

Medium-Chain Acyl-CoA Dehydrogenase (MCAD) Mutations Identified by MS/MS-Based Prospective Screening of Newborns Differ from Those Observed in Patients with Clinical Symptoms: Identification and Characterization of a New, Prevalent Mutation That Results in Mild MCAD Deficiency*

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Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is the most frequently diagnosed mitochondrial β -oxidation defect, and it is potentially fatal. Eighty percent of patients are homozygous for a common mutation, 985A→G, and a further 18% have this mutation in only one disease allele. In addition, a large number of rare disease-causing mutations have been identified and characterized. There is no clear genotype-phenotype correlation. High 985A→G carrier frequencies in populations of European descent and the usual avoidance of recurrent disease episodes by patients diagnosed with MCAD deficiency who comply with a simple dietary treatment suggest that MCAD deficiency is a candidate in prospective screening of newborns. Therefore, several such screening programs employing analysis of acylcarnitines in blood spots by tandem mass spectrometry (MS/MS) are currently used worldwide. No validation of this method by mutation analysis has yet been reported. We investigated for MCAD mutations in newborns from US populations who had been identified by prospective MS/MS-based screening of 930,078 blood spots. An MCAD-deficiency frequency of 1/15,001 was observed. Our mutation analysis shows that the MS/MS-based method is excellent for detection of MCAD deficiency but that the frequency of the 985A→G mutant allele in newborns with a positive acylcarnitine profile is much lower than that observed in clinically affected patients. Our identification of a new mutation, 199T→C, which has never been observed in patients with clinically manifested disease but was present in a large proportion of the acylcarnitine-positive samples, may explain this skewed ratio. Overexpression experiments showed that this is a mild folding mutation that exhibits decreased levels of enzyme activity only under stringent conditions. A carrier frequency of 1/500 in the general population makes the 199T→C mutation one of the three most prevalent mutations in the enzymes of fatty-acid oxidation.

Introduction

In humans, medium-chain acyl-CoA dehydrogenase (MCAD [E.C.1.3.99.3; MIM 201450]) deficiency is the

most frequently diagnosed defect of mitochondrial β -oxidation (Roe and Ding 2001). Disease presentation may occur at any time of life, from the neonatal period (Andresen et al. 1993a; Wilcken et al. 1993) to adulthood (Marsden et al. 1992; Ruitenbeek et al. 1995; Yang et al. 2000). The vast majority of patients present with metabolic crisis during the first years of life when metabolically challenged by fasting and/or viral illness. Usually, the phenotype includes hypoketotic hypoglycemia, lethargy, coma, seizures, and death (Roe and Ding 2001). As many as 20% of patients die during their first metabolic crisis (Iafolla et al. 1994). In sharp contrast, patients diagnosed with MCAD deficiency who comply with a treatment regimen that includes the avoidance of fasting, a low-fat diet, and carnitine supplementation either dramatically reduce or completely eliminate recurrent disease episodes (Iafolla et al. 1994). It is also

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recognized that undiagnosed affected individuals may remain asymptomatic for decades and possibly throughout life (Kelly et al. 1990; Andresen et al. 1997).

The majority (80%) of patients with clinically manifested MCAD deficiency are homozygous for a common mutation, 985A→G, and a further 18% have this mutation in one disease allele (Gregersen et al. 1991; Yokota et al. 1991; Pollitt and Leonard 1998). So far, no other prevalent mutations have been identified, but a large number of different mutations have been detected and characterized in patients with clinical presentation of MCAD deficiency (Andresen et al. 1997; B. S. Andresen, unpublished data). There is no clear correlation between mutation type and clinical phenotype (Andresen et al. 1997, and authors' unpublished data). The incidence of MCAD deficiency in newborns, in most European countries, Japan, and the United States, has been estimated by determination of carrier frequencies for the prevalent 985A→G mutation in blood spots from newborns. The prevalent 985A→G mutation was not found in 1,000 Japanese newborns, and the carrier frequencies were low in southern European newborns (Tanaka et al. 1997). High carrier frequencies (1/64–1/101) have been determined for newborns from the northwestern part of Europe and from the white population of the United States. The high carrier frequency in populations of European descent suggests that MCAD deficiency may be a candidate in screening of newborns.

During periods of acute metabolic decompensation, patients with MCAD deficiency show urinary excretion of C₆–C₁₀ dicarboxylic acids, acylglycines, and acylcarnitine conjugates. In particular, identification of acylglycines by gas chromatography–mass spectrometry (GC/MS) has been used for diagnosis during metabolic decompensation (Rinaldo et al. 1988; Gregersen et al. 1994; Roe and Ding 2001). Because urinary excretion of these metabolites is much lower when patients are not undergoing metabolic decompensation, GC/MS is more difficult and thus unsuitable for screening of newborns. Instead, the diagnosis of MCAD deficiency in asymptomatic newborns can be made through the analysis of acylcarnitines in blood spots by tandem mass spectrometry (MS/MS) (Van Hove et al. 1993; Ziadeh et al. 1995; Chace et al. 1997). Because the MS/MS-based method is fast, automatable, highly sensitive, and specific, it has been the basis worldwide for several newborn-screening programs for acylcarnitines and amino acids in blood spots. To date, no validation of this method by full mutation analysis has been reported, but preliminary data from the first 80,371 blood spots of newborns prospectively screened by MS/MS-based acylcarnitine analysis have been reported (Ziadeh et al. 1995). Analysis of these blood spots resulted in identification of nine newborns with a positive screening result (an MCAD-deficiency frequency of 1/8,930), and

