Maple Syrup Urine Disease in Cypriot Families: Identification of Three Novel Mutations and Biochemical Characterization of the p.Thr211Met Mutation in the E1 α Subunit

Theodoros Georgiou,^{1,*} Jacinta L. Chuang,^{2,*} R. Max Wynn,² Goula Stylianidou,^{3,†} Mark Korson,⁴ David T. Chuang,² and Anthi Drousiotou¹

We report five mutations, three of them novel, responsible for maple syrup urine disease in four unrelated Cypriot families. The five children studied are the first cases of classic maple syrup urine disease to be reported among Cypriots. The first novel mutation identified is a single-base deletion in exon 6 of the E1 α gene (c.718delG), which leads to a frameshift after Ala240 and to a stop codon 89 residues further downstream. The other two novel mutations identified are in the E1 β subunit: a two-base deletion in exon 6, c.662_663delCC, which leads to a frameshift after Ala221 and creates a stop codon 17 residues further downstream, as well as a splice mutation, IVS3[+3]delA, which results in the skipping of exon 3. The two known mutations identified are in the E1 α gene: the G > C transversion at the 3'-splice acceptor site, (IVS5-1G > C), which results in the deletion of the entire exon 6, and the missense mutation in exon 5 (c.632C > T), which corresponds to a p.Thr211Met substitution. The p.Thr211Met substitution is located in a potassium-ion pocket in the E1 component required for stability of the bound cofactor thiamine diphosphate. The mutant E1 protein harboring the p.Thr211Met substitution was shown unable to bind thiamine diphosphate, leading to undetectable E1 activity.

Introduction

MAPLE SYRUP URINE DISEASE (MSUD; OMIM 248600) is a genetically heterogeneous metabolic disorder of pan ethnic distribution inherited in an autosomal recessive manner. The worldwide frequency based on data from neonatal screening is approximately 1 in 185,000 newborns (Chuang and Shih, 2001).

MSUD results from a deficiency in one of the three catalytic components of the mitochondrial branched chain α -keto acid dehydrogenase (BCKD) complex: the branched chain α -keto acid decarboxylase or E1 (EC 1.2.4.4.), the dihydrolipoamide branched chain transacylase or E2 (EC 2.3.1.168), and the dihydrolipoyl dehydrogenase or E3 (EC 1.8.1.4.). The E1 component consists of two α and two β subunits encoded by the *BCKDHA* gene on chromosome 19 and the *BCKDHB* gene on chromosome 6, respectively. The E2 subunit is encoded by the *DBT* gene on chromosome 1. The E3 subunit is encoded by the

DLD gene on chromosome 7 and is a common component of three mitochondrial multienzyme complexes. As a result, E3-deficient MSUD presents as combined deficiencies of the BCKD, pyruvate dehydrogenase, and α -ketoglutarate dehydrogenase complexes (Munnich *et al.*, 1982). Apart from the three catalytic components, the BCKD complex contains two regulatory enzymes, the BCKD kinase and the BCKD phosphatase (Chuang and Shih, 2001). The E2 subunit forms the 24-meric cubic core of the complex; to this core multiple copies of E1 and E3 as well as the BCKD kinase and the BCKD phosphatase are attached through noncovalent bonds (Pettit *et al.*, 1978; Chuang, 1998; Åvarsson *et al.*, 2000; Chuang *et al.*, 2001; Harris *et al.*, 2001). The metabolic block in the BCKD complex, which occurs in MSUD results in the accumulation of the branched chain amino acids (BCAA) leucine, isoleucine, and valine and the corresponding branched chain α -keto acids. The elevated branched chain α -keto acid concentrations are responsible for severe clinical consequences, including

¹Department of Biochemical Genetics, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus.

²Departments of Biochemistry and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas.

³Department of Pediatric Neurology, Archbishop Makarios III Hospital, Nicosia, Cyprus.

⁴Children's Hospital, Boston, Massachusetts.

[†]Deceased.

*These two authors contributed equally to this work.

often-fatal ketoacidosis, mental retardation, and neurological impairment (Chuang and Shih, 2001).

There are presently five known MSUD clinical phenotypes based on clinical presentation, tolerance to dietary protein, and response to thiamine and enzyme levels, that is, classic, intermediate, intermittent, thiamine-responsive, and E3-deficient forms (Chuang and Shih, 2001). Classic MSUD has a neonatal onset of encephalopathy and is the most severe and most common form accounting for 75% of MSUD patients. Symptoms include poor feeding, lethargy, seizures, and coma. The majority of untreated classic patients die within the first few months of life from recurrent metabolic crisis and neurologic deterioration. Treatment involves both long-term dietary management and aggressive intervention during acute metabolic decompensation.

