

Biochemical Basis of Type IB (E1 β) Mutations in Maple Syrup Urine Disease

A PREVALENT ALLELE IN PATIENTS FROM THE DRUZE KINDRED IN ISRAEL*

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Maple syrup urine disease (MSUD) is a metabolic disorder associated with often-fatal ketoacidosis, neurological derangement, and mental retardation. In this study, we identify and characterize two novel type IB MSUD mutations in Israeli patients, which affect the E1 β subunit in the decarboxylase (E1) component of the branched-chain α -ketoacid dehydrogenase complex. The recombinant mutant E1 carrying the prevalent S289L- β (TCG \rightarrow TTG) mutation in the Druze kindred exists as a stable inactive $\alpha\beta$ heterodimer. Based on the human E1 structure, the S289L- β mutation disrupts the interactions between Ser-289- β and Glu-290- β' , and between Arg-309- β and Glu-290- β' , which are essential for native $\alpha_2\beta_2$ heterotetrameric assembly. The R133P- β (CGG \rightarrow CCG) mutation, on the other hand, is inefficiently expressed in *Escherichia coli* as heterotetramers in a temperature-dependent manner. The R133P- β mutant E1 exhibits significant residual activity but is markedly less stable than the wild-type, as measured by thermal inactivation and free energy change of denaturation. The R133P- β substitution abrogates the coordination of Arg-133- β to Ala-95- β , Glu-96- β , and Ile-97- β , which is important for strand-strand interactions and K $^+$ ion binding in the β subunit. These findings provide new insights into folding and assembly of human E1 and will facilitate DNA-based diagnosis for MSUD in the Israeli population.

Maple syrup urine disease (MSUD)¹ or branched-chain ketoaciduria is an autosomal recessive metabolic disorder in the catabolism of branched-chain α -ketoacids (BCKAs) derived from branched-chain amino acids (BCAAs) leucine, isoleucine,

and valine (1). The accumulated BCKAs and BCAAs are secreted in the urine, giving rise to a distinct maple syrup odor and hence the name of the disease (2). Based on variations of clinical presentation, there are currently five different forms of MSUD (1). The classic form, which accounts for 75% of MSUD patients, is manifested within the first 2 weeks of life by poor feeding, lethargy, seizures, coma, and death if left untreated. Intermediate MSUD is associated with elevated levels of BCAAs and BCKAs, with progressive mental retardation and developmental delay without a history of catastrophic illness. An intermittent form of MSUD has normal levels of BCAAs, normal intelligence, and development until a stress (e.g. infection) precipitates in decompensation with ketoacidosis without seriously affecting intelligence and development. Thiamine-responsive MSUD is similar to the intermediate or intermittent phenotype but responds to pharmacologic doses of thiamine with returns to the normal levels of BCAAs (3). The E3-deficient MSUD is caused by defects in the dihydrolipoyl dehydrogenase (E3) (see below). Patients with E3 deficiency have combined enzyme impairments in α -ketoacid dehydrogenase complexes and usually die in infancy with severe lactic acidosis (4).

The enzyme affected in MSUD, the mitochondrial branched-chain α -ketoacid dehydrogenase (BCKD) complex, is a multienzyme complex of 4–5 million daltons. It is organized about a 24-meric cubic core of dihydrolipoyl transacylase (E2). Attached to the E2 core are multiple copies of branched-chain α -ketoacid decarboxylase (E1), E3, BCKD kinase, and BCKD phosphatase (5, 6). The kinase and the phosphatase tightly regulate activity of the BCKD complex by reversible phosphorylation (inactivation)/dephosphorylation (activation) (7). The E1 component is a TDP-dependent enzyme consisting of two α and two β subunits. The E3 component is a homodimeric flavoprotein and is common among α -ketoacid dehydrogenase complexes comprising pyruvate dehydrogenase, α -ketoglutarate dehydrogenase, and BCKD complexes. Therefore, there are six genetic loci that contribute to the BCKD complex, and mutations in the four catalytic subunits (E1 α , E1 β , E2, and E3) have been reported in MSUD patients (1). On the basis of the affected subunit in the BCKD complex, MSUD is classified into six genetic subtypes (1). Among them, type IA MSUD affects the E1 α subunit; type IB affects the E1 β subunit; type II affects the E2 subunit; and type III affects the E3 subunit. Type IV and type V MSUD involve the kinase and the phosphatase, respectively, in which the disease-causing mutations have not been detected.