only 56% (5/9) of them were found to be homozygous for the 985A→G mutation. Because of the small number of positive samples and because, in the pilot study, only one of the four prospectively identified newborns not homozygous for the 985A→G mutation had the second mutation identified, both the reported frequency of 1/8,930 and the ratio—only 56%—of newborns with a positive screening result who were homozygous for the 985A→G mutation have been the subject of much controversy.

In the present article, we investigated the spectrum of mutations in the *MCAD* gene in newborns identified by observation of a diagnostic acylcarnitine profile by prospective MS/MS-based screening of >900,000 blood spots. We investigated whether the observed mutations were polymorphic in the general population, and we characterized the molecular consequences of the identified missense mutations, using our *Escherichia coli*-based expression system with and without co-overexpression of the chaperonins GroEL and GroES. Finally, by testing 1,000 blood spots, we determined the carrier frequency of a new common MCAD mutation that causes a mild enzyme deficiency.

Material and Methods

MS/MS-Based Screening of Newborns

Samples for the present study were identified by MS/MS-based screening of 930,078 blood spots collected <72 h after birth from newborns from Pennsylvania, Ohio, New Jersey, Illinois, Florida, and North Carolina. The 80,371 blood spots reported in the study by Ziadeh et al. (1995) were included in the present sample. The acylcarnitine profiles were obtained by analysis of butylated acylcarnitines by MS/MS. Screening for MCAD deficiency was achieved by detection of “diagnostic” acylcarnitine profiles (i.e., elevated C₆, C₈, C₁₀, and C_{10:1}). The analysis of all blood spots with a positive screening result was repeated at least once. Details of the MS/MS protocol have been described elsewhere (Chace et al. 1997). Sixty-two newborns with pathological acylcarnitine profiles consistent with MCAD deficiency were prospectively detected by screening of newborns. Infants identified as having MCAD deficiency were maintained on either breast milk or regular formula, with no fat restriction during the 1st year of life. Parents were instructed that their infants should avoid prolonged fasting. Most patients were given a supplement with oral carnitine, in varied doses. Only two infants detected by screening of newborns died as a result of complications associated with MCAD deficiency; these two infants were the first in whom MCAD deficiency had been detected by routine screening of newborns in 1992 (Ziadeh et al. 1995). None of the patients

identified since then have had any serious or permanent sequelae. As a precaution, some patients have been admitted for glucose infusion in connection with episodes of poor oral intake/viral illness. There have been no deaths, seizures related to hypoglycemia, or comas. All 14 children from North Carolina have normal neurological development, to date.

Identification of Mutations in the MCAD Gene

Samples having acylcarnitine profiles indicative of MCAD deficiency were assayed for the common 985A→G mutation. DNA was extracted from dried blood spot, by Generations reagents (Gentra Systems). The assay for the 985A→G mutation employed amplification in a LightCycler (Roche Molecular Systems), by a forward primer internally labeled with LC red 640 and an assay utilizing melting-temperature analysis and fluorescence-resonance energy transfer (FRET). The fluorescein-labeled probe perfectly matches the mutant (985G) sequence and mismatches the wild-type (985A) sequence. The details of assay for the 985A→G mutation are described elsewhere (J. K. Smith, S. F. Dobrowolski, M. G. Herrmann, C. T. Wittwer, personal communication); figure 1A displays typical results obtained by this assay. Samples that had acylcarnitine profiles indicative of MCAD deficiency and were either (a) identified as being heterozygous for the 985A→G mutation or (b) had a normal sequence in exon 11 corresponding to cDNA position 985 were analyzed by sequencing of the entire protein-coding region of the gene. PCR amplifications of all 12 exons—including part of the flanking intron sequences—of the human *MCAD* gene were performed as described elsewhere (Andresen et al. 1997), with intron-located primers under standard conditions in an automated Thermal Cycler 480 (Applied Biosystems). Amplification of a 628-bp fragment (from 22 nt downstream of the translation-initiation ATG to 606 nt upstream) harboring the *MCAD* promoter region (Zhang et al. 1992) used nested amplification (first-PCR primers were 5'-GGACAAGTCATAAACAGCC-TTGGGAATACGCGGAAAGGT-3' and 5'-CCTGCA-GCATCGCCCGAACCCACAGCCAT-3', and second-PCR primers were 5'-TGCTTTCGCTTTAGGTAGGG-CATTTGAGAGCAA-3' and 5'-ATCGCCCGAACCCGCTGCCATGT-3') under standard conditions, except that 4 μ l deaza dGTP was added to both amplification reactions. PCR products were subjected to direct bidirectional sequencing using DNA BigDye terminator-sequencing kits (Applied Biosystems). Sequence reactions were run on semiautomated ABI 373A and ABI 377 sequencers (Applied Biosystems).

