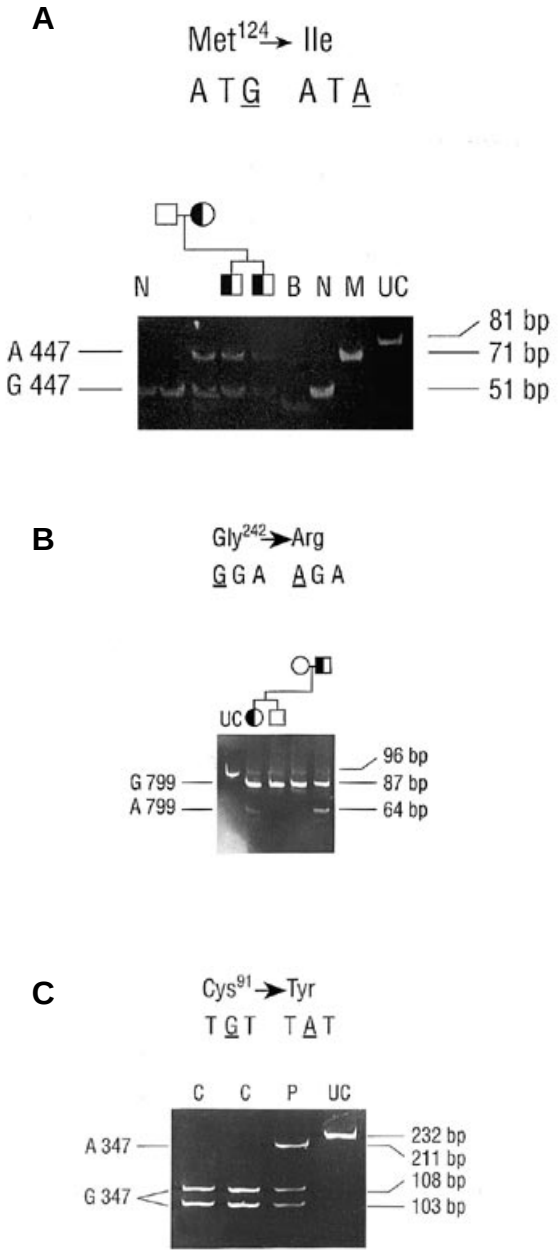

Figure 1. (**A**) Testing for the A447 mutation. The results from testing for the A447 mutation in Family 1 is shown together with a normal control (N) and an artificially homozygous mutant control (M). Uncleaved (UC) PCR product is 81 bp. Cleaved A447 mutation bearing fragments are 51 bp and cleaved fragments with normal sequence are 71 bp. The presence of the A447 mutation is indicated by black shading in the pedigree. (**B**) Testing for the A799 mutation. The results from testing for the A799 mutation in Family 3 is shown. Uncleaved (UC) PCR product is 96 bp. Cleaved A799 mutation bearing fragments are 64 bp and cleaved fragments with normal sequence are 87 bp. The presence of the A799 mutation is indicated by black shading in the pedigree. (**C**) Testing for the Δ343-48 deletion. The results from testing for the Δ343-48 deletion are shown. Normal control (C). Uncleaved (UC) wild-type PCR product is 232 bp. Uncleaved PCR product harboring the Δ343-348 deletion would be 226 bp. Cleaved wild-type fragments are 108/103/21 bp and cleaved fragments with the Δ343-48 deletion would be 205/21 bp. No patients with the Δ343-48 deletion were identified. Lane P shows the patient who was first suspected to be heterozygous for the Δ343-348 deletion. Sequence analysis showed that the observed pattern was instead caused by a 347G→A mutation that abolished the diagnostic *Rsa*I site. The 347G→A mutation changes cysteine^{91} to tyrosine.

a protein would be truncated by nearly two thirds (234 amino acids) of the coding region.

In a Czech family (Family 9) sequence analysis of the index patient, who is heterozygous for the G985 mutation, showed her to be heterozygous for a deletion of a T (Δ474) corresponding to position 474 or 475 of the cDNA sequence. The Δ474 mutation changes the TGT codon encoding cysteine[134] to GTG (valine) and leads to a premature stop codon only one codon downstream. If translated such a protein would be truncated by nearly two thirds (232 amino acids) of the coding region. The Δ474 mutation has not previously been reported.

In a French family (Family 10) sequence analysis of the index patient showed that he was heterozygous for a 577A→G transition (G577). We have previously reported that this patient, his father and his healthy sister are heterozygous for a 13 bp insertion mutation (∇999-12) in exon 11 (15). The mother was found to be heterozygous for the G577 mutation. The G577 mutation has not previously been reported. It changes the first nucleotide of the ACC codon encoding threonine[168] of the mature MCAD protein to GCC for alanine.

Sequencing of exon 11

We have previously reported the results from sequencing of exon 11 from compound heterozygous patients from 36 unrelated families with MCAD deficiency (18). In the present study we sequenced exon 11 from the remaining 16 of our 52 families. In all the investigated persons the results from the G985 assay and the sequencing were in agreement. Only one of the seven known non-G985 mutations located in exon 11 (18,22) was identified. In an American family (Family 11) we identified the previously described 4 bp deletion (Δ1100-03) (30,31), corresponding to cDNA position 1100–1103 or 1102–1105. The mutation was identified in heterozygous form in the index patient and his father. The index patient is heterozygous for the G985 mutation. Interestingly, his mother is homozygous for the G985 mutation, but she has always been asymptomatic.

The encoded mutant protein would be truncated by 37 amino acids. The deleterious nature of this mutation has previously been verified by expression of recombinant mutant protein in *E.coli* (30).