The genomic changes that impair BCKD activity can occur in any of the catalytic components of the complex, but both alleles at a single gene locus must harbor mutations (Chuang and Shih, 2001; Henneke *et al.*, 2003; Rodriguez *et al.*, 2006). More than 107 disease-causing mutations in all four molecular phenotypes of MSUD have been identified (HMGD-Cardiff mutation database) of which 36 (34%) are in the E1 α subunit, 31 (29%) in the E1 β subunit, 26 (24%) in the E2 subunit, and 14 (13%) in the E3 subunit. Many of these mutations have been characterized, for example, the p.Tyr438Asn substitution in the E1 α , which is present in Mennonite patients with classic MSUD, was shown to impede E1 assembly resulting in the preferential degradation of E1 β (Fisher *et al.*, 1991). The crystallographic studies (Ævarsson *et al.*, 2000) showed that the p.Tyr438Asn mutation disrupts the E1 α -E1 β subunit interface, preventing the dimerization of heterodimeric intermediates into a functional E1 component.

In the present report, we describe the first five Cypriot patients with classic MSUD. All disease alleles have been characterized. Two known mutations in the E1 α subunit, one novel mutation in the E1 α subunit, and two novel mutations in the E1 β subunit have been identified. The c.632C > T mutation in exon 6 of the E1 α subunit has been biochemically characterized.

Materials and Methods

Subjects

The present study includes all known cases of MSUD that have been diagnosed in Cyprus, a country with a population of 780,000 and a birth rate of approximately 10,000 per year. The families gave informed consent for this study. All four families are of Greek Cypriot origin with no consanguinity, and unrelated between them. Basic clinical and biochemical data are summarized in Table 1.

Tissue cultures

Lymphoblastoid and/or fibroblast cell lines were established from the patients or their parents according to standard procedures (Miller and Lipman, 1973).

Sequence analysis

DNA was extracted from peripheral blood leukocytes or cell cultures using standard procedures (Miller *et al.*, 1988). Total RNA was extracted from cultured lymphoblasts or fibroblasts using the RNeasy kit (Cat. No. 74104) by Qiagen

(Chatsworth, CA). To synthesize the first-strand E1 α cDNA and E1 β cDNA from total RNA, the Omniscript™ reversed transcriptase from Qiagen (Cat. No. 205111) was used. The cDNAs obtained were used as template for PCR amplification using standard protocols and the Taq DNA polymerase (Qiagen).

The procedure for every fragment started with a 3-min denaturation step at 94°C, followed by 36 cycles of 60 s denaturation, 30 s annealing time at 55°C, and 60 s extension at 72°C. After the last cycle, the samples were incubated for 5 min at 72°C for final extension. PCR products were sequenced using an ABI Prism™ model 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). The sequences of the primers used to synthesize and sequence the E1 α cDNA and E1 β cDNA were previously reported (Chuang *et al.*, 2004).

Western blot analysis

Western blot analysis was performed on homogenates from cultured lymphoblasts using previously published procedures (Chuang and Chuang, 2000). After washing, the blots were incubated for 1 h with a secondary antibody, rabbit IgG-horseradish peroxidase conjugated (Biorad Cat. No. 172-1019, Hercules, CA), at a dilution of 1:50,000. The bound antibodies were detected by Pierce SuperSignal Substrate (Cat. No. 34080; Pierce, Rockford, IL).

Production of wild-type and mutant proteins

The Altered Site *in vitro* mutagenesis system (Promega, Madison, WI) was used to introduce the p.Thr211Met (A $\overline{\text{C}}$ G to A $\overline{\text{T}}$ G) mutation into the cDNA for the mature E1 α subunit. Detailed protocols for the mutant vector construction and subsequent mutagenesis were described previously (Chuang *et al.*, 1995). Briefly, oligonucleotides for the desired mutations and the β -lactamase repair primer were annealed to the single-stranded form of pAlter- α or pAlter- β vector. After the second strand synthesis and two rounds of ampicillin selection, clones harboring the correct mutations were isolated for plasmid preparation. DNA segments containing the mutations were used for cassette replacements of the expression vector pHis-TEV-E1 for wild-type E1, which contained a tobacco-etch virus protease cleavage site after the 6xHis tag (Chuang *et al.*, 1995).