A mutation-specific assay for the 199T→C mutation was also developed. An asymmetric amplification using the forward primer (5'-ATACTGACTTCATAGGACA-

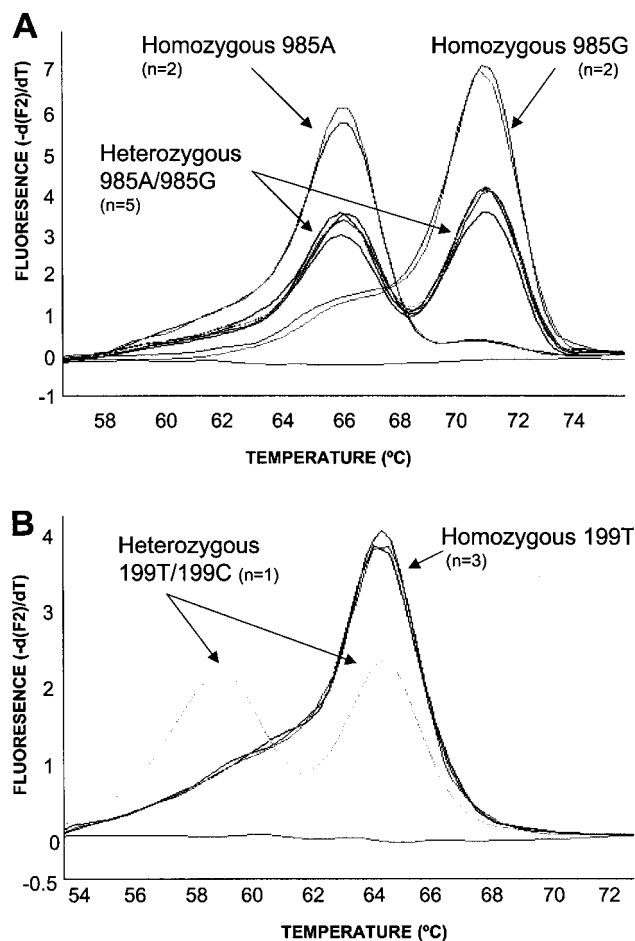


Figure 1 LightCycler assay for the 985A→G mutation and the 199T→C mutation. A, Assay-melting curves for the 985A→G mutation, for 985A homozygotes (two samples), 985A/985G heterozygotes (five samples), and 985G homozygotes (two samples). B, Assay-melting curves for the 199T→C mutation, for 199T homozygotes (three samples) and a 199T/199C heterozygote (one sample).

TTTTTC-3') at 0.5 μ M and the reverse primer (5'-AGA-TTTTTCCCTCTTTAAAATGTATA-3') at 0.25 μ M produces a 198-bp fragment from the *MCAD* gene. A strategy of paired hybridization probes is used to detect the 199T→C mutation. The anchor probe (5'-LC-red640-GGGATGATTTTCCTCTCTGGCAAATTTAC-GAGCAGT-(0P03)-3'), used at 0.2 μ M, hybridizes to a nonpolymorphic region, whereas the detection probe (5'-CAGTTTTATCATATTCTGCAGCCA-fluorescein-3'), used at 0.1 μ M, overlays the site of the mutation. The detection probe matches the wild-type (199T) sequence and mismatches the mutant (199C) sequence. The assay was validated by DNA from dried blood spots shown, by sequence analysis, to harbor the 199T→C mutation. Subsequently, DNA from dried blood spots from 1,000 randomly selected, anonymous newborns from the U.S. population screened were genotyped with the assay. Fig-

ure 1B shows profiles from specimens that are wild type (199T) and from specimens that are heterozygous for the 199T→C allele.

Site-Directed Mutagenesis and Expression of Wild-Type and Mutant MCAD in E. coli JM109 Cells

The mutations 199T→C, 362C→T, 734C→T, and 928G→A were introduced by standard PCR-based site-directed mutagenesis into the expression vector pWt (Bross et al. 1995) using *Pfu* polymerase (Stratagene). A fragment harboring the 233T→C mutation was obtained by digesting a plasmid harboring the entire MCAD protein-coding region cloned from PCR-amplified cDNA from a patient. The PCR products were digested with restriction enzymes (*Pst*I/*Eco*RI or *Eco*RI/*Hind*III), and the relevant fragment was 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. Growth—at 31°C and at 37°C—and disruption of bacterial cells and analysis by SDS-PAGE, native PAGE, and western blot were performed essentially as described elsewhere (Bross et al. 1993, 1995). Enzyme activity was assayed by ferricinium ion-based analysis using octanoyl-CoA as substrate (Lehman et al. 1990). Measurements of thermal-inactivation profiles were performed as described elsewhere (Bross et al. 1995; Andresen et al. 1997). The concentration of protein in *E. coli* JM109 cell extracts was determined by a modified Bradford assay kit (BioRad). All experiments were performed at least twice, with enzyme-activity measurements performed in duplicate each time.