In an Australian family (Family 12) sequence analysis of the index patient, who is heterozygous for the G985 mutation, showed that this patient was heterozygous for a 1055A→G transition (G1055). The G1055 mutation has not previously been reported. It changes the second nucleotide of the TAT codon encoding tyrosine[327] of the mature MCAD protein to TGT for cysteine.

In a family from Argentina (Family 13) sequence analysis of the index patient, who is heterozygous for the G985 mutation, showed that she was also heterozygous for a 1 bp insertion of a T (∇1189) in exon 11 corresponding to cDNA position 1189 or 1190. The ∇1189 mutation will lead to a shifted reading frame from tyrosine[362] ending with a premature stop codon only four codons downstream. The encoded mutant protein would be truncated by 31 amino acids. This mutation has not previously been reported.

In a Scottish family (Family 14) direct sequence analysis of the amplified fragment from the index patient showed a normal sequence through exon 11, except that the nucleotides AT corresponding to position 955–956 or 957–958 or alternatively the nucleotides TA corresponding to position 956–957 of the cDNA were deleted. Sequence analysis of her parents revealed

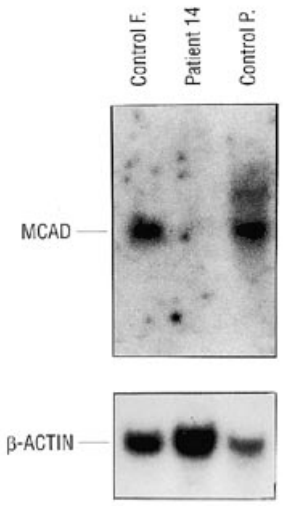

Figure 2. Northern blot analysis of patient homozygous for the Δ955-56 deletion. Northern blot analysis of 10 μg total RNA from control fibroblasts (Control F), 15 μg total RNA from patient 14 and 10 μg total RNA from control placenta (Control P) was performed as described previously using a MCAD probe (53) and a β-actin probe (Clonetech, Palo Alto, CA).

that they were both heterozygous for this 2 bp deletion (Δ955-56), agreeing well with the fact that they are first cousins, and showing that the index patient is homozygous for the Δ955-56 mutation. The Δ955-56 deletion changes the reading frame from serine[295] and leads to a premature stop codon only four codons downstream. If the mutant MCAD mRNA is translated the resulting protein would be truncated by 99 amino acids.

In order to investigate if the premature stop codon had any effect on the steady state MCAD mRNA amount in patient cells we analyzed MCAD mRNA from the patient by Northern blot analysis (Fig. 2). The results from the Northern blot analysis document that the steady state amounts of mutant MCAD mRNA are drastically decreased to a level not detectable by this type of analysis.

This is the first MCAD deficient patient identified who is homozygous for a non-G985 mutation, and at the same time she is the first patient with two mutations that result in a complete lack of functional MCAD enzyme.

Deletion of exons 11 and 12

In addition to the mutations dealt with above, a deletion covering the entire exon 11 and 12 of the MCAD gene has been reported (23). By examination of our total patient material, including both families where the patient is homozygous for the G985 mutation and the 52 families where the patient is not homozygous for the G985 mutation, we excluded the exon 11–12 deletion mutation in 114 families with MCAD deficiency. In 65 families with patients who were diagnosed as being homozygous for the G985 on the basis of results from the G985 assay, hemizygosity, and thus the presence of the deletion, could be ruled out because both parents were found to be heterozygous for the G985 mutation. In 49 non-G985 homozygous patients the deletion could be ruled out either because they were found to be heterozygous for the G985 mutation or because they were found to be heterozygous for the polymorphic silent 1161A→G mutation in exon 11 (32). These results document that the deletion mutation is very rare.

Table 1. Mutations in the MCAD gene

cDNA pos.[a]	Exon	residue	1991 I/T[a]	This study[b] I/T	Total I/T	Ethnicity	Family no.	Reference[d]
157C→T	3	R28C	1/2	2/57	2/70	France, Holland		(29)
Δ343-48	5	ΔG90-C91	Nd	0/57	1/70	USA		(24)
347G→A	5	C91Y	Nd	1/57	1/70	USA		(Present study)
447G→A	6	M124I	1/20	2/57	2/70	UK	1,2	(20)
474T→G	7	Y133X	Nd	1/57	1/70	France	8	(Present study)
Δ474	7	Truncation	Nd	1/57	1/70	Czech Rep.	9	(Present study)
577A→G	7	T168A	Nd	1/57	1/70	France	10	(Present study)
583G→A	7	G170R	Nd	4/57	6/70	USA, UK, Eire	4,5,6,7	(21)
730T→C	9	C219R	1/20	0/57	1/70	USA		(20)
799G→A	9	G242R	2/20	1/57	3/70	Australia, USA	3	(20)
Δ955-56	11	Truncation	Nd	2/57	2/70	UK	14	(Present study)
977T→C	11	M301T	Nd	1/57	1/70	USA		(18)
∇999-12	11	Truncation	3/38	2/57	3/70	Spain, France, Germany	10	(15,20)
1008T→A	11	S311R	Nd	1/57	1/70	UK		(18)
1045C→T	11	R324X	Nd	1/57	1/70	USA		(18)
1055A→G	11	Y327C	Nd	1/57	1/70	Australia	12	(Present study)
Δ1100-03	11	Truncation	2/23	1/57	3/70	USA	11	(30,31)
1124T→C	11	I350T	1/23	0/57	1/70	USA		(20)
1150G→T	11	E359X	Nd	1/57	1/70	UK		(18)
∇1189	11	Truncation	Nd	1/57	1/70	Argentina	13	(Present study)
			11/38	24/57	34/70			

[a]All nucleotide numbering is according to Kelly and co-workers (3).
[b]Includes results from references 15,18 and 29
[c]I/T = Identified alleles/Tested alleles
[d]The reference for the initial identification of the mutation is given.
Nd = Not detected.

