Maple syrup urine disease (MSUD) results from mutations affecting different subunits of the mitochondrial branched-chain α-ketoacid dehydrogenase complex. In this study, we identified seven novel mutations in MSUD patients from Israel. These include C219W- and H391R substitutions in the E1 subunit; H156Y- (CAT to TAT), V69G- (GTT to GGT), IVS 9 del (+7−4), and 1109 ins 8bp (exon 10) in the E1β subunit; and H391R (CAC to CGC) and S133stop (TCA to TGA) affecting the E2 subunit of the branched-chain α-ketoacid dehydrogenase complex. Recombinant E1 proteins carrying the C219W-α or H156Y-β mutation show no catalytic activity with defective subunit assembly and reduced binding affinity for cofactor thiamin diprophosphate. The mutant E1 harboring the V69G-β substitution cannot be expressed, suggesting aberrant folding caused by this mutation. These E1 mutations are ubiquitously associated with the classic phenotype in homozygous-affected patients. The H391R substitution in the E2 subunit abolishes the key catalytic residue that functions as a general base in the acyltransfer reaction, resulting in a completely inactive E2 component. However, wild-type E1 activity is enhanced by E1 binding to this full-length mutant E2 in vitro. We propose that the augmented E1 activity is responsible for robust thiamin responsiveness in homozygous patients carrying the H391R E2 mutation and that the presence of a full-length mutant E2 is diagnostic of this MSUD phenotype. The present results offer a structural and biochemical basis for these novel mutations and will facilitate DNA-based diagnosis for MSUD in the Israeli population.

Maple syrup urine disease (MSUD) is an autosomal recessive metabolic disorder caused by deficiency in the mitochondrial branched-chain α-ketoacid dehydrogenase (BCKD) complex. The BCKD complex catalyzes the oxidative decarboxylation (Reaction 1) of three branched-chain α-ketoacids (BCKAs) derived from branched-chain amino acids (BCAAs) leucine, isoleucine, and valine (1).

\[ \text{R-COOK} + \text{CoA-S} + \text{NAD}^+ \rightarrow \text{R-CO-S-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \]

**Reaction 1**

The metabolic block in the BCKD complex results in the inability of MSUD patients to degrade BCKA. The elevated BCKA levels produce severe clinical consequences including often-fatal ketoacidosis, mental retardation, and neurological impairment. There are presently five known MSUD clinical phenotypes (i.e. classic, intermediate, intermittent, thiamin-responsive, and E3-deficient forms) (2). The classic form with the most severe phenotype, was originally reported by Menke et al. in 1954 (3) and manifests within the first 2 weeks of life by poor feeding, lethargy, seizures, coma, and death if left untreated. The classic form accounts for 75% of MSUD patients (1). Intermediate MSUD is associated with progressive mental retardation and developmental delay without a history of catastrophic illness. An intermittent form of MSUD has normal levels of BCAA as well as normal intelligence and development until a stress such as infection precipitates metabolic decompensation with ketoacidosis. Thiamin-responsive MSUD is similar to the intermediate or intermittent phenotype but responds to pharmacological doses of thiamin with normalization of the BCAA levels (2). The E3-deficient MSUD is caused by defects in the dihydrolipamide dehydrogenase (E3) component of the BCKD complex, which is common to that of the pyruvate and α-ketoglutarate dehydrogenase complexes. Patients with E3 deficiency display a combined dysfunction of the three α-ketoacid dehydrogenase complexes (4). Two prevailing mutations (G229C and Y335stop) in the E3 gene have been identified in the Ashkenazi Jewish community in Israel (5). Most of the Israeli patients who were homozygous for the G229C E3 mutation presented in early childhood or later with recurrent episodes of vomiting, encephalopathy, and prolonged prothrombin time, occasionally associated with lactic acidosis and ketoacidosis. Patients who were compound-heterozygous for these two mutations presented neonatally with more severe sequelae.