Results

MS/MS-Based Screening of Newborns

Prospective screening of newborns identified 62 blood spots with a pathological acylcarnitine profile from 930,078 analyzed in the 8-year period from December 1, 1992, through January 31, 2001. All results with a pathological acylcarnitine profile were verified in at least two separate analyses of the blood spot and, in most cases, also in a repeat blood spot specimen obtained at a later date. This indicates a frequency in the population screened of 1/15,001 newborns with a pathological acylcarnitine profile. The profiles observed were rated according to the amounts of acylcarnitines present, and the results are listed in table 1; a “mild” profile was defined as an octanoylcarnitine concentration of 0.5–2.0 $\mu\text{mol/liter}$ and an octanoylcarnitine:decanoylcarnitine ratio of 2–4, and a “severe” profile was defined as an octanoylcarnitine concentration of $>2.0 \mu\text{mol/liter}$ and an octanoylcarnitine:decanoylcarnitine ratio of >4 .

Blood spots from newborns homozygous for the 985A→G mutation always showed a severe profile.

Identification of Mutations in the MCAD Gene

The 62 acylcarnitine-positive blood spots identified by MS/MS-based screening of newborns were first tested for the presence of the common 985A→G mutation, with the LightCycler-based mutation-specific assay. In 60 of these 62 newborns, at least one copy of the 985G→A mutation could be identified: 39 (63%) were homozygous for the 985A→G mutation, 21 (34%) were heterozygous for it, and 2 (3%) did not harbor it at all (table 1). To search for mutations in the 25 alleles not harboring the 985A→G mutation, we performed PCR followed by direct sequencing of the entire protein-coding region and of the 5' UTR harboring the *MCAD* promoter. This analysis was possible for 20 of the 23 blood spots found not to be homozygous for the 985A→G mutation. The results of the sequence analysis are listed in table 1. We identified a non-985A→G mutation in 17 of the 20 analyzed samples. In a sample from an African American baby, we did not identify any mutations, and, in blood spots from three newborns, we identified only one mutation (the prevalent 985A→G mutation in two of the newborns and a 734C→T mutation in the other). In three of these four newborns, the acylcarnitine profile repeatedly showed a pathological profile, indicating that these newborns have a defect in *MCAD* but that we failed to identify the causal mutation. The fourth case is an apparent normal carrier with a single copy of the 734C→T mutation and had a mild acylcarnitine profile that normalized with time. Also the newborn heterozygous for the 985A→G and 351A→C mutations (table 1) had a mild acylcarnitine profile that normalized with time; a completely normal acylcarnitine profile and normal organic acid analysis was obtained from this newborn during an illness when he was admitted because of high fever and poor oral intake. This indicates that the silent mutation 351A→C is neutral and, thus, that this newborn is to be considered only as a normal carrier with a single copy of the 985A→G mutation.

The frequency of all identified mutations was checked in 100 chromosomes from normal controls, by sequence analysis of the relevant exons (exons 3–5, 7, 9, and 10, including part of the flanking introns) from genomic DNA. Four control samples were heterozygous for the 351A→C mutation in exon 5, showing that this mutation is polymorphic in the general population. None of the other sequence variations were identified in controls. None of the newborns had any nonpolymorphic sequence variations in the sequenced part of the 5' UTR. Our sequence analysis of the 100 control chromosomes also revealed three intronic single-nucleotide polymorphisms (SNPs)—IVS3+10T/C, IVS5+32G/C, and

Table 1**Mutations in Asymptomatic Newborns Identified by Prospective MS/MS-Based Screening of Blood Spots**

STATUS OF COMMON MUTATION AND NO. OF BLOOD SPOTS	MCAD MUTATIONAL GENOTYPE						OBSERVED IN PATIENTS	HAPLOTYPE ^b	MS/MS PROFILE	COMMENTS
	985A→G			Second Mutation						
	cDNA	Location	Protein	cDNA	Location	Protein ^a				
Homozygous: 39	985G→A	Exon 11	Lys ³⁰⁴ →Glu	985G→A	Exon 11	Lys ³⁰⁴ →Glu	Yes	A	Severe	One died, two hospitalized with mild hypoglycemia
Heterozygous: 7	985G→A	Exon 11	Lys ³⁰⁴ →Glu	199T→C	Exon 3	Tyr ⁴² →His	No	A	Mild	
1	985G→A	Exon 11	Lys ³⁰⁴ →Glu	233T→C	Exon 4	Ile ⁵³ →Thr	Yes	A	Severe	
1	985G→A	Exon 11	Lys ³⁰⁴ →Glu	244insT	Exon 4	PTC	Yes	C	Severe	
1 ^c	985G→A	Exon 11	Lys ³⁰⁴ →Glu	343-348del	Exon 5	DelGly ⁹⁰ -Cys ⁹¹	No	ND	Severe	Died
1	985G→A	Exon 11	Lys ³⁰⁴ →Glu	351A→C	Exon 5	Silent	Yes	B	Mild	Profile normalized over time
1	985G→A	Exon 11	Lys ³⁰⁴ →Glu	362C→T	Exon 5	Thr ⁹⁶ →Ile	Yes	C	Severe	
1 ^c	985G→A	Exon 11	Lys ³⁰⁴ →Glu	IVS5+1G→A	Intron 5	Splicing	No	A	Severe	Died
1	985G→A	Exon 11	Lys ³⁰⁴ →Glu	489T→G	Exon 7	Silent	No	A	Mild	
1	985G→A	Exon 11	Lys ³⁰⁴ →Glu	IVS8+6G→T	Intron 8	Splicing?	No	A	Mild	
1	985G→A	Exon 11	Lys ³⁰⁴ →Glu	928G→A	Exon 10	Gly ²⁸⁵ →Arg	No	A	Mild	
2	985G→A	Exon 11	Lys ³⁰⁴ →Glu				Yes	C	Severe	
Absent: 1				734C→T	Exon 9	Ser ²²⁰ →Leu	No	A	Mild	Profile normalized over time
1							Yes	A	Severe	