The results from the mutation specific assays and sequencing of exon 7 and 11 are listed in Table 1 and 2. Also the data from the 1991 Workshop report (33) have been included, and a compilation of the data from 1991 and the data from the present study is also listed. By comparing patient lists from the laboratories involved, all duplicates have been excluded from the list. Moreover, only one sibling from each family is counted in the tallies.

Characterization of the identified mutations

The 12 different non-G985 mutations identified in patients investigated in the present study (Table 1) can be divided into six missense mutations and six mutations that either directly or indirectly results in creation of a premature stop codon (PSC). There can be little doubt that all six of the identified PSC mutations are disease-causing (34). In contrast, the disease-causing nature of the identified missense mutations is more complicated to predict.

Therefore they were characterized by expression of recombinant mutant protein in *E.coli*. A total of 10 missense mutations (including the G985 mutation) have been identified in our sample of MCAD-deficient patients (Table 1 and 2). We have previously analyzed four of them (G985, T157, C977 and A1008) using an *E.coli* based expression system with or without chaperonin co-overexpression and a eukaryotic expression system in *COS-7* cells (18,25–27,29).

In the present study we have analyzed five of the remaining six missense mutations present in our patients. Expression of mature wild-type MCAD protein and the mature forms of the mutant MCAD proteins resulting from the A447, G577, A583, A799, G1055 and G985 mutations were performed in *E.coli* JM109 cells. Transformed JM109 cells harbored expression plasmids encoding the mature form of wild-type or one of the mutant MCAD proteins and the plasmid pGroESL encoding the chaperonins GroEL and GroES or the control plasmid pCaP which lack the GroESL chaperonin genes (see Materials and Methods). The A347 mutation was not tested because it was identified after we had finished testing all the other mutations. Our results from expressing the mutant proteins M124I (A447 mutation), T168A (G577 mutation), G170R (A583 mutation), G242R (A799 mutation) and Y327C (G1055 mutation) without GroESL co-overexpression show that they all exhibit a severely decreased enzyme activity when compared with the wild-type protein (Fig. 3). This indicates the disease-causing nature of these mutations. Moreover, our results show that the enzyme activity of all the mutant proteins, except G170R and T168A, to a varying degree can be increased by co-overexpression of the GroESL chaperonins, and thus that compromised folding and/or tetramer formation is part of the molecular defect mechanism.

Table 2. Summary of clinical, biochemical and molecular data

Pat.	Sex	Mutation	Parents	1 st episode	Recurrent	Metabolic stress	Symptoms	Metabolites	Dead sibs	Affected sibs	Status
1	M	G985/**A447**	**Y**	24mo	No		Reye-like	DA,Gly	No	1 asympt	Well
2	F	G985/**A447**	**N**	26mo	No	Fever	Reye-like Unconcious,Hypotonia, Cerebral oedema	DA,Gly	No	1 asympt	Well, 13 years
3	F	G985/**A799**	**Y**	23mo	No	Fever, Vomiting, Diarroeha	Coma,Hypotonia, Hypoketosis,Hypoglycemia	DA,Gly	No	No	Well,14 years
4	M	G985/**A583**	**Y**	11.5mo	Yes, 23 mo	Vomiting, Diarrhea	Floppy,drowsy Hypoketosis,Hypoglycemia	DA,Gly	No	No	Well, 2 years
5	F	G985/**A583**	**Y**	7.5mo	No	Fever, Vomiting, Diarrhea,	Coma,Hypotonia, Hypoketosis,Hypoglycemia, Hepatomegaly	DA,Gly	No	No	Well, 6 years
6	M	G985/**A583**	**N**	7mo	Yes, Several for 12 day period	Diarrhea, Phenobarbital	Lethargy,convulsions	DA,Gly	No	No	Neur def. 2 years
7	F	G985/**A583**	**N**	4mo	No	Fever	Reye-like, Hypoglycemia	DA,Gly	No	No	Well at 2 years
8	M	G985/**G474**	**Y**	17mo	Yes, 24mo	Fasting	Coma,Hypotonia, Hypoketosis,Hypoglycemia, Hepatomegaly	DA,Gly,Car	No	No	Well, 13 years
9	F	G985/**Δ474**	**Y**	30mo	No	Fasting	Coma,Hypoketotis, Hypoglycemia,Hepatomegaly	DA,Gly	1 †day 3	No	Neur def. 5 years
10	M	**∇999-12/G577**	**Y**	12mo	Yes 30;35mo	Vomiting	Coma,Hypotonia Hypoglycemia,Hepatomegaly	DA,Gly	1 †day 3	No	Well, 15 years
11	M	G985/**Δ1100-03**	**Y**	12years	Yes,	Gastroenteritis	Hypoglycemia	Da,Gly,Car	1 †13mo, 1 Still birth	No	Well, 18 years
12	M	G985/**G1055**	**Y**	36 hours	No	------	Floppy,Hypoglycemia Seizures	DA,Gly	No	No	Well, 5 years
13	F	G985/**∇1189**	**Y**	15mo	No	Fever, Vomiting, Diarreha, Phenobarbital	Somnolence->Coma, Apnea,Hypotonia, Lethargy,Seizures	DA,Gly	No	No	Severe neur def., 2 years
14	F	**Δ955-56/Δ955-56**	**Y**	20mo	Yes; 29mo	Fever, Vomiting, Diarreha	Drowsiness,hypotonia, Hepatomegaly	DA,Gly	No	No	Well, 5 years

DA, dicarboxylic aciduria; Gly, glycine conjugates present in urine; Car, carnitine conjugates present in plasma/urine; mo, months; †, dead.