Both wild-type and mutant recombinant His₆-tagged E1 heterotetramer were efficiently expressed in *Escherichia coli* strain CG-712 (ES^{ts}) by cotransformation of the pGroESL plasmid overproducing chaperonins GroEL and GroES as described previously (Wynn *et al.*, 1992, 1998). Wild-type and mutant His₆-tagged E1 heterotetramers were isolated from cell lysates using a Ni²⁺-NTA-derivatized Sepharose CL-6B column (Qiagen) as also described previously (Chuang *et al.*, 2000). E1 proteins were further purified on a Superdex-200 gel filtration column (2.6×60 cm) connected to an FPLC system (Amersham Pharmacia Biotech, now GE Healthcare, Piscataway, NJ). The column buffer consisted of 50 mM potassium phosphate (pH 7.5), 250 mM KCl, 10% (v/v) glycerol, 5 mM dithioerythritol, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. E1 activity during purification was assayed spectrophotometrically (see below). Protein concentrations were determined using the Coomassie Plus

TABLE 1. CLINICAL AND BIOCHEMICAL DATA OF CYPRIOT MAPLE SYRUP URINE DISEASE PATIENTS

Family	Patient/sex	Clinical features	Age at diagnosis	BCAA μM	Urine organic acids	BCKD nmol/h/mg prot.	Outcome
I	1/M	Lethargy, feeding problems, brain edema, delayed motor development, speech delay, nystagmus, bilateral cataract, seizures, abnormal CT scan	13 days	Alleu: 213 Ileu: 337 (NR: 26–100) Leu: 3855 (NR: 65–220) Val: 621 (NR: 90–300)	N.D.	0.009 (NR: 0.177) (Lymphoblasts)	Died 4½ years
II	2/M	Weakness, feeding problems, dyspnea, seizures	1½ months	Ileu: 963 (NR: 41–93) Leu: 4966 (NR: 85–169) Val: 1106 (NR: 161–285)	Increased: 2-OH-isovaleric 2-keto-isovaleric 2-keto-3-methylvaleric 2-keto-iso-caproic	N.D.	Died 4 months
II	3/F	Weakness, feeding problems, dyspnea, seizures	9 days	Increased Leu, Ileu & Valine	N.D.	N.D.	Died 12 days
III	4/F	Lethargy, feeding problems, hypotonia/hypertonia, tachypnea, grunting, abnormal EEG	19 days	Alleu: Oileu: 220 (NR: 26–100) Leu: 3270 (NR: 65–220) Val: 459 (NR: 90–300)	Increased:lactate 2-keto-glutarate 2-keto-isocaproic 2-OH-isovaleric 2-keto-isovaleric 2-keto-3-methylvaleric 4-OH-phenylacetate 4-OH-phenylpyruvate	0.03 (NR: 0.4–3.4) (Fibroblasts)	Died 1 month
IV	5/F	Feeding problems, hypotonia, poor head control, seizures, mitral insufficiency, anemia, abnormal MRI	22 days	Alleu: 185 Ileu: 410 (NR: 26–100) Leu: 2873 (NR: 65–220) Val: 445 (NR: 90–300)	Increased:lactate 2-keto-isocaproic 2-OH-isovaleric 2-keto-isovaleric 2-keto-3-methylvaleric 2-OH-3-methylvaleric 3-methylvaleric 4-OH-phenylacetate 4-OH-phenylpyruvate	Undetectable (NR: 0.4–3.4) (Fibroblasts)	Died at 3½ months

BCAA, branched chain amino acids; BCKD, branched chain α -keto acid dehydrogenase; NR, normal range; N.D., not done; CT, computed tomography; EEG, electroencephalogram; MRI, magnetic resonance imaging.

protein reagent from Pierce with absorbance read at 595 nm. Alternatively, during enzyme purification, protein concentrations were determined by the direct measurement of absorbance at 280 nm using a calculated molar extinction coefficient of $1.15 \text{ cm}^{-1} \text{ mg}^{-1} \text{ mL}$ for the $\alpha_2\beta_2$ heterotetramer.

Enzyme assays

The residual activity of the BCKD complex in lymphoblastoid cell lines or fibroblasts from MSUD patients was assayed based on the rate of decarboxylation of α -keto ($1\text{-}^{14}\text{C}$) isovalerate (KIV) using intact cells as described previously (Chuang *et al.*, 2000).