^a PTC = premature termination codon.

^b Alleles with C also had the silent polymorphism 1161G (Andresen et al. 1993b), whereas alleles with A or B had 1161A; ND = not determined.

^c From the pilot study (Ziadeh et al. 1995).

IVS6–22A/C. The frequencies of these SNPs are listed in table 2. Analysis for the three intronic SNPs and 351A/C in individuals who are homozygous for the prevalent 985A→G mutation, in informative families, and in controls showed that these polymorphisms represented only three different haplotypes. The haplotype of the chromosome harboring the non-985A→G mutation in the newborns is also listed in table 1. In all seven heterozygous individuals, the 199T→C mutation is located on a chromosome with the same haplotype (i.e., haplotype A), indicating a common origin of the 199T→C mutant chromosome.

Because the 199T→C mutation in exon 3 was present in as many as 7 (35%) of the 20 sequenced samples with non-985A→G mutations, we decided to estimate the carrier frequency for this mutation in the general U.S. population. For this purpose, we developed a 199T→C mutation-specific LightCycler assay. First, the assay was used to confirm the presence of the 199T→C mutation in the samples from newborns with a pathological acylcarnitine profile for whom this mutation had already been identified by sequence analysis. Next, we tested genomic DNA from dried blood spots from 1,000 randomly selected, anonymous newborns from the U.S. population screened by the LightCycler assay. Two samples were found to be carriers of the 199T→C mutation, indicating that the carrier frequency may be as high as 1:500.

Evaluation of the Effect of the Identified Mutations

We identified 12 different mutations (including 985A→G) in samples from newborns; of these mutations, 5—985A→G, 233T→C, 244insT, 351A→C, and 362C→T—have previously been identified in patients with clinically manifested MCAD deficiency (B. S. Andresen, unpublished data), and the other 7—199T→C, 343–348del, IVS5+1G→A, 489T→G, IVS8+6G→T, 734C→T, and 928G→A—have not previously been identified in patients with a clinical phenotype. That the patient with the IVS5+1G→A mutation died from a metabolic crisis shows, however, that this mutation may result in clinical presentation. Three of the identified mutations—the two silent mutations, 351A→C and 489T→G, and the IVS8+6G→T mutation—are impossible to evaluate at present. We speculate that these three mutations and the IVS5+1G→A mutation affect MCAD mRNA splicing, but it is also possible that 489T→G, IVS8+6G→T, and, in particular, 351A→C are neutral sequence variations. The IVS5+1G→A mutation changes the highly conserved +1G in the splice consensus sequence; and mutations at this position have, to the best of our knowledge, always affected mRNA processing. The 244insT mutation results in a shifted reading frame from codon 57 of the mature MCAD protein,

Table 2

SNPs and Haplotypes Obtained by Sequence Analysis of >100 Controls

POLYMORPHISM	NO. (%) OF ALLELES	HAPLOTYPE		
		A	B	C
IVS3+10T/C:		T	C	C
T	88 (77%)			
C	26 (23%)			
IVS5+32G/C:		C	G	G
G	36 (33%)			
C	72 (67%)			
IVS6–22A/C:		C	A	A
A	25 (21%)			
C	95 (79%)			
351A/C:		A	C	A
A	4 (4%)			
C	108 (96%)			

ending with a premature termination codon 23 codons downstream, and it thus is clearly deleterious. The 343–48del (delGly⁹⁰–Cys⁹¹) could be predicted to be deleterious, because we have shown, by overexpression experiments, that mutation of Cys91 to either tyrosine or glycine leads to a complete lack of functional MCAD (B. S. Andresen and S. Udvari, unpublished data). Furthermore, we have, in clinically affected patients (Andresen et al. 1997; B. S. Andresen, unpublished data), identified mutations that change the codon for Cys91.