Parents: Y = one mutation identified in each parent. N = material not available.

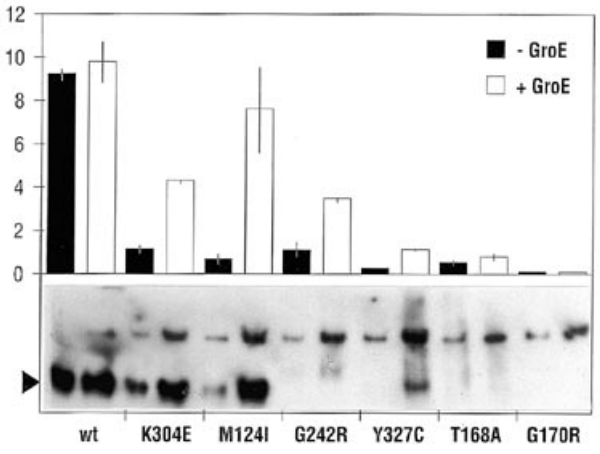

Figure 3. Tetramer formation and enzyme activity of mutant MCAD proteins. Transformed JM109 cells harboring expression plasmids encoding the mature part of wild-type or one of the mutant MCAD proteins and the plasmid pGroESL encoding the chaperonins GroEL and GroES (open bars) or the control plasmid pCaP which lacks the GroESL chaperonin genes (shaded bars) were grown and induced as described (26). Extracts (duplicates) were investigated by measuring enzyme activity (upper panel) and by PAGE followed by Western blot analysis (lower panel). The designations of the mutant M124I (A447 mutation), T168A (G577 mutation), G170R (A583 mutation), G242R (A799 mutation), Y327C (G1055 mutation) and wild-type (wt) MCAD proteins are indicated below the corresponding lanes of the gel in the lower panel. The bars in the upper panel corresponds to the lanes in the lower panel. (Upper panel) MCAD enzyme activity in the soluble fraction was measured with the ferricenium assay (56). Samples with GroESL co-overexpression are indicated with open bars and samples without GroESL expression are indicated with shaded bars. The scale is micromoles of ferricenium reduced per hour per mg of total soluble protein. Columns represents the average from two independent experiments where enzyme activity measurements were performed in duplicates in each experiment. The lines on top of the bars indicate the range of the values obtained in the two experiments. (Lower panel) 1.25µg of total protein of the soluble extracts were subjected to PAGE in a 7% gel without SDS followed by Western blotting with rabbit antiporcine MCAD antibodies. The position of the tetrameric form of MCAD protein is indicated by an arrow. The band observed above the band from the tetramer has previously been shown to cross react with GroESL antibodies, indicating that it represents complexes between GroESL chaperonins and MCAD protein (25).

To further characterize the molecular defects resulting from the different mutations we analyzed lysates from the *E.coli* cells expressing wild-type MCAD and the mutant proteins by native PAGE followed by Western blotting (Fig. 3). Considerable amounts of tetrameric MCAD protein was present from cells expressing wild-type, K304E and M124I MCAD, whereas very low amounts or no tetrameric protein was present from cells expressing the T168A, G170R and Y327C proteins. The observed amounts of tetrameric MCAD protein corresponded well with the measured enzyme activities in all cells except in those expressing the G242R mutant protein. Despite the fact that the enzyme activity measured from cells expressing the G242R mutant was comparable to that of cells expressing the K304E protein, the tetramer band observed in the native gel was much weaker than that from K304E, and migrated slightly slower. However, when the wild-type and mutant MCAD proteins were analyzed by SDS-PAGE and Western blotting of pellet and supernatant fractions (not shown) the size of the wild-type and all mutant proteins, including G242R, were correct. Moreover, the amount of G242R and K304E proteins were comparable and corresponded well with the measured enzyme activity.

As a first approach to establish an explanation for the observed discrepancy between the enzyme activity of the G242R mutant protein and the observed amount of tetrameric protein in the native gel we investigated whether this mutation affects the thermal stability of the tetrameric mutant enzyme by comparing its thermal inactivation profile to that of wild-type MCAD protein (data not shown). Furthermore, we also compared the thermal inactivation profiles of the mutant proteins M124I, Y327C and K304E to that of wild-type MCAD protein (data not shown). Measurements of the thermal inactivation profiles were performed as previously described for the K304E mutant protein (26). Consistent with our previous experiments we found the thermal stability of the K304E mutant protein to be moderately decreased as compared to the wild-type. In contrast the thermal stability of the M124I and G242R mutant proteins were similar to that of the wild-type MCAD. This shows that for these two mutations instability of the functional mutant protein does not contribute to the molecular defect mechanism, and consequently that a decreased thermal stability is not the explanation for the altered mobility and decreased intensity of the band from the G242R mutant protein in native-PAGE. Therefore, at present we have no simple explanation for the observed behavior of the G242R mutant protein.

The thermal stability of the Y327C mutant protein was significantly decreased as compared to wild-type, indicating that this is a major molecular defect mechanism of this mutation.

In summary our results from the expression studies show that the molecular defect of the M124I mutant protein (resulting from the A447 mutation) resembles that previously described for the R28C protein (resulting from the T157 mutation) (25–27,29), namely an isolated defect in the folding process which may be partially rescued by increasing the amount of chaperonins. The G242R mutant protein (resulting from the A799 mutation) behaves similarly to the K304E protein (resulting from the G985 mutation), namely with two molecular defects. One that affects the folding process (like M124I, R28C and K304E), which can be partly alleviated by increasing the amount of chaperonins and a second effect, which cannot be corrected. Based on this the T157, A447, A799 and G985 mutations may be considered as being milder than the other missense mutations.