The mammalian BCKD multi-enzyme complex is a 4 × 10^6 dalton metabolic machine organized around a cubic core comprising 24 lipoate-bearing dihydrolipoyl transacylase (E2) subunits, to which multiple copies of branched-chain α-ketoacid
decarboxylase (E1), E3, a specific kinase, and a specific phosphatase are attached through ionic interactions. The kinase and the phosphatase are responsible for the regulation of BCKD complex by a reversible phosphorylation (inactivation)/dephosphorylation (activation) cycle (6). The E1 component is a thiamin diphosphate (ThDP)-dependent enzyme consisting of two E1α and two E1β subunits. The E3 component is a homodimeric flavoprotein. There are in total six genetic loci that encode subunits of the BCKD complex. Mutations in the four different catalytic subunits (E1α, E1β, E2, and E3) have been described in MSUD patients (2). Genetic subtypes of MSUD have been proposed to indicate the altered subunit in the BCKD complex (2). These include type IA MSUD affecting the E1α subunit, type IB affecting the E1β subunit, type II affecting the E2 subunit, and type III affecting the E3 subunit. Types IV and V, which have not been reported, are reserved for MSUD in which the kinase and the phosphatase, respectively, are affected. Except for type II and III MSUD, which are linked to the thiamin-responsive (2, 7, 8) and E3-deficient phenotypes (4), respectively, a tight correlation between a specific genetic subtype and a particular clinical phenotype of MSUD has not been demonstrated.

Reaction steps catalyzed by the three enzyme components, based largely on those elucidated for the related pyruvate dehydrogenase complex (9), are as follows.

R-CO–COOH + E1-ThDP → E1–R-CO–OH-ThDP + CO₂

E1–R–CO–COO⁻ + ThDP + E2–lipSH₂ → E2–R–CO–SH + E1–ThDP

E2–R–CO–SH + CoASH → E2–CoASH + E1–thiolase

E2–(SH)₂ → E2–(lipSH)₂ + E3–(FAD–SH)₂

E3–(FAD–SH)₂ + NAD⁺ → E3–(FAD–S)₂ + NADH + H⁺

REACTIONS 2–6

The E1 component binds ThDP and catalyzes a ThDP-mediated decarboxylation of α-ketoacids (Reaction 2), and subsequent reduction of the lipoyl moiety, which is covalently attached to E2 (Reaction 3). The lipoyl-bearing domain (LBD) carrying the S-acyl dilehydrodiamine serves as a "swinging arm" (10) to transfer the acyl group from E1 to the E2 active site giving rise to acyl-CoA (Reaction 4). Finally, the E3 component with a tightly bound FAD moiety reoxidizes the didehydrodiamine residue on E2 (Reaction 5) with NAD⁺ as the ultimate electron acceptor (Reaction 6). The overall reaction is the production of branched-chain acyl-CoA, CO₂, and NADH from BCKAs (Reaction 1).

We previously described two novel missense mutations in MSUD patients from the non-Jewish Druze kindred in Israel (11). The incidence of MSUD in Israel is relatively high, presumably as a result of consanguinity (12). Toward the goal of providing a wider spectrum of MSUD mutations in this region, we continue to analyze cell cultures derived from Israeli patients with MSUD. Lymphoblasts were prepared from blood samples by infection with the Epstein-Barr virus, and lymphoblastoid cell cultures were grown and assayed for the decarboxylation of α-keto-1-4-cislosovalerate by intact cells as described previously (15).

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Cultures—**Blood samples (15 ml) were withdrawn from classic MSUD patients B.R., W.A., D.M., K.Y., and C.R. and from classic MSUD patients H.C. and H.S., all from Israel. Lymphoblasts were prepared from blood samples by infection with the Epstein-Barr virus, and lymphoblastoid cell cultures were grown and assayed for the decarboxylation of α-keto-1-4-cislosovalerate by intact cells as described previously (15).