To evaluate their effects, the identified missense mutations were characterized by our *E. coli*-based expression system, with and without co-overexpression of the chaperonins GroEL and GroES (Bross et al. 1993, 1995; Andresen et al. 1997). Expression of the recombinant mutant proteins Y42H (199T→C), I53T (233C→T), T96I (362C→T), S220L (734C→T), G285R (928G→A), and K304E (985A→G), at 31°C without GroESL co-overexpression, showed that all except Y42H exhibit a considerably decreased enzyme activity, when compared to the wild-type protein (fig. 2). When the GroESL chaperonins were co-overexpressed, the enzyme activity of I53T, T96I, S220L, G285R, and K304E was increased, indicating that compromised folding is part of the molecular defect mechanism. Analysis by native PAGE/western blotting of the lysates from the *E. coli* cells expressing wild-type MCAD or the mutant proteins showed that the amounts of tetrameric MCAD protein formed corresponded well with the amount of enzyme activity for all mutations except T96I, in which the amount of tetramers was comparable to that in the wild-type protein (results not shown). Analysis of the three-dimensional structure of the MCAD monomer (Kim et al. 1993) shows that T96I is located in the binding site of the acyl moiety of the substrate, indicating that this mutation may affect enzyme activity by interfering with the structure of the active site. That, at 31°C, the activity

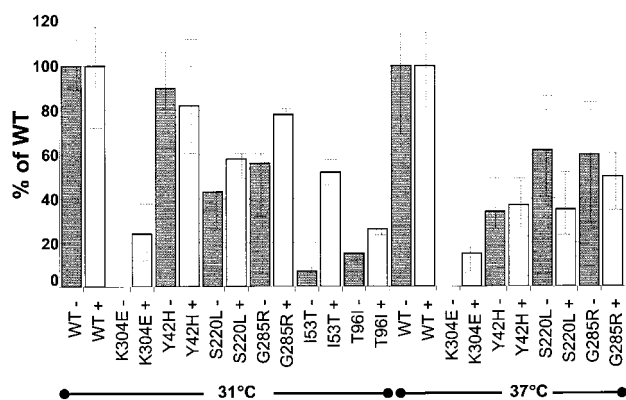


Figure 2 Enzyme activity of mutant MCAD proteins. Transformed JM109 cells harboring expression plasmid encoding the mature part of either wild-type (WT) MCAD protein or one of the mutant MCAD proteins and either the plasmid pGroESL, encoding the chaperonins GroEL and GroES (*unshaded bars*), or the control plasmid pCaP, which lacks the GroESL chaperonin genes (*shaded bars*), grown and induced as described by Andresen et al. (1997). Extracts were investigated by measurement of enzyme activity. The designations of the mutant are indicated below the corresponding bars; + and – respectively indicate whether the chaperonins GroEL and GroES were co-overexpressed. The culture temperature is indicated. MCAD enzyme activity in the soluble fraction was measured by the ferricinium assay (Lehman et al. 1990). Columns represent the average from two to five independent experiments in which enzyme-activity measurements were performed in duplicate in each experiment. The vertical lines on top of the bars indicate the ranges of the values obtained in the experiments.

of cells overexpressing Y42H was nearly indistinguishable from that of cells overexpressing the wild-type protein indicates that this is a mild mutation. We therefore repeated the overexpression experiments for this mutant and the other mild mutations—S220L and G285R—at 37°C, with K304E and the wild-type protein as references (fig. 2). This repetition showed that, at physiological temperature, these mutations all may lead to a decreased enzyme activity. That the residual enzyme activity of the Y42H mutant enzyme at 37°C was slightly increased when the chaperonins GroESL were overexpressed and that a lower culture temperature (31°C) restores enzyme activity to near wild-type levels indicate that this is a mild folding mutation. We also investigated the thermal inactivation profiles of Y42H, S220L, and G285R (fig. 3), in the manner elsewhere described for K304E (Bross et al. 1995), and three other MCAD mutant proteins (Andresen et al. 1997). The thermal stability of G285R was markedly lower than that of wild-type protein, whereas the thermal stabilities of Y42H and S220L were not significantly affected.

Discussion

The primary goal of this study was to validate, by molecular genetic methods, the MS/MS-based method rou-

tinely being used, in several countries, for screening of newborns. Second, we wanted to determine if differences in the biochemical phenotypes of MCAD deficiency, monitored as an acylcarnitine profile in blood spots of newborns, could be explained by differences in the MCAD genotype and, thus, if a correlation between biochemical phenotype and MCAD genotype exists during the asymptomatic period.

From 930,078 newborns screened at NeoGen Screening, we identified 62 newborns with elevated acylcarnitine in their blood spots that was indicative of MCAD deficiency. This is an MCAD-deficiency frequency of 1/15,001, which is much lower than the frequency of 1/8,930 that was reported in the pilot study, in which only 80,371 samples were studied (Ziadeh et al. 1995). The frequency of 1/15,001 is, however, significantly higher than previous estimates based on studies of the 985A→G carrier frequency in the United States (Matsubara et al. 1991; Gregersen et al. 1993). In Texas, molecular testing of 536 blood spots of newborns indicated a carrier frequency of 1/107 in a population of diverse ethnicities (Matsubara et al. 1991); likewise, testing of 3,595 blood spots of newborns from North Carolina indicated a carrier frequency of 1/108 (Gregersen et al. 1993). Subdivision of the blood spots tested in North Carolina showed a carrier frequency of 1/84 in whites and a carrier frequency of 1/492 in nonwhites. In light of the diverse ethnicities of the populations of Pennsylvania, Ohio, Illinois, Florida, New Jersey, and North Carolina, it is reasonable to estimate the carrier frequency to be $\leq 1/80$ in the diverse-ethnicity population screened in the present study, indicating a 985A→G-homozygous frequency of 1/25,000. Thus, we would expect 36–38 985A→G homozygotes in the 930,078 samples screened, and our identification of 39 985A→G homozygotes in the 62 acylcarnitine-positive blood spots tested shows that the MS/MS-based screening method detects the expected number of 985A→G-homozygous newborns. Moreover, this indicates that all 985A→G-homozygous newborns have a diagnostic acylcarnitine profile in their blood spots. We therefore conclude that the MS/MS-based screening method is excellent for detection of individuals homozygous for the prevalent MCAD deficiency-causing mutation 985A→G.