The Y327C mutant protein (resulting from the G1055 mutation) has two separate defects. One folding defect, which can only be alleviated to a minor extent by chaperonin co-overexpression and a defect resulting in a decreased thermal stability of the mutant protein. The molecular defects of the Y327C mutant protein are, however, more severe and result in a much lower residual enzyme activity than those of the K304E mutant protein, which also has defects in folding/tetramerization as well as in thermal stability of the functional tetramer. Finally, our results show that the G577 and A583 mutations both lead to severe defects in the mutant proteins (T168A and G170R), which cannot be modulated by chaperonin co-overexpression.

DISCUSSION

One of the original goals of the present study was to establish whether any of the known mutations (15,18,21,23,24,29) are prevalent among the non-G985 chromosomes, and thus, if testing with one or a few mutation specific assays would improve the diagnostic efficiency. Our results show that this is not the case. None of the 14 mutations previously described, nor the seven new

mutations identified in the present study are prevalent among the non-G985 chromosomes (Table 1).

The second aim of this study was to investigate if or to what extent the different MCAD genotypes can be correlated to the variable clinical phenotypes of the disease.

In an attempt to investigate this we evaluated the molecular defect of all mutations identified in the patients of the present study. Firstly, our results from *E.coli* expression of the mutant proteins document the disease-causing nature of all the missense mutations investigated. It is noteworthy that the disease-causing nature of the A447 and A799 mutations have not previously been verified by expression studies, despite the fact that these mutations were identified already in 1991 (20,30). Secondly, our results show that the different non-G985 mutations may in fact cause different and variable levels of residual MCAD enzyme activity. Furthermore, the amount of active mutant enzyme is for some of the mutant variants dependent on the level of available chaperonins. In line with the results of our previous reports (18,26,27,29) the G985 mutation was shown to cause a milder defect than the other mutations, except for the A447 mutation (characterized herein) and the T157 mutation (characterized in refs 26,27 and 29).

It is interesting to note that *E.coli* cells expressing the M124I mutant protein, resulting from the A447 mutation, or the G242R mutant protein, resulting from the A799 mutation, display up to 80 and 40%, respectively, of the MCAD enzyme activity levels measured from cells expressing wild-type MCAD, when the folding defect is alleviated by chaperonin co-overexpression. Thus, the residual MCAD enzyme activity of the G242R mutant protein is similar to the K304E mutant protein and that of M124I is nearly twice as high. It could thus be hypothesized that the seriousness of the A447, A799 and G985 mutations could be influenced by the capacity of the folding machinery in patient cells.

In contrast to this the mutant proteins (T168A, G170R and Y327C) resulting from the G577, A583 and G1055 mutations show very little (Y327C) or no (T168A and G170R) response to chaperonin co-overexpression. This indicates that for these mutations the defect in enzyme biogenesis/stability is so severe that the capacity of the folding machinery in patient cells would have a much smaller (if any) impact on the residual MCAD enzyme activity. It is interesting to note that the G577 mutation is the first mutation in the MCAD protein that directly affects an amino acid (threonine^{168}) known to be functionally important. Threonine^{168} is located in the β-sheet domain and forms a hydrogen bond with the flavin adenine dinucleotide ring of the bound FAD (9). It could be speculated that replacement of this important residue would affect FAD binding and/or perhaps disturb electron transfer. Despite this it appears that the main molecular defect of the G577 mutation is on enzyme biogenesis, like all the other identified missense mutations. Results by Saijo and Tanaka corroborate this observation, as they have shown that FAD cofactor binding is crucial for folding/oligomerization of the MCAD tetramer (35). In relation to the possible phenotypic effect of the G577 mutation in the index patient, it should however be noted that we have previously observed that in patient cells the steady state amounts of MCAD mRNA from G577 mutation bearing alleles were for unknown reasons drastically decreased (15). The G577 mutation should not by itself interfere with pre-mRNA processing, and sequence analysis of all exon/intron junctions of the MCAD gene from the index patient did not reveal any abnormalities.

Interestingly, glycine^{170}, which is changed to an arginine by the A583 mutation, is located in the same turn in the β-sheet domain close to the FAD molecule (9) as threonine^{168}. It may thus seem reasonable to suggest that the drastic substitution of glycine^{170} with the much larger and charged arginine in this part of the enzyme may disturb FAD-cofactor binding and thus the folding/oligomerization of the MCAD tetramer. The fact that a similar mutation of the homologous glycine residue to a valine in isovaleryl-CoA dehydrogenase has been observed in a patient with IVD deficiency (36), corroborates the notion that disturbance of the molecular architecture of this part of the acyl-CoA dehydrogenase enzymes may affect their folding pathway. In this connection it is also interesting to note that a mutation of glutamate^{365} (E365K) in glutaryl-CoA dehydrogenase (GCD), which is homologous to tyrosine^{327} (changed to a cysteine by the G1055 mutation) in MCAD, has recently been reported to cause GCD enzyme deficiency, resulting in glutaric-aciduria in patients (37). Moreover, we have previously reported that arginine^{28} (changed to a cysteine by the T157 mutation) in MCAD is homologous to the mutated arginine^{22} (R22W) in the short-chain acyl-CoA dehydrogenase (SCAD), causing SCAD deficiency (29,38). Thus, mutations in the homologous amino acids for as many as three of the amino acids changed by the 12 missense mutations identified in MCAD deficiency have been found in other acyl-CoA dehydrogenase deficiencies. This indicates that the mutational pattern in the acyl-CoA dehydrogenase enzymes is similar, and that mutations often affect residues which do not seem to be directly involved in enzyme function, but rather are important for enzyme biogenesis.