**Nucleotide Sequencing for MSUD Mutations—**The first-strand E1α cDNA was synthesized from the total RNA prepared from patients’ cells using the primer 5'-GAAGACAGTGTTGCTGCT-3' (cDNA sequence 1461–1442) and the Omniscript™ reverse transcription from Qiagen (Chatsworth, CA). The subsequent PCR amplification was carried out using the forward primer 5'-CAGACCGCTGATGTGCTG-3' (positions 1–18) and the reverse primer 5'-CTTACATGGGCTACTCTG-3' (positions 1425–1404). For the first-strand E1β cDNA synthesis, the primer 5'-GTGAAAACTTTTGACAGCAATATCAGTGG-3' (positions 1367–1339) was employed. The first round PCR was carried out using the forward primer 5'-TGGCGGCTGATAGCTAGG-3' (positions 8–27) and the reverse primer: 5'-AAAAGGAGTAACTGCGAGGAAG-3' (positions 1312–1295). To amplify the 5' segment of the E1β cDNA, a second round PCR was performed using the forward primer 5'-ATGGCGGTGGTACGCGG-3' (positions 48–64) and the reverse primer 5'-CAGGCAAATAGATTAAAAGACT-3' (positions 878–858). The 3' region of the E1β cDNA was amplified in a parallel second round PCR utilizing the forward primer 5'-ATACCCCCATTGTTAGACAAAGATTT-3' (positions 409–438) and as a reverse primer the above first-strand primer for the E1β cDNA. To synthesize the first-strand E2 cDNA, the primer 5'-CAGCTAGGTCTAGATACCT-3' (positions 1523–1504) was utilized. The ensuing PCR amplification was performed with the forward primer 5'-TCCCAGTGAAATGCTCG-3' (positions 5–22) and the reverse primer 5'-GTTCAAAATGTCTAAAGGCTCTACAG-3' (positions 1496–1477). PCR products were sequenced with an ABI Prism™ model 377 automated DNA sequencer manufactured by Applied Biosystems (Foster City, CA).

**Production of Wild-type and Mutant Proteins—**The pTrcHisB expression plasmid (Invitrogen) for N-terminally His₆-tagged wild-type E1 was constructed as described previously (16). The same plasmids carrying MSUD mutations were produced using the QuickChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions. Wild-type and mutant E1 proteins were expressed in Escherichia coli by co-transformation with the pGroESL plasmid over expressing chaperonins GroEL and GroES, as described previously (17, 18). His₆-tagged wild-type and mutant E1 were purified with Ni²⁺-nitrilotriacetic acid resin and Resource Q and Superdex-200 columns (17) and used for biochemical studies without removal of the His₆-tag. The E2 24-mer was expressed in XL1-Blue cells and lipoylated in vitro with bacterial lipoyl ligase as described previously (19). C-terminal His₆-tagged E2 domain constructs LBD2 (residues 1–99) containing the lipoyl domain and the C-terminal linker and the di-domain (DD) (residues 1–167) containing the LBD and subunit-binding domain (SBD) were prepared as described previously (20). Lipoylated LBD2 (lip-LBD2) and DD (lip-DD) were prepared as described above. To produce the SBD, a tobacco-etch virus protease site (LENLYFQ) was cleaved by exogenously added NtdCI (21). The E3 construct was inserted into the linker between the lipoyl-bearing and subunit binding domains. The purified C-terminally His₆-tagged DD was digested with the tobacco-etch virus protease and the SBD was extracted by Ni²⁺-nitrilotriacetic acid resin.
H.C. is the third child of first generation Arabs. He was delivered uneventfully after a normal pregnancy. The parents reported irritability of the child since the first week of life. Brain MRI reveals leukodystrophy, which in combination with elevated levels of BCAAs led to the diagnosis of MSUD. The patient underwent hemodialysis and was placed on a MSUD formula supplemented with thiamin at the dosage of 100 mg/day. The BCAA levels decreased to normal after 3 days. On follow-ups, the plasma BCAA levels remained in the normal range (50–100 nmol/ml) for the next 4 years. The patient consumed 4 times the leucine levels that were allowed for MSUD patients, but during acute infections his BCAA and allosleucine levels rose only slightly above the normal range. At age 5, he had spastic cerebral palsy and his IQ was 80. H.S. was also born uneventfully after a normal pregnancy. Parents of H.S. and H.C. were first-degree cousins; therefore, dietary restriction was instituted for H.S after birth. She was diagnosed with MSUD at 2 days of age, and thiamin supplement (100 mg/day) was added to the restricted diet. At 1 week of age, H.S. was discharged and given breast milk at home. Three days later, she was readmitted with vomiting, apathy, and encephalopathy with the plasma leucine level at 1,800 nmol/ml, and underwent hemodialysis. Since this episode, the regimen of restricted diet with thiamin supplements was reinstated and maintained. At 18 months of age, H.C. showed normal growth and development.