The BCKD complex was reconstituted with E1, lipoylated E2 (lip-E2), and E3 at a molar ratio of 12:1:55 as described previously (Chuang *et al.*, 2000). The oxidative decarboxylation of KIV catalyzed by the reconstituted complex was monitored by the increase of absorbance at 340 nm.

Thiamine diphosphate (ThDP)-mediated decarboxylation by the isolated E1 component was assayed at 25°C with KIV as substrate in the presence of an artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP) as described previously (Wynn *et al.*, 1992). The oxidation of enamine-ThDP ($\text{RCOH} = \text{ThDP}$) by DCPIP converts enamine-ThDP to a free acid (RCOOH), which promotes the turnover of enamine-ThDP with concomitant release of the product acid from E1. Reduction of DCPIP was monitored by the decrease in absorbance at 600 nm.

Binding measurements based on tryptophan fluorescence quenching

Steady-state fluorescence quenching upon ThDP binding to wild-type and p.T211M- α mutant E1 was measured using an LS50 B luminescence spectrometer (PerkinElmer Life Sciences, Waltham, MA) in the photon counting mode (Hennig *et al.*, 1966). Fluorescence intensities were recorded at 25°C using a 3-mL quartz cuvette at an excitation wavelength of 290 nm and an emission wavelength of 335 nm. Slit widths were set at 5 nm for excitation and 10 nm for emission. A 290 nm cut-off emission filter was installed to reduce light scattering effects. Protein concentrations for E1 and ThDP ($A_{235} \text{ nm} = 11,300 \text{ M}^{-1} \text{ cm}^{-1}$, $\text{pH} > 7.0$) were determined spectrophotometrically. The concentration for all protein samples was $0.25 \mu\text{M}$ (as heterotetramers) in 50 mM HEPES buffer, $\text{pH} 7.5$, 200 mM KCl, and 1 mM MgCl_2 . Fluorescence readings were

corrected for dilution and inner filter effects using Equation 1 (Lakowicz *et al.*, 1983).

$$F_{\text{corr}} = F_{\text{obs}}(V/V_0) \times 10^{0.5d(A_{\text{ex}} + A_{\text{em}})} \quad (1)$$

where F_{corr} is the corrected fluorescence intensity value, F_{obs} is the experimentally measured fluorescence intensity, V_0 is the initial volume of the sample, V is the volume after adding ThDP, d is the path length of the cuvette, A_{ex} is the absorption of the sample at the excitation wavelength, and A_{em} is the absorption of the sample at the emission wavelength. Three different readings were taken and averaged, and the experiment was repeated three times ($n = 3$). The binding data were fitted by nonlinear regression using the program Prism 4 (GraphPad Software, San Diego, CA) according to Equation 2 for a bimolecular reaction (Nemeria *et al.*, 2001).

$$(-\Delta F/F_0) = (-\Delta F_{\text{max}}/F_0) \times [\text{ThDP}] / (K_d + [\text{ThDP}]) \quad (2)$$

where ΔF is the corrected fluorescence change, F_0 is the fluorescence intensity before the addition of ThDP, ΔF_{max} is the maximal fluorescence change, K_d is the dissociation constant, and $[\text{ThDP}]$ is the concentration of ThDP in the cuvette. The parameters determined by the fitting procedure were ΔF_{max} and K_d .

Results and Discussion

In this study, we present the clinical, biochemical, and molecular characteristics of the first five reported cases of MSUD in Cyprus. All five patients, from four unrelated families, were of the classic phenotype with age at presentation ranging from 9 days to 1.5 months. The diagnosis was based on their clinical features, elevated branched chain amino acids/organic acid levels, and/or residual activities of the BCKD complex (Table 1). Patient 1 developed bilateral cataract at the age of 17 months, a finding that has only been reported twice before for MSUD patients (le Roux *et al.*, 2006; Bellini *et al.*, 2007).