Whereas most of the missense mutations may result in residual MCAD activity above background, the identified PSC mutations, which would result in premature termination if translated, would not be expected to give any residual MCAD enzyme activity. Firstly, we have shown that for two of the mutations [Δ955-56 in the present study and ∇999-12 in our previous study (15)] where patient cells were available, that they both result in drastically decreased amounts of steady state mutant MCAD mRNA. This is in itself sufficient to explain the enzyme defect, and it is in good agreement with the observation that this type of mutation usually results in severely decreased amounts of mutant mRNA (34). Secondly, for those mutations where the truncated enzymes have been tested by expression (1045C→T, Δ1100-03 and 1150G→T) they were shown to result in very low amounts of immunoreactive protein and no residual enzyme activity, when compared to wild-type MCAD (18,30). Finally, all eight PSC mutations in the MCAD gene (Table 2) encode proteins lacking the active site glutamate^{376} (39,40).

We have thus shown that the different non-G985 mutations may cause different levels of residual MCAD enzyme activity. Furthermore, our results from the present and the results from previous studies (15,18,21,29) document that the entire clinical spectrum of MCAD deficiency, from remaining without symptoms for several years (Family 11) to patients who die suddenly in the neonatal period (21,29, and Families 9 and 10 of the present study), can be observed in patients who are not homozygous for the G985 mutation (Table 2). Based on this it may seem reasonable to expect that differences in MCAD genotype may be important in determining the clinical phenotype. In both Families 1 and 2 there exists a sibling who is also compound heterozygous with the A447 and the G985 mutations, but only the index patients

have experienced clinical symptoms (Table 2). Moreover, neither of the index patients in these two families has suffered from more than one episode, and there were no dead siblings. This is in good agreement with the observation from our expression experiments that the A447 mutation is rather mild, and the folding of the resulting mutant protein (M124I) may be influenced by the available levels of chaperonins. It could be speculated that in these two families a good capacity of the folding machinery is able to ensure a rather high residual MCAD activity from the two mutant proteins. This is further corroborated by the fact that measurements of the β-oxidation activity towards tritium labeled myristic acid in cultured fibroblasts from the index patient in Family 2 repeatedly have shown the highest activity that we have ever observed among MCAD patients (results not shown). On the other hand, the index patient in Family 11, who is compound heterozygous with the severe Δ1100-03 deletion in one allele and the G985 mutation in the other allele, remained without clinical symptoms until 12 years of age (Table 2), despite the fact that he suffered from metabolic stress several times during the first two years of life. Moreover, we have recently reported that individuals who harbor the severe ∇999-12 insertion mutation or the severe 1045C→T stop codon mutation in one allele, together with the G985 mutation in the other allele, may remain without symptoms (15,18). These data clearly show that patients compound heterozygous with the G985 mutation in one of the alleles may remain without clinical symptoms, irrespective of the nature of the second mutation.

Thus the residual activity from the K304E mutant protein expressed from only one G985 mutation bearing allele may, under some circumstances, be sufficient to avoid disease precipitation.

Table 3. Primers and restriction enzymes

T157 assay:	Sense: 5′-GCAGAAAGAATTTCAAGCTAGAGCT-3′ (pos. 132–156)
	Antisense: 5′-AAATATATTTTGAGCTCCAGATAGTTTGAT-3′ (intron 3)
	Enzyme: *Eco*ICRI
A447 assay:	Sense: 5′-CAAATGCCGATTCTTATTGCTGGA-3′ (pos. 388–411)
	Antisense: 5′-ACACATCAATGGCTCCTGAGT-3′ (pos. 468–448)
	Enzyme: *Hin*fI
C730 assay:	Sense: 5′-AGGAATTAAACATGGGCCATCGA-3′ (pos. 707–729)
	Antisense: 5′-CCATTGCATCGATGAAACCAGC-3′ (pos. 823–802)
	Enzyme: *Cla*I
A799 assay:	Sense: 5′-CGATGTTCAGATCTTAGAGGAATTGTC-3′ (pos. 727–753)
	Antisense: 5′-CATTGCAACTTTGAAACCAGATC-3′ (822–800)
	Enzyme: *Bgl*II
Exon 5:	Sense: 5′-ACCTTTATTTCTATTGTGATGTACTAC-3′ (intron 4)
(Δ343-48/347G→A assay)	Antisense: 5′-TCTTCTAAAAATCCAACTTCTTCAGG-3′ (intron 5)
	Enzyme: *Rsa*I
Exon 1:	Sense: 5′-GGGAGTATGTCAAGGCCGTG-3′ (pos. –60 to –41)
	Antisense: 5′-CCACAATACCCATGTTCCAGC-3′ (intron 1)
Exon 2:	Sense: 5′-ACCACTTGCTGTACTCACTTATG-3′ (intron 1)
	Antisense: 5′-CCAAACAAACATATAAAGCTTCA-3′ (intron 2)
Exon 3–4:	Sense: 5′-TTCTACATACTGACTTCATAGG-3′ (intron 2)
	Antisense: 5′-ATGACTGAGTAGAGTTCCACA-3′ (intron 4)
Exon 6:	Sense: 5′-CATTTTGAATTATAGCATCTCTGA-3′ (intron 5)
	Antisense: 5′-TGAAAAGGAGAAATAGCACCT-3′ (intron 6)
Exon 7:	Sense: 5′-CAATCCTGTTTCCAAACAGTCA-3′ (intron 6)
	Antisense: 5′-CAAACATACCTTAACTGTAGTGGTT-3′ (intron 7)
Exon 8:	Sense: 5′-GCATTCACCATGTGTTATTTGCC-3′ (intron 7)
	Antisense: 5′-CAAACAAATGTTTTTATTAAGGAAAGT-3′ (intron 8)
Exon 9:	Sense: 5′-TGCAGCAAGTCATGAAACAGTG-3′ (intron 8)
	Antisense: 5′-GATCCTATAAAGGCTATGAACCCA-3′ (intron 9)
Exon 10:	Sense: 5′-CTCCCAAACATAGACACTTAGGC-3′ (intron 9)
	Antisense: 5′-GAATATGAGGGTCTTTACATTTGAACA-3′ (intron 10)
Exon 11:	Sense: 5′-ACATAGCAAGCCCGTCACT-3′ (intron 10)
	Antisense: 5′-ATCAGAAATCCACGTTGTCA-3′ (intron 11)
Exon 12:	Sense: 5′-ATTACAACACACCTTATGCTACTG-3′ (intron 11)
	Antisense: 5′-AGTAAAGTGGTACTAAAGAAAACACATC-3′ (pos.1490–1463)