Identification of the Affected BCKD Subunit in MSUD Patients—Since the human BCKD complex consisted of six different subunits, it was necessary for mutational analysis to identify which subunit was affected in the MSUD patient of interest. Homogenates of cultured lymphoblasts from the eight Israeli patients were subjected to Western blotting using antibodies against E1 (both E1α and E1β subunits) or E2 as a probe. Cells from the classic patient B.R. showed reduced levels of both the E1α and the E1β subunit, with the level of the E2 subunit in the normal range. Levels of both E1α and E1β subunits were below the detection limit in cells from classic patients W.A., D.M., and K.Y. The data strongly suggested that either the E1α or the E1β locus was affected in these Israeli MSUD patients. Individual E1 subunits are not stable in cells, and a mutation that impedes the stability of either the E1α or the E1β subunit usually results in reduced levels or the absence of both subunits in cells from MSUD patients (7). Levels of both E1α and E1β subunits were below the detection limit in cells from classic patients W.A., D.M., and K.Y. The data strongly suggested that either the E1α or the E1β locus was affected in these Israeli MSUD patients. Individual E1 subunits are not stable in cells, and a mutation that impedes the stability of either the E1α or the E1β subunit usually results in reduced levels or the absence of both subunits in cells from MSUD patients (7). Levels of both E1α and E1β subunits were below the detection limit in cells from classic patients W.A., D.M., and K.Y. The data strongly suggested that either the E1α or the E1β locus was affected in these Israeli MSUD patients. Individual E1 subunits are not stable in cells, and a mutation that impedes the stability of either the E1α or the E1β subunit usually results in reduced levels or the absence of both subunits in cells from MSUD patients (7).

Novel MSUD Mutations in the BCKD Complex—Samples of total RNA isolated from cells of MSUD patients were amplified by reverse transcriptase-PCR using primers corresponding to the cDNA sequence of the affected subunit. Nucleotide sequencing identified mutations in different subunits of the BCKD complex. All mutations in mRNA were confirmed by sequencing of the corresponding gene of the human BCKD complex in these patients. Table I shows seven novel MSUD mutations (types IA, IB, and II) identified in the eight Israeli patients investigated. A homozygous C to G type IA missense mutation C219W (TGC → TGG) is present in classic patient B.R. Two homozygous type IB mutations, H156Y (CAT → TAT) and V69G-β (GTT → GGT), occur in classic patients W.A. and D.M., respectively. Another classic patient, K.Y., harbors two compound heterozygous type IB alleles: a 4-base pair deletion in intron 9 (IVS9 del[7–4]), resulting in the deletion of entire exon 10, and an 8-base pair insertion in exon 10 (1,109

\[
\Delta F = \frac{F_0 - F_{\text{m}}}{F_0} \times [\text{ThDP}]/K_D + \text{ThDP})
\]

where \(\Delta F\) represents the fluorescence change corrected for dilution and inner filter effects (26), \(F_0\) is the fluorescence intensity prior to the addition of ThDP, \(\Delta F_{\text{m}}\) is the maximal fluorescence change, \(K_D\) is the dissociation constant, and [ThDP] is the concentration of ThDP in the cuvette. The parameters determined by the fitting procedure were \(\Delta F_{\text{m}}\) and \(K_D\).

Treatment of Mutant E1 Proteins with Natural Osmylate Trimethylamine N-Oxide—Wild type or mutant E1 proteins (200 μg/ml) were incubated with different concentrations of trimethylamine N-oxide (TMAO) at 23 °C for 16 h in buffer A that contained 50 mM potassium phosphate, pH 7.5, 100 mM KCl, 2 mM ThDP, 2 mM MgCl₂, and 2 mM dithiothreitol, 0.6 μM lysine, and a mixture of protease inhibitors (Roche Applied Science, Indianapolis, IN). Activity of the BCKD complex reconstituted with TMAO-treated wild-type or mutant E1 was assayed as described above.