Five mutations were found to be responsible for MSUD in the Cypriot families, three of which are novel (Table 2). The first patient was found to be homozygous for a previously described mutation in the E1 α subunit (IVS5-1G > C), which results in the deletion of the entire exon 6 in the mRNA. This mutation was first identified in a Muslim family from Jordan

TABLE 2. MAPLE SYRUP URINE DISEASE MUTATIONS DETECTED IN THE *BCKDHA* AND *BCKDHB* GENES

Family	Affected locus	Nucleotide change	Location exon/intron	Protein prediction	Type of mutation
I	Father	E1 α	IVS5-1G>C	Intron 5	Exon 6 deletion
	Mother	E1 α	IVS5-1G>C	Intron 5	Exon 6 deletion
II	Father	E1 β	IVS3[+3]delA ^a	Intron 3	Exon 3 deletion
	Mother	E1 β	c.662_663delCC ^a	Exon 6	p.Ala221fr17X Deletion
III	Father	E1 α	c.718delG ^a	Exon 6	p.Ala240fr89X Deletion
	Mother	E1 α	c.632C>T	Exon 5	p.Thr211Met Missense
IV	Father	E1 α	c.718delG ^a	Exon 6	p.Ala240fr89X Deletion
	Mother	E1 α	IVS5-1G>C	Intron 5	Exon 6 deletion Splicing

DNA mutation numbering is based on GenBank accession number NM_00709.2 for *BCKDHA* and NM_000056.2 for *BCKDHB* considering nucleotide +1 as the A of the ATG translation-initiation codon.

^aNovel mutations.

FIG. 1. Western blot analysis using homogenated lymphoblasts. The membranes (300 μ g of lymphoblast homogenate/lane) were probed either with E1 α and E1 β antibodies (upper panel) or with E2 antibodies (lower panel). Markers of bovine E1 α and E2 subunits are shown in the first lane. Patient 1 is an maple syrup urine disease patient homozygous for the E1 α (IVS5-1 G > C) mutation.

(Chuang and Chuang, 2000). Western blot analysis showed the absence of both the E1 α and the E1 β subunit of the protein (Fig. 1). The absence of the E1 α protein is expected. Interestingly, the E1 β subunit that does not carry a mutation is also absent, a finding similar to that observed with the p.Tyr438-Asn missense mutation in the E1 α subunit of the Mennonite classic MSUD patients (Fisher *et al.*, 1989, 1991; Chuang *et al.*, 1994). The absence of the E1 β subunit due to mutations in the E1 α subunit likely results from the fact that the unassembled E1 β subunit is largely insoluble and is degraded in the cell. We have shown that in the chaperonin-assisted E1 assembly pathway, the E1 β subunit maintains its solubility through assembly with the E1 α subunit as a heterodimeric intermediate, which subsequently dimerizes into an active heterotetramer (Chuang *et al.*, 1999; Wynn *et al.*, 2000). This thesis is corroborated by our previous studies that showed that transfection of the wild-type E1 α subunit into the E1 α -affected MSUD cells restores the assembly and presence of the normal E1 β subunit in cultured cells from Mennonite MSUD patients (Fisher *et al.*, 1991; Koyata *et al.*, 1993). A total of five prenatal diagnoses were performed for this family (family I), and all five fetuses were found to be affected (four of the five prenatal diagnoses were performed both by DNA analysis and by measurement of the activity of the BCKD complex activity in chorionic villi).

Since no biological samples were available from the MSUD patients of the second family, we performed mutational analysis on the parents. The father was found to carry a novel mutation in E1 β subunit, IVS3[+3]delA, which affects the splicing mechanism. As a result, the entire exon 3 is skipped in the mRNA (Fig. 2). Sequencing of the E1 α cDNA and the E1 β cDNA in the mother showed no mutations. After the identification of the mutation in the E1 β subunit in the father, exons of the E1 β subunit from the mother were sequenced using genomic DNA. A novel frameshift mutation, c.662_663delCC,

FIG. 2. (A) Nucleotide sequencing of the E1 β subunit (cDNA) showing (a) normal and (b) heterozygous for IVS3[+3]delA. (B) Schematic description of the mutation.

was identified in exon 6. This mutation leads to a frameshift after Ala221 and creates a stop codon 17 residues further downstream of the sequence (Table 2). The reason that this mutation was not identified in the cDNA was probably due to the production of an unstable mutant mRNA, which could not possibly be amplified by RT-PCR. Since the mother was heterozygous for this specific mutation, one was only able to sequence the cDNA from the normal allele. Extensive carrier screening in this family identified nine carriers, three with the E1 β c.662_663delCC mutation and six with the E1 β IVS3[+3]delA mutation.

Analysis of the cDNA of the proband of the third MSUD family revealed a missense mutation in exon 5 of the E1 α subunit, replacing a threonine by a methionine residue at position 211 of the precursor sequence. The p.Thr211Met mutation (Thr166Met according to the mature sequence) was reported earlier by us in a homozygous Thai classic MSUD patient (Ævarsson *et al.*, 2000; Chuang and Shih, 2001). The recombinant mutant E1 protein carrying the p.Thr211Met substitution shows no detectable activity of E1-catalyzed decarboxylation and no overall activity of the BCKD complex when reconstituted with this E1 variant (Table 3). The results establish that the p.Thr211Met missense mutation is a disease-causing mutation for classic MSUD.