The crystal structure of the human E1 $\alpha_2\beta_2$ heterotetramer was recently determined to 2.7-Å resolution (8). Each of the two

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¹ The abbreviations used are: MSUD, maple syrup urine disease; BCKA, branched-chain α -ketoacid; BCAA, branched-chain amino acid; BCKD, branched-chain α -ketoacid dehydrogenase; E1, branched-chain α -ketoacid decarboxylase; E2, dihydrolipoyl transacylase; E3, dihydrolipoamide dehydrogenase; FPLC, fast protein liquid chromatography; GdnHCl, guanidine hydrochloride; KIV, α -ketoisovalerate; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; TDP, thiamine diphosphate.

binding sites for cofactor TDP is located in the interface between α and β subunits. The E1 structure also discloses that the extended small C-terminal region protruding from the bulk of the E1 α subunit is essential for the interaction between heterologous α and β subunits. This segment is referred to as the "Menonite region" because it contains the type IA Y393N- α mutation, which is prevalent in the Menonite population (9, 10). The tyrosine to asparagine conversion at position 393 of the α subunits abrogates the interaction between α and β' as well as α' and β subunits, thereby preventing heterotetramer assembly, with the mutant E1 locked in an inactive heterodimeric conformation (11). The other two type IA mutations in the Menonite region, Y368C- α and F364C- α , also disrupt the heterologous α and β subunit interactions, resulting in the inability to assemble into the native heterotetrameric conformation of E1.

We have recently studied MSUD mutations in Israeli patients, in particular those from the non-Jewish Druze kindred. The incidence of MSUD in the kindred is relatively frequent due to consanguinity. We report a homozygous mutation in the Druze MSUD patients, which affects the E1 β subunit. This novel type IB mutation apparently disrupts β/β' subunit interactions, resulting in the formation of inactive E1 heterodimers, similar to the Menonite Y393N- α type IA substitution (11). The second type IB mutation, which occurs in Jewish patients in Israel and the U.S., affects the folding and stability of the mutant E1 in a temperature-sensitive manner. The genetic and biochemical information presented here provides structural insights into folding and assembly of the E1 heterotetramer and will facilitate DNA-based detection of these type IB MSUD alleles in the Israeli population.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Cultures—Blood samples (15 ml) were withdrawn from classic MSUD patients A. S., M. N., and F. N. and an intermittent MSUD patient C. G. from the non-Jewish Druze kindred in Israel as well as from a classic Ashkenazi-Jewish Israeli patient (N. P.) in Israel (provided by Dr. O. N. Elpeleg, Shaare Zedek Medical Center, Jerusalem, Israel) and a classic Jewish patient (H. D.) in the United States. Lymphoblasts were prepared from blood samples by infection with Epstein-Barr virus (12). Lymphoblast cell cultures were grown as described previously (13).

Western Blotting—Homogenates from cultured lymphoblasts were subjected to SDS-PAGE separation and then transferred to Immobilon-P membranes. The membranes were probed with either anti-E2 or anti-E1 (with titers against both E1 α and E1 β subunits) antibodies, followed by detection with ^{125}I -labeled protein A as described previously (14).

DNA Sequencing for Type IB MSUD Mutations—The first strand cDNA was synthesized from the total RNA prepared from patients' cells using the OmniscriptTM Reverse Transcriptase from Qiagen (Chatsworth, CA). The reverse primer B1, 5'-GTAGAACTTTTCAGCCAATATCATGATGG-3', was designed from the 3'-noncoding region of the human E1 β cDNA (15). The first round polymerase chain reaction was carried out using the forward primer B2 (5'-GTGCGGCTGCATAGCCTGAG-3') and the reverse primer B3 (5'-AAAAGAGGTAAGTCGAGGA-3'). To amplify the 5' segment of the E1 β cDNA, a second round polymerase chain reaction was performed using the forward primer B4 (5'-ATGGCGTTGTAGCGGC-3') and the reverse primer B5 (5'-CCAGGCAACTAGAGTAACATC-3'). To amplify the 3' region of the E1 β cDNA, the forward primer B6 (5'-ATACCCCATGTGTGAA-CAAGGAATTGTTG-3') and the reverse primer B3 (see above) were employed. The polymerase chain reaction products were sequenced using an ABI PrismTM model 377 automated DNA sequencer from Applied Biosystems (Foster City, CA).