RESULTS

Clinical Phenotypes—B.R., W.A., D.M., and K.Y. of Israeli origin manifested the classic MSUD phenotype as described above. T.G. and C.R. were from the Druze kindred in Israel, who suffered also from the classic MSUD phenotype with a turbulent neonatal presentation. Both exhibited mild to moderate developmental retardation with leukodystrophy in brain MRI. Furthermore, both patients had recurrent encephalopathic episodes during viral infections or suspected dietary noncompliance. T.G. underwent hemodialysis at age 2 and 4, which may have also resulted from dietary noncompliance.

\[
R - COO\cdot COOH + DCPIP(\text{oxidized}) + H_2O \\
\rightarrow R - COO\cdot CO_2 + DCPIP(\text{reduced}) + H^+ \\
\]

Reaction 7

The assay mixture contained 100 mM potassium phosphate, pH 7.5, 2.0 mM MgCl₂, 0.2 mM ThDP, and 0.1 mM DCPIP. The rate of decarboxylation at 30 °C was measured by monitoring the reduction of the dye at 600 nm. The acyltransferase activity (Reaction 4) of E2 was assayed using a model reaction (Reaction 8) with [1-14C]isovaleryl-CoA and dihydrolipoamide (Lip(SH)₂) as substrates.

\[
[1-14C]R - COO\cdot S - CoA + Lip(SH)₂ \rightarrow [1-14C]R - S - Lip - SH + CoA
\]

Reaction 8

The assay mixture contained 100 mM MOPS, pH 7.5, 2.5 mM tr-dihydrolipoamide, and 2.5 mM [1-14C]isovaleryl-CoA. The reaction product S-[1-14C]isovaleryl dihydrolipoamide (R-S-Lip-SH) was extracted with benzene as also described previously (22).
Activity Measurement of MSUD Mutant Proteins—The E1 component of the human BCKD complex carrying one of the above MSUD missense mutations was expressed in E. coli co-transformed with the pGroESL plasmid overexpressing chaperonins GroEL and GroES. The yield for the mutant E1 protein carrying the C219W-α mutation was 87% of the wild type. The H156Y-β mutant E1 protein was poorly expressed with a yield of 18% of the wild-type. The V69G-β mutant E1 was not expressed in the soluble fraction of transformed BL-21 cells. Mutant E2 harboring the H391R missense mutation was as efficiently expressed as the wild-type without the co-transformation of chaperonins GroEL and GroES and existed as a stable homo-24-mer as determined by gel filtration.

The activity of the BCKD complex (Reaction 1) reconstituted with stoichiometric amounts of E1, E2, and E3 components was measured using α-keto[1-[14]C]isovalerate as a substrate. The wild-type human BCKD complex when reconstituted with normal E1, E2, and E3 exhibits a decarboxylation rate of 196 min\(^{-1}\). A substitution of the wild-type E1 with a mutant carrying the C219W-α or H156Y-β mutation does not produce BCKD activity in the presence of normal E2 and E3. Direct measurements of E1 component activity using 2,6-dichlorophenolindophenol as an electron acceptor (Reaction 7) confirmed the absence of E1 activity in the C219W-α and H156Y-β E1 variants (data not shown). Similarly, no BCKD activity was reconstituted when the wild-type E2 was replaced by a mutant containing the H391R alteration. The activity of the E2 component alone, based on the transfer of the radiolabeled acyl moiety from [1-[14]C]isovaleryl-CoA to dihydriodiolamide (Reaction 8), was measured. The wild-type E2 showed a specific activity of 1.97 nmol/min/mg, whereas the mutant E2 harboring the H391R substitution exhibited no acyltransferase activity. The above data establish that the C219W-α and H156Y-β substitutions are the cause of E1-deficient or type IA MSUD, whereas the H391R-E2 mutation is the culprit for E2-deficient or type II MSUD. The remaining three mutations in Table I (i.e. intron 9 (IVS9 del[7–4]) and exon 10 (1,109 ins 8bp) type IB gene alterations and S133stop type II subunit truncation) are obligatory null mutants and therefore also responsible for MSUD.