Amino acid sequence analysis using the Ensemble database showed that p.Thr211 is invariant across species, which suggests that a substitution by methionine is likely to have profound effects on the structure of the protein. The conserved p.Thr211 is one of the amino acid ligands (others are

TABLE 3. TURNOVER NUMBERS (k_{CAT}) FOR THE E1-CATALYZED DECARBOXYLATION OF α -KETOISOVALERATE AND THE OVERALL REACTION OF THE BRANCHED CHAIN α -KETO ACID DEHYDROGENASE COMPLEX FOR WILD-TYPE E1 AND THE P.T211M-AMUTANE E1

E1 protein	Decarboxylation ^a k_{cat} (min^{-1})	BCKDC overall reaction k_{cat} (min^{-1})
Wild-type	12 ± 0.5	198 ± 9.8
p.T211M- α	0	0

The reduction of DCPIP or NAD^+ was determined by monitoring the decrease in absorbance at 600 nm and the increase in absorbance at 340 nm, respectively, as described in Materials and Methods section.

^aKinetic values are averages of three independent experiments. DCPIP, 2,6-dichlorophenolindophenol.

p.Ser206, p.Ser207, p.Leu209, and p.Gln212) in the potassium-binding pocket of the E1 α subunit, which maintains a loop conformation through interactions between main chain oxygen atoms and the potassium ion (Fig. 3A). This loop conformation carries Leu209, an essential hydrophobic determinant that stabilizes the aminopyrimidine ring of the bound cofactor ThDP. The introduction of a bulky methionine residue likely

impedes the access of the potassium ion to the binding pocket. This could also lead to a collapse of the potassium-binding pocket and the resultant absence of bound ThDP in the p.Thr211Met E1 variant. This hypothesis was corroborated by ThDP-binding studies based on tryptophan fluorescence quenching, which shows nonmeasurable binding of the ThDP cofactor by the mutant protein (Fig. 3B). Under the same experimental conditions, wild-type E1 shows a hyperbolic-responsive curve for fluorescence quenching as a function of increasing ThDP concentrations, with a dissociation constant (K_d) of 1.3 μM for ThDP. Thus, the inability to bind the cofactor essential for the decarboxylation reaction explains the absence of overall activity of the BCKD complex caused by the p.Thr211Met mutation. These results provide the biochemical mechanism underlying the classic MSUD phenotype caused by the p.Thr211Met mutation in the E1 α subunit. Moreover, studies of this missense mutation substantiate the critical role that the residue p.Thr211 of the E1 α subunit plays in fostering cofactor ThDP binding by the E1 component of the BCKD complex.

The proband of family III appeared to be homozygous for the p.Thr211Met mutation. However, when genomic DNA from this patient was examined, the mutation was found to be present in the heterozygous state. Sequencing of the genomic DNA from the parents showed the mother to be a carrier for

FIG. 3. Absence of thiamine diphosphate (ThDP) binding caused by the p.Thr211Met mutation in the E1 α subunit. **(A)** The location of the p.Thr211Met mutation in the potassium-ion binding pocket of the E1 α subunit. The potassium ion (purple spheres) is held in the pocket by an octahedral coordination imparted by the ligands provided by either side chains or main chain oxygen atoms (red sticks in the stick model) of amino acids Ser206, Ser207, Leu209, Thr211, and Gln212 (labeled in single-letter codes) and two water molecules (red spheres), W1 and W2. The side chain of Leu209 on the loop forms hydrophobic interactions with the aminopyrimidine ring of ThDP (in pink) to stabilize the cofactor. In the p.Thr211Met mutation, the side chain of the substituted methionine residue (yellow for the sulfur atom) is rotated away from the potassium ion and likely hinders the access of the metal ion to the binding pocket. The collapse of the potassium-ion binding pocket in turn destabilizes the bound cofactor ThDP, resulting in an inactive mutant E1 protein. The molecular graphics of the 1.88-Å E1 structure (PDB code 2j9f) of the human branched chain α -keto acid dehydrogenase complex was generated using PyMol. **(B)** The absence of ThDP binding by the p.Thr211Met E1 variant determined by fluorescence quenching. Wild-type or the p.Thr211Met E1 mutant in 0.25 μM (as heterotetramer) was titrated with increasing concentrations of ThDP (0–25 μM). The fluorescence emitted by Trp136 of the E1 β subunit upon ThDP binding was recorded at 335 nm and corrected for inner filter effect of ThDP (see Materials and Methods section). Decreases in tryptophan fluorescence from that of apo-E1 in the absence of ThDP are expressed as % quenching and plotted against the ThDP concentration. Curve fitting using Prism 4 produces a dissociation constant (K_d) of 1.3 μM for ThDP of wild-type E1 (■). The p.Thr211Met mutant E1 protein shows negligible ThDP binding as depicted by the horizontal trace (▼).