The proportion of the acylcarnitine-positive samples found not to be homozygous for the prevalent 985A→G mutation (i.e., 23 [37%] of the 62 detected) is, however, nearly twice as high as among patients (~20%) who have experienced clinical symptoms of MCAD deficiency (Gregersen et al. 1991; Yokota et al. 1991; Pollitt and Leonard 1998). This skewed ratio could occur if some of the samples that were not homozygous for the 985A→G mutation (mainly 985A→G heterozygotes) were false positives, a possibility that was not addressed

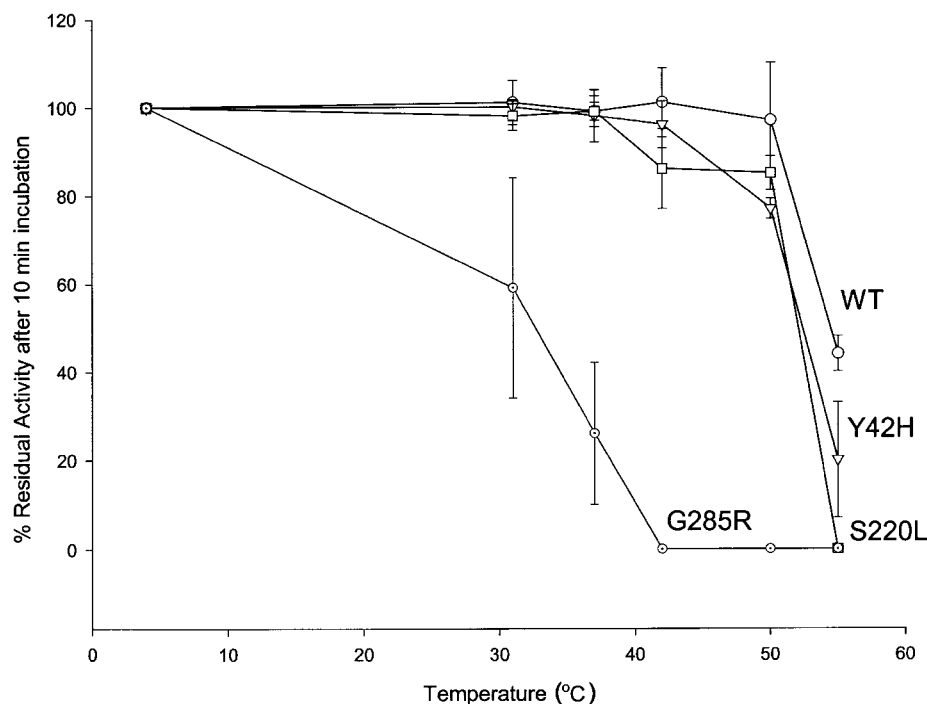


Figure 3 Thermal inactivation profiles of the mutant proteins Y42H, S220L, and G285R and of wild-type (WT) MCAD protein. Investigation of the thermal-inactivation profiles of the mutant proteins Y42H, S220L, and G285R and of wild-type MCAD was performed as described elsewhere (Bross et al. 1995; Andresen et al. 1997). Lysed samples from cells overexpressing the mutant proteins were incubated for 10 min at the temperatures indicated, before MCAD enzyme activity in the soluble fraction was measured by the ferricinium assay. Each value represents the mean of four samples, and the bars represent the ranges of the highest and lowest measurements.

in the pilot study by Ziadeh et al. (1995). So far, preliminary studies have indicated that the acylcarnitine profiles in blood spots from older children who were either 985A→G heterozygotes or 985A→G homozygotes are not always clearly distinguishable (Clayton et al. 1998), whereas the situation seems to be more clear-cut for newborns (Van Hove et al. 1993; Clayton et al. 1998). That, in the present study, we have been able to identify two mutations in all but 4 of the 20 sequenced samples that were not homozygous for the 985A→G mutation strongly suggests that false positives are not the reason for the skewed ratio. Sequencing was not performed on three samples. In three of the four sequenced samples in which two mutations could not be identified, there was a consistent elevation of acylcarnitines on repeated screening, and it is therefore most likely that our lack of identification of two mutations in these samples is caused by the mutations being located outside the examined region of the gene. This is further indicated by the fact that the only sample in which we failed to identify both mutations was from an African American. We have previously investigated a single African American patient for MCAD mutations, and in that case we failed also to identify two disease-causing mutations (B. S. Andresen, unpublished data).