Assembly State of Wild-type and Mutant E1 Proteins—We have shown previously that the folded E1α and E1β subunits assemble into a heterodimeric intermediate that subsequently dimerizes to form a functional native E1 heterotetramer (16, 27). To dissect the effect of these novel MSUD mutations on E1 assembly, purified wild-type and mutant E1 proteins were subjected to a 10–30% sucrose density gradient centrifugation, and collected fractions were analyzed by SDS-PAGE coupled with Coomassie Blue staining. Both α and β subunits of wild-type E1 migrate as a single species consistent with a 170-kDa heterotetramer (Fig. 1). The mutant E1 carrying the C219W-α substitution exists as two major species, one corresponding to the heterotetramer and the other migrating as a soluble high molecular weight form. A small amount of heterodimeric species that sediments close to the top of the gradient is also present in this mutant. The H156Y-β mutant E1 occurs in solution in multiple species ranging from heterodimers to heterotetramers to the higher molecular weight form.

Affinity of Mutant E1 for Cofactor ThDP—Effects of the above MSUD mutations on the ability of mutant E1 to bind cofactor ThDP were studied. The heterotetrameric fractions of wild-type and mutant E1 proteins separated on a FPLC Superdex-200 column were collected and used for cofactor binding studies. The heterodimeric species does not bind ThDP, since the cofactor-binding pocket is formed at the interface between two heterodimers. \(K_d\) values for ThDP independent of the E1 concentration were determined by measuring tryptophan fluorescence quenching upon cofactor binding. Wild-type E1 shows a \(K_d\) value of 1.52 \(\mu M\) (Table II). \(K_d\) values of 60.4 \(\mu M\) and 104

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### Table I

| Patient  | Genetic subtype | Altered subunit | Residual protein | MSUD allele | Gene/protein alteration | Genotype | Clinical phenotype |
|----------|-----------------|-----------------|------------------|-------------|-------------------------|----------|-------------------|
| B.R.     | IA              | E1a             | Yes              | C219W-α     | TGC to TGG              | --/--    | Classic           |
| W.A.     | IB              | E1b             | No               | H156Y-β     | CAT to TAT              | --/--    | Classic           |
| D.M.     | IB              | E1b             | No               | V69G-β      | GTT to GGT              | --/--    | Classic           |
| K.Y.     | IB              | E1b             | No               | IVS 9 del[7–4] | Exon 10 deleted | +/--    | Classic           |
| H.C.     | II              | E2              | Yes              | H391R       | CAT to CGC              | --/--    | Thiamin-responsive |
| H.S.     | II              | E2              | Yes              | H391R       | CAT to CGC              | --/--    | Thiamin-responsive |
| T.G.     | II              | E2              | No               | S133stop    | TCA to TGA              | --/--    | Classic           |
| C.R.     | II              | E2              | No               | S133stop    | TCA to TGA              | --/--    | Classic           |

* IVS, intervening sequence or intron; intronic deletion beginning at 7 bases and ending at 4 bases upstream of the intron/exon 10 junction (see Ref. 30 for nomenclature).

* ins, insertion; 8-bp insertion at base 1,109 of the human E1β cDNA sequence corresponding to exon 10 (GenBank accession number U51015).


**Structural and Biochemical Basis for MSUD**

**TABLE II**

| E1 protein   | $K_d$ | Maximal quenching |
|--------------|-------|-------------------|
| Wild type    | 1.52 ± 0.01 | 60.1 ± 1.7 |
| C219W-α      | 60.4 ± 8.9   | 29.1 ± 1.5   |
| H156Y-β’     | 104 ± 6.8    | 47.4 ± 1.7    |

* Dissociation constants represent the average of three independent measurements.

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**DISCUSSION**

In the present study, we were able to produce sufficient quantities of mutant E1 proteins for biochemical characteriza-
tion using the chaperonin-augmented expression system (17, 18), with the exception of the V69G-β mutation. The cotransformation with chaperonins GroEL and GroES is essential for proper folding and assembly of the native wild-type E1 heterotetramer in E. coli. The yield for the H156Y-β mutation was 20% of the wild type, and the yield for the C219W-α mutation was comparable with the wild type. The inability to express the V69G-β E1 protein suggests that this mutation causes a misfolding of the E1β subunit in E. coli, which cannot be corrected by the overexpression of chaperonins GroEL and GroES. The wild-type E2 24-mer can be readily expressed in XL1-Blue E. coli cells without cotransformation with the chaperonins, indicating that the intrinsic chaperonins are sufficient for the folding and assembly of E2. The H391R E2 mutation does not appear to affect folding and assembly of E2, since the yield of this mutant is comparable with that of the wild-type E2.