Mismatching nucleotides are indicated by underlining. The cDNA positions of primers located in the coding region are indicated relative to the start codon. The enzymes used for detection of the different mutations in the mutation specific assays are indicated.

In light of this, it is interesting to note that Brackett and co-workers (21), based on their observation of sudden neonatal death in two unrelated families, suggested that compound heterozygosity with the A583 and G985 mutations represents a particularly severe genotype (A583/G985) which correlates to a severe, early presentation of the disease. Although our results from *E.coli* expression of the mutant protein (G170R) resulting from the A583 mutation corroborate the notion that it is one of the most severe missense mutations, the clinical/biochemical data from the four unrelated patients of the present study (Families 4–7, Table 2) with the A583/G985 MCAD genotype do not indicate that this genotype is particularly severe. None of the four index patients with the A583/G985 genotype have suffered from an early or particularly severe presentation (Table 2). Moreover, Kelly and co-workers (31) reported a patient who died suddenly at 22 months of age and had an identical MCAD genotype as the index patient in Family 11, and we have previously reported two unrelated families where the index patients were compound heterozygous for the T157 and G985 mutations, but presented very differently (e.g. one mild episode versus sudden neonatal death) (29). These data clearly document the fact that G985 compound heterozygous patients with identical MCAD genotypes may have very different clinical presentations. Taken together with the fact that G985 compound heterozygous patients with the most severe non-G985 mutations may remain asymptomatic for years, and the fact that G985 compound heterozygous patients with the mildest MCAD mutations (i.e. T157) may present with the most severe symptoms i.e. sudden unexpected death at day 3 (29) our data show that it is questionable whether it is reasonable, like Brackett and co-workers do, to state that a particular MCAD genotype may explain a severe form of the disease.

However, in all the patients discussed above the effect of the non-G985 mutant allele may be masked by the simultaneous presence of an allele with the relatively mild G985 mutation. It could be speculated that in these patients interindividual variation in the folding capacity of their cells, by modulating the residual activity of the K304E protein resulting from the G985 mutation, is an equally important factor to consider.

The identification of the index patient in Family 14 is in this connection particularly interesting. She is the first patient identified who is homozygous for a mutation other than the G985 mutation. Despite the fact that she does not produce any functional MCAD, she has only experienced two mild episodes at 20 and 29 months of age. Obviously, the nature of her mutation (Δ955-56), for which she is homozygous, implies that her (lack of) MCAD enzyme activity would not be responsive to any factors capable of modulating the biogenesis of the MCAD protein. Thus, she may illustrate that other endogenous factors may also be decisive for the risk of disease precipitation. The overlapping substrate activity (2,41,42) from the other acyl-CoA dehydrogenases is an obvious candidate to provide the enzyme activity sufficient to overcome the 'enzyme block' imposed by the lack of MCAD and to ascertain sufficient flow through the β-oxidation in a normal situation. Also, interindividual variations in enzymes involved in the metabolism of potentially toxic intermediates of fatty-acid oxidation accumulating due to the MCAD enzyme defect, could be speculated to be of importance for disease precipitation.

In conclusion, the present study suggests that variation in the MCAD genotype, despite the fact that different MCAD mutations may contribute with different susceptibilities for disease precipitation, is probably not the primary explanation for the observed clinical variation in this potentially severe disease. Finally, it should be emphasized that in the vast majority of patients disease presentation occurs during or following a period of metabolic stress (Table 2), for instance after periods of fasting or due to viral infections. Thus, environmental factors are crucial determinants of disease precipitation and the frequency of exposure to metabolic stress and the severity of the metabolic stress are therefore probably the most important determinants of the clinical phenotype, irrespective of the MCAD genotype.

MATERIALS AND METHODS

Patients

A total of 52 unrelated families with patients in whom MCAD deficiency was not caused by homozygosity for the prevalent G985 mutation were studied. The PCR/restriction enzyme digestion based assay for the prevalent mutation, G985, was performed as described elsewhere (19). In 45 of the families the index patients were diagnosed as being heterozygous for the G985 mutation, five index patients did not possess the G985 mutation at all, and in two families material from the index patient was not available for DNA analysis, but investigation of the parents indicated that the index patients should be expected to be heterozygous for the G985 mutation. The number of alleles with non-G985 mutations in the 52 families is therefore 57. In all studied families, the index patient had experienced clinical symptoms of MCAD deficiency, and excreted urine organic acids, including acylglycines, indicative of MCAD deficiency (14,15). In the majority of the patients, further evidence for the MCAD defect was provided by at least one of the following methods: *in vitro* enzyme assays (43–46), β-oxidation activity measurements (47,48) or the phenylpropionic acid loading test (49).