this mutation but the father to be normal. This result suggested that the father's mutation did not allow the production of a stable mutant mRNA, and therefore the paternal mutation could not be detected in the proband's cDNA, making the p.Thr211Met mutation appear homozygous. Subsequent sequencing of the genomic DNA of the proband revealed a novel single base deletion in exon 6, c.718delG, which leads to a frameshift after Ala240 and creates a stop codon further downstream of the amino acid sequence (Table 2). The presence of the c.718delG mutation was confirmed in the father's E1 β genomic DNA.

The proband of the fourth family was found to be a compound heterozygote for the IVS5-1G > C mutation found in family I and the novel c.718delG mutation found in family III (Table 2). It is interesting that five different mutations were found in the four families without evidence for a founder effect. The most frequent mutation, the IVS5-1G > C (deletion of exon 6), found in three out of eight alleles, might prove to be common in the Middle East since it has previously been reported in a family originating from Jordan (Nemeria *et al.*, 2001 plus personal communication). Population screening for this mutation in Cyprus and other neighboring countries might prove rewarding in this respect.

Acknowledgments

This work was supported in part by the Ministry of Health of the Republic of Cyprus, a training grant from the Cyprus Human Resources Authority (to T.G.) and Grant DK26758 from the National Institutes of Health (to D.T.C.).

Electronic-Database Information

The URLs for data presented in this article are as follows:
HDGM: www.hgmd.cf.ac.uk/hgmd0.html
OMIM 248600.

Disclosure Statement

No competing financial interests exist.