The crystal structure of human E1 was previously determined at 2.7-Å resolution (29). The overall structure is organized in a tetrahedral arrangement with each lobe roughly corresponding to one of the four subunits. The two active sites that accommodate cofactor ThDP are at the interfaces of α-β' and α' -β subunits. Novel structural K⁺ ion-binding pockets are present in both α and β subunits. Our laboratory has recently refined the human E1 structure to 1.8-Å resolution (24). This high resolution structure is used to provide a structural basis for the present novel MSUD mutations in Israeli patients. As shown in Fig. 4A, the Mn²⁺ ion in the ThDP-binding pocket coordinates to side chain Asn²²²-α and Glu¹⁹³-α and the main-chain carbonyl oxygen atom of Tyr²²⁴-α (not shown) as well as two phosphate oxygen atoms of cofactor ThDP. The Cys²¹⁹-α residue is in the immediate vicinity of the ThDP-binding pocket. The replacement of Cys²¹⁹-α by a large bulky Trp residue in the C219W-α mutation is likely to produce a clash with Glu¹⁹³-α and Asn²²²-α, thereby disrupting the octahedral coordination of the Mn²⁺ ion. This explains the significant reduction in the affinity of this mutant for cofactor ThDP (Table II). The strained conformation surrounding the ThDP-binding site probably accounts for the loss of E1 activity in the C219W-α variant. However, the binding of ThDP cannot correct this structural defect, as indicated by the absence of activity for the BCKD complex reconstituted with this mutant and measured at a saturating cofactor concentration. Since this mutation is away from the subunit interface, the structural basis for the appearance of high molecular weight species in addition to the heterotetramer in this E1 mutant is not immediately clear.

The H156Y-β mutation occurs at a tight interface between β and β' subunits along a pseudo-2-fold symmetry (Fig. 4B). In the wild-type structure, His¹⁵⁶-β in the β subunit is hydrogen-bonded to Thr²⁵⁴-β' on the opposite β' subunit. A substitution of the His¹⁵⁶-β by a tyrosine residue disrupts this hydrogen bond and therefore impedes the β-β' subunit interactions required for heterotetrameric assembly. This results in the formation of soluble heterodimer and soluble high molecular weight aggregates in addition to the heterotetramer. The defective subunit interactions leading to improper assembly apparently accounts for loss of E1 activity in this mutant. The assembly defect is confirmed by the restoration of the H156Y-β mutant BCKD activity from null to 6% of the wild-type by incubating with the naturally occurring osmolyte TMAO (Fig. 2). We have shown previously that TMAO forces the assembly of otherwise permanently locked inactive heterodimers caused by MSUD mutations into a partially active heterotetramer (28). TMAO promotes protein folding by preferential hydration of the exposed peptide backbone of an unfolded protein (31).
This entropically unfavorable situation promotes the folding of the unfolded protein into a native conformation. The H156Y-β mutation is 15–20 Å away from the ThDP-binding site, and therefore the impaired β-β’ subunit interactions may also have an adverse effect on the cofactor binding. This is confirmed by the 2-order of magnitude increase in the binding constant (an adverse effect on the cofactor binding. This is confirmed by the crystal structure of dihydrolipoyl acetyltransferase from Azotobacter vinelandii (33). The main-chain carbonyl oxygen from His391 forms a hydrogen bond to the N-1 nitrogen atom of its own side chain to increase the basicity of the N-3 atom in the same imidazole ring. The nucleophilic attack by the N-3 nitrogen atom on the thiol group of CoA produces a tetrahedral intermediate involving S-acyldihydrolipoamide carried by the E2 subunit, with the intermediate stabilized by Ser338 through hydrogen bonding. The abstraction of the N-3 atom of His391 by the tetrahedral intermediate results in the production of dihydrolipoamide on E2 and free acyl-CoA.