In six of the families mutations in exon 11 have previously been reported (15,18), and in two of the families the T157 mutation has previously been reported (29). The case history and the biochemical characterization, as well as the identification of one of the disease-causing mutations (∇999-12 insertion in exon 11) in Family 10 has previously been described (15). The index patient in Family 12 has previously been reported as case 4 elsewhere (50). A summary of the clinical and biochemical data on all the 14 patients in whom both disease-causing mutations are described for the first time are listed in Table 2, together with the disease causing mutations identified in each patient. More detailed case histories, and clinical-biochemical data are available upon request.

Preparation of DNA

Genomic DNA was isolated from blood samples and from cultured skin fibroblasts by standard methods (51). DNA from blood spots and whole cells were liberated as described elsewhere (19,52).

Sequencing of amplification products

PCR amplification and subsequent purification of a 421 bp fragment spanning the entire exon 11 and part of the flanking introns of the MCAD gene was performed as described elsewhere (18). Bi-directional solid-phase dideoxy-sequencing was performed using a prism T7 kit (Applied Biosystems) and a

semiautomated ABI 373A sequencer (Applied Biosystems). PCR amplification, purification and sequence analysis of a 232 bp fragment spanning the entire exon 5 and part of the flanking introns and of a 305 bp fragment spanning the entire exon 7 and part of the flanking introns was carried out as described for exon 11. PCR, purification and sequence analysis of all exons of the MCAD protein coding region was performed as described for exons 5, 7 and 11. All primers used are listed in Table 3.

Mutation specific assays

We designed PCR/restriction enzyme digestion based mutation specific assays for the T157, A447, C730 and A799 mutations (20,29), all four of which are located outside exon 11. These assays are all based on the principle of PCR-based introduction of diagnostic restriction enzyme sites like the G985 assay (19). In addition to the diagnostic site, control sites for enzyme cleavage efficiency are introduced in both the variant and control alleles in all assays. The restriction enzymes and primers used for the different assays are listed in Table 3. It should be noted that the assay for the T157 mutation has been changed from that originally described (29), so that the antisense primer is now located in intron 3. In addition, we have substituted the use of *Sac*I with the neoschizomeric enzyme *Eco*ICRI, which is more efficient and less expensive.

The 6 bp deletion mutation (Δ343-48) in exon 5 (24) was tested for by *Rsa*I digestion of the 232 bp PCR amplified fragment spanning the entire exon 5 and part of the flanking introns. From normals the 232 bp fragment can be cut into fragments of 21, 103 and 108 bp with *Rsa*I. If the Δ343-48 deletion is present, *Rsa*I digestion should instead produce two fragments of 21 and 205 bp.

The PCRs for the different mutation specific assays were performed with the following program for 35 cycles: denaturation for 1 min at 94°C, annealing for 2 min at either 50°C (157C→T assay) or 57°C (447G→A assay; 730T→C assay; 799G→A assay) and chain elongation at 74°C for 3 min. The PCRs were carried out in an automated thermal cycler (Perkin Elmer Cetus, Norwalk, CT) with either genomic DNA, boiled blood spots or boiled whole cells as DNA source in 100 µl with 15 pmol of each primer and 2 U recombinant *Taq* polymerase (Perkin Elmer Cetus, Norwalk, CT or Northumbria Biologicals, Northumberland, UK). After digestion with the appropriate restriction enzyme, samples were electrophoresed in 12% or 14% polyacrylamide gels together with uncleaved samples. The bands were visualized by staining with ethidium bromide.

RNA extraction and Northern blot

Total RNA was prepared from frozen patient fibroblasts using either a RNeasy kit (Qiagen, Hilden, Germany) or a RNAzol kit (WAK-Chemie, Bad Homburg, Germany). Northern blot analysis of 15 µg total RNA from patient and 10 µg total RNA from control fibroblasts and 10 µg total RNA from control placenta was performed as described previously using the MCAD probe (53), a VLCAD probe (54) and a β-actin probe (Clonetech, Palo Alto, CA).

Site-directed mutagenesis, and expression of wild-type and mutant MCAD in *E.coli* JM109 cells

The mutations A447, G577, A583, A799 and G1055 were all introduced by PCR based site-directed mutagenesis into the previously described expression vector pWt (26), which harbors a gene encoding the mature part of human MCAD under the control of a *lac* promoter. PCR based site-directed mutagenesis was performed according to the method described by Sarkar and Sommer (55) using Pfu polymerase (Stratagene, La Jolla, CA) and mutagenic primers for each of the mutations with the pWt plasmid as template. The PCR-products harboring the respective mutations, were digested with restriction enzymes (*Pst*I/*Eco*RI or *Eco*RI/*Hin*dIII) purified and cloned back into the pWT plasmid, replacing the corresponding fragment with the wild-type sequence. To confirm that no PCR-derived errors were present in the exchanged fragments, all the constructed plasmids were sequenced.

Analysis of expression of wild-type and mutant MCAD cDNA in *E.coli* JM109 cells

Growth (at 31°C) and disruption of bacterial cells, SDS-PAGE, native PAGE and Western blot analysis was performed as described previously (25,26). Measurements of the thermal inactivation profiles were performed as previously described for the K304E mutant protein (26). The activity measurements were performed by the ferricenium ion based assay (56). The concentration of protein in *E.coli* JM109 cell extracts was determined with a modified Bradford assay kit (BioRad, Richmond, CA). All experiments were performed at least two times, with enzyme activity measurements performed in duplicates each time.

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ABBREVIATIONS

ETF, Electron transfer flavoprotein; FAD, flavin adenine dinucleotide; GC/MS, Gas chromatography/mass spectrometry; GCD, glutaryl-CoA dehydrogenase; IVD, isovaleryl-CoA dehydrogenase; PAGE, polyacrylamide gel electrophoresis; MCAD,

medium-chain acyl-CoA dehydrogenase; PCR, polymerase chain reaction; SCAD, short-chain acyl-CoA dehydrogenase.

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