References

- Ævarsson A, Chuang JL, Wynn RM, *et al.* (2000) Crystal structure of human branched-chain alpha-ketoacid dehydrogenase and the molecular basis of multienzyme complex deficiency in maple syrup urine disease. *Structure* 8:277–291.
- Bellini LP, Brum GS, Coelho J (2007) Cataract in MSUD. *J Inher Metab Dis* 30:123.
- Chuang D, Shih V (2001) *The Metabolic and Molecular Basis of Inherited Disease*. McGraw-Hill, New York, pp 1971–2005.
- Chuang DT (1998) Maple syrup urine disease: it has come a long way. *J Pediatr* 132:S17–S23.
- Chuang DT, Chuang JL, Wynn RM, Song JL (2001) *Encyclopedia of Molecular Medicine*, volume 5. John Wiley & Sons, Inc., New York, pp 393–396.
- Chuang JL, Chuang DT (2000) Diagnosis and mutational analysis of maple syrup urine disease using cell cultures. *Methods Enzymol* 324:413–423.
- Chuang JL, Davie JR, Chinsky JM, *et al.* (1995) Molecular and biochemical basis of intermediate maple syrup urine disease. Occurrence of homozygous G245R and F364C mutations at the E1 alpha locus of Hispanic-Mexican patients. *J Clin Invest* 95:954–963.
- Chuang JL, Davie JR, Wynn RM, Chuang DT (2000) Production of recombinant mammalian holo-E2 and E3 and reconstitution of functional branched-chain alpha-keto acid dehydrogenase complex with recombinant E1. *Methods Enzymol* 324:192–200.
- Chuang JL, Fisher CR, Cox RP, Chuang DT (1994) Molecular basis of maple syrup urine disease: novel mutations at the E1 alpha locus that impair E1(alpha 2 beta 2) assembly or decrease steady-state E1 alpha mRNA levels of branched-chain alpha-keto acid dehydrogenase complex. *Am J Hum Genet* 55:297–304.
- Chuang JL, Wynn RM, Moss CC, *et al.* (2004) Structural and biochemical basis for novel mutations in homozygous Israeli maple syrup urine disease patients: a proposed mechanism for the thiamin-responsive phenotype. *J Biol Chem* 279:17792–17800.
- Chuang JL, Wynn RM, Song JL, Chuang DT (1999) GroEL/GroES-dependent reconstitution of alpha2 beta2 tetramers of human mitochondrial branched chain alpha-ketoacid decarboxylase. Obligatory interaction of chaperonins with an alpha beta dimeric intermediate. *J Biol Chem* 274:10395–10404.
- Fisher CR, Chuang JL, Cox RP, *et al.* (1991) Maple syrup urine disease in Mennonites. Evidence that the Y393N mutation in E1 alpha impedes assembly of the E1 component of branched-chain alpha-keto acid dehydrogenase complex. *J Clin Invest* 88:1034–1037.
- Fisher CW, Chuang JL, Griffin TA, *et al.* (1989) Molecular phenotypes in cultured maple syrup urine disease cells. Complete E1 alpha cDNA sequence and mRNA and subunit contents of the human branched chain alpha-keto acid dehydrogenase complex. *J Biol Chem* 264:3448–3453.
- Harris RA, Huang B, Wu P (2001) Control of pyruvate dehydrogenase kinase gene expression. *Adv Enzyme Regul* 41:269–288.
- Henneke M, Flaschker N, Helbling C, *et al.* (2003) Identification of twelve novel mutations in patients with classic and variant forms of maple syrup urine disease. *Hum Mutat* 22:417.
- Hennig J, Kern G, Keef H, *et al.* (1966) *Biochemistry and Physiology Of Thiamin Diphosphate Enzymes*. AuC Intemann, Prien, Germany.
- Koyata H, Cox RP, Chuang DT (1993) Stable correction of maple syrup urine disease in cells from a Mennonite patient by retroviral-mediated gene transfer. *Biochem J* 295 (Pt 3):635–639.
- Lakowicz JR, Maliwal BP, Cherek H, Balter A (1983) Rotational freedom of tryptophan residues in proteins and peptides. *Biochemistry* 22:1741–1752.
- le Roux C, Murphy E, Lilburn M, Lee PJ (2006) The longest-surviving patient with classical maple syrup urine disease. *J Inher Metab Dis* 29:190–194.
- Miller G, Lipman M (1973) Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. *Proc Natl Acad Sci USA* 70:190–194.
- Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215.
- Munnich A, Saudubray JM, Taylor J, *et al.* (1982) Congenital lactic acidosis, alpha-ketoglutaric aciduria and variant form of maple syrup urine disease due to a single enzyme defect: dihydrolipoyl dehydrogenase deficiency. *Acta Paediatr Scand* 71:167–171.
- Nemeria N, Yan Y, Zhang Z, *et al.* (2001) Inhibition of the *Escherichia coli* pyruvate dehydrogenase complex E1 subunit and its tyrosine 177 variants by thiamin 2-thiazolone and thiamin 2-thiothiazolone diphosphates. Evidence for reversible tight-binding inhibition. *J Biol Chem* 276:45969–45978.

- Pettit FH, Yeaman SJ, Reed LJ (1978) Purification and characterization of branched chain alpha-keto acid dehydrogenase complex of bovine kidney. *Proc Natl Acad Sci USA* 75:4881–4885.
- Rodriguez-Pombo P, Navarrete R, Merinero B, *et al.* (2006) Mutational spectrum of maple syrup urine disease in Spain. *Hum Mutat* 27:715.
- Wynn RM, Davie JR, Chuang JL, *et al.* (1998) Impaired assembly of E1 decarboxylase of the branched-chain alpha-ketoacid dehydrogenase complex in type IA maple syrup urine disease. *J Biol Chem* 273:13110–13118.
- Wynn RM, Davie JR, Cox RP, Chuang DT (1992) Chaperonins groEL and groES promote assembly of heterotetramers (alpha 2 beta 2) of mammalian mitochondrial branched-chain alpha-keto acid decarboxylase in *Escherichia coli*. *J Biol Chem* 267:12400–12403.
- Wynn RM, Song JL, Chuang DT (2000) GroEL/GroES promote dissociation/reassociation cycles of a heterodimeric intermediate during alpha(2)beta(2) protein assembly. Iterative annealing at the quaternary structure level. *J Biol Chem* 275:2786–2794.

Address correspondence to:

Anthi Drousiotou, Ph.D.

Department of Biochemical Genetics

The Cyprus Institute of Neurology and Genetics

P.O. Box 23462

1683 Nicosia

Cyprus

E-mail: anthidr@cing.ac.cy