Fig. 5. His391 in the E2 subunit as a general base in the acyltransfer reaction catalyzed by E2. The proposed reaction scheme involving His391 and Ser338 is based on the crystal structure of dihydrolipoyl acetyltransferase from A. vinelandii (33). The main-chain carbonyl oxygen from His391 forms a hydrogen bond to the N-1 nitrogen atom of its own side chain to increase the basicity of the N-3 atom in the same imidazole ring. The nucleophilic attack by the N-3 nitrogen atom on the thiol group of CoA produces a tetrahedral intermediate involving S-acyldihydrolipoamide carried by the E2 subunit, with the intermediate stabilized by Ser338 through hydrogen bonding. The abstraction of the N-3 atom of His391 by the tetrahedral intermediate results in the production of dihydrolipoamide on E2 and free acyl-CoA.
studied previously (8) including the first patient (WG-34) described by Scriver et al. (35), who was found to contain one allele expressing a full-length F215C mutant E2 (36) (Table III). In contrast, the nine type II patients that manifest the classic MSUD phenotype including the two in this study do not contain at least one allele that produces a full-length mutant E2 protein. These null mutations, as defined by the absence of a full-length E2 protein, are accompanied by the complete absence of residual decarboxylation activity in intact cells derived from these patients.

**Table III**

Mutant alleles and clinical phenotypes of type II MSUD patients

| Patient | Allele*          | Residual intact cell activity % | Residual E2 protein | Clinical phenotype | Reference/source |
|---------|------------------|--------------------------------|---------------------|--------------------|------------------|
| H.C.    | H391R (homozygous) | 11                             | Yes                 | Thiamin-responsive | This study       |
| H.S.    | H391R (homozygous) | 11                             | Yes                 | Thiamin-responsive | This study       |
| WG-34   | F215C*/IVS4(del[-3.2kb:-15]) | 30–40                         | Yes                 | Thiamin-responsive | 8, 36            |
| MGL-290 | R240C/H281T       | 30                             | Yes                 | Thiamin-responsive | 2                |
| RD1     | P73R/G292R        | 18                             | Yes                 | Thiamin-responsive | 8                |
| MGF-497 | R230G/IVS4(del[-3.2kb:-15]) | 14                             | Yes                 | Thiamin-responsive | 8                |
| C.R.    | S133stop (homozygous) | 0                             | No                  | Classic            | This study       |
| T.G.    | S133stop (homozygous) | 0                             | No                  | Classic            | This study       |
| S.G.    | IVS4(del[-3.2kb:-15]) (homozygous) | 0                             | No                  | Classic            | 8                |
| S.B.    | IVS4(del[-3.2kb:-15])/IVS4(del[-15:-4]) | 0                             | No                  | Classic            | 8                |
| A.L.    | Δ27E/T7T          | 0                              | No                  | Classic            | 2                |
| DaRa    | 354 del 7 (homozygous) | 0                             | No                  | Classic            | 2                |
| C.E.    | 90delAT/IVS5(del[−1−1]) | 0                             | No                  | Classic            | 8                |
| W.J.    | IVS10(del[−9+1]) (homozygous) | ND*                           | No                  | Classic            | 8                |
| A.E.    | IVS9–7A–G (homozygous) | 0                             | No                  | Classic            | 8                |

* The allele name conforms to the recommendations of the Ad Hoc Committee on Mutations Nomenclature (30).

† Phenylalanine to cysteine substitution.

‡ IVS, intervening sequence or intron; del, deletion; intronic deletion beginning at 3.2 kb and ending at 15 bases upstream of the intron 4/exon 5 junction.

§ Deletion of the last base of exon 5 and the first base of intron 5 at the exon 5/intron 5 junction.

* Not determined.
Thiamin-responsive MSUD is unique among vitamin-treatable metabolic disorders in that the E1 component binds ThDP derived from thiamin but does not carry disease-causing mutations (37). In light of the apparent requirement for the presence of a full-length mutant E2 and the data from E1 activity measurements, a mechanism for the thiamin-responsive phenotype can be proposed (Fig. 6). In the E2 null mutations such as S133stop, the E1 component alone is capable of decarboxylating BCKAs according to Reaction 1. This explains the high (30–40%) residual decarboxylation activity obtained with WG-34 cells, despite the presence of only one full-length E2-expressing allele (Table III).

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Structural and Biochemical Basis for Novel Mutations in Homozygous Israeli Maple Syrup Urine Disease Patients: A PROPOSED MECHANISM FOR THE THIAMIN-RESPONSIVE PHENOTYPE

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