This may indicate that an MCAD mutation exists in African Americans that escapes detection by our procedure.

All identified mutations except two silent mutations and one splice mutation may be predicted to be deleterious. Confirmation of the enzyme defect in three newborns harboring silent/presumed splice mutations must await analysis of cells by specific enzyme assay and/or RNA/cDNA analysis. The 351A→C polymorphism is, however, likely to be neutral, since the newborn with the 985A→G/351A→C genotype showed a normal acylcarnitine profile and normal organic acids during a feverish illness. Our results for *E. coli* expression of the missense mutant proteins proved the deleterious nature of all the missense mutations investigated. The results also illustrate, as described elsewhere (Andresen et al. 1997; Bross et al. 1998), that there is considerable variation in the severity of the different mutations and that the residual enzyme activity of many of the missense mutants may be modulated by endogenous factors, such as the number of available chaperones and the temperature. Despite this, the intrinsic severity of the mutations was reflected in the biochemical phenotype of the newborns and was expressed by differences in their acylcarnitine profile.

Identification of a new mutation, 199T→C, in seven of the samples was particularly interesting. Its presence in such a high proportion of the acylcarnitine-positive samples suggested to us that this mutation must have a rather high carrier frequency in the general population. We therefore developed a mutation-specific assay and, in an analysis of 1,000 blood spots of newborns, determined that the 199T→C mutation had a frequency of 1/500. With a 985A→G carrier frequency of 1/80 and a 199T→C carrier frequency of 1/500, we would expect to detect five to six newborns with a 199T→C/985A→G genotype when 930,078 newborns are screened—if they all exhibit a diagnostic acylcarnitine profile. Although the number of samples is too low to allow any firm conclusions to be drawn, our results indicate that all individuals with this genotype are detected. Because the frequency of 199T→C homozygotes would be ~1/1,000,000, it is not possible to determine, from the present data, whether such individuals also exhibit a diagnostic acylcarnitine profile. A carrier frequency of 1/500 makes the 199T→C mutation one of the three most common mutations in enzymes of mitochondrial fatty-acid oxidation. Its frequency is comparable with that of the common 1528G→C long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) mutation (i.e., 1/175–1/680) (Ibdah et al. 1999; den Boer et al. 2000), which is found in 87% of alleles of patients with LCAD deficiency. The high frequency of the 199T→C mutation in samples from newborns with a diagnostic acylcarnitine profile is also illustrated by our recent identification of a newborn (not included in this study) with a 985A→G/199T→C genotype who was identified by MS/MS-based screening of newborns in New England. In addition, two newborns with this same genotype have recently been identified in a German MS/MS-based newborn-screening program (Lindner et al. 2000), indicating that it is also prevalent in populations outside the United States. Despite this high frequency, the 199T→C mutation has never been observed in our sample of >80 clinically affected patients who were not homozygous for the 985A→G mutation (B. S. Andresen, unpublished data), nor has it been reported in the literature by others. In fact, the large proportion of samples of newborns with the 985A→G/199T→C genotype that are detected by the MS/MS-based screening method is sufficient to explain the lower frequency of the 985A→G mutant allele in this group, as compared to its frequency in the group of patients with clinically manifested MCAD. This, together with the fact that it is a mild missense mutation (as judged both from our overexpression experiments in *E. coli* and from the observation of a mild acylcarnitine profile in the samples), makes it difficult to evaluate the likelihood of clinical symptoms in newborns with the 985A→G/199T→C genotype. Likewise, this evaluation is difficult in newborns with the other

mild mutations (i.e., G285R and S220L). We have previously shown that there is no correlation between the severity of the mutations and the clinical phenotype in patients (Andresen et al. 1997; B. S. Andresen, unpublished data). Patients homozygous for mutations that leave no residual enzyme activity (i.e., frameshift and stop mutations) may remain without clinically manifested disease for years (Andresen et al. 1997; B. S. Andresen, unpublished data), whereas a patient who is compound heterozygous for the 985A→G mutation and a mild mutation—157C→T (R28C), which, in its molecular pathology, is similar to the 199T→C (Y42H) mutation—may die suddenly and unexpectedly (Andresen et al. 1993a). Moreover, individuals with MCAD deficiency may present the disease in manners other than those usually expected (Marsden et al. 1992; Beekman et al. 1994; Ruitenbeek et al. 1995). It could therefore be speculated both (1) that individuals with the mild 985A→G/199T→C genotype—and, perhaps, also individuals with some of the other apparently mild genotypes identified in the present study—may present with clinical symptoms under circumstances other than those usually observed in patients with MCAD deficiency and (2) that this is the reason why no individuals with the mild genotypes have so far been identified among patients with “classical” MCAD. So far, none of the newborns identified by MS/MS-based screening (except for the two newborns described elsewhere [Ziadeh et al. 1995] who died) have had any serious or permanent sequelae. In light of the potential morbidity of the disease, this clearly illustrates the advantages of the MS/MS-based newborn-screening program.

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Electronic-Database Information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for MCAD [MIM 201450])

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