Construction of Expression Plasmids for Mutant His₆-tagged E1—The Altered SiteTM *in vitro* mutagenesis system (Promega, Madison, WI) was used to introduce desired mutations into the cDNA of the human E1 β subunit. Detailed protocols for the mutant vector construction and subsequent mutagenesis were described previously (16). Briefly, oligonucleotides for the desired mutations and the β -lactamase repair primer were annealed to the single-stranded form of pAlter-E1 β vector. After the second strand synthesis and two rounds of ampicillin

selection, clones harboring the correct mutations were isolated. DNA segments containing the mutations were used for cassette replacements of the expression vector pHis-TEV-E1 for wild-type E1, which contained a His₆ affinity tag linked to the N terminus of the E1 α subunit (5' to 3') (11).

Expression and Purification of His₆-tagged Wild-type and MSUD Mutant E1s—The recombinant His₆-tagged E1 heterotetramer was expressed in *Escherichia coli* strain CG-712 (ES⁺) by co-transformation of the pGroESL plasmid overproducing chaperonins GroEL and GroES as described previously (17, 18). Wild-type and mutant His₆-tagged E1s were isolated from cell lysates using a Ni²⁺-NTA-derivatized Sepharose CL-6B column (Qiagen) as described previously (11). E1 proteins were further purified on a Superdex-200 gel filtration column (1 × 30 cm) in an FPLC system from Amersham Pharmacia Biotech. The column buffer consisted of 50 mM potassium phosphate, pH 7.5, 250 mM KCl, 5% (v/v) glycerol, 5 mM dithioerythritol, 1 mM benzimidazole, and 1 mM phenylmethylsulfonyl fluoride. E1 activity during purification was assayed radiochemically by reconstitution with E2 and E3 (see below). Protein concentrations were determined using the Coomassie Plus 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 molar extinction coefficient of 1.15 cm⁻¹ mg⁻¹ ml⁻¹ for the $\alpha_2\beta_2$ heterotetramer.

Temperature-dependent Folding and Assembly of Mutant E1—Cultures (1 liter in size) for the expression of His₆-tagged wild-type and mutant E1 were grown at 37 °C until A₅₉₀ = 0.6 was reached. Aliquots of 50 ml were placed in 100-ml flasks and induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside. Cultures were subsequently grown overnight at 23, 28, 33, or 37 °C. Cells were harvested and lysed by sonication in a lysis buffer comprising 50 mM potassium phosphate, pH 8.0, 500 mM NaCl, 2 mM MgCl₂, 0.2 mM TDP, 0.1% (v/v) Triton X-100, 0.01% (w/v) Na₃N, 0.1 mM EDTA, 20 mM β -mercaptoethanol, lysozyme (1 mg/ml), and protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 mM benzimidazole). Lysates were clarified by ultracentrifugation at 50,000 × g for 30 min to sediment unbroken cells and debris. The supernatants (10 ml) were extracted with 100 μ l of Ni²⁺-NTA, which was washed three times (1.5 ml each time) with the above FPLC column buffer containing 15 mM imidazole. The washed Ni²⁺-NTA resin containing the bound E1 was eluted with the FPLC buffer containing 1 M imidazole, and the eluted proteins were separated on 12% SDS-PAGE gels (19). The radiochemical assay based on activity of the reconstituted BCKD complex (20) was used to determine wild-type and mutant E1 activities following elution of E1 proteins from Ni²⁺-NTA with 100 mM imidazole.

Measurements of Kinetic Constants— K_m and k_{cat} for TDP and substrate α -keto[1-¹⁴C]isovalerate (KIV) were determined using the spectrophotometric assay (see below) as reported previously (21). The computer program Curve Fit version 0.7e was used to fit the kinetic data and obtain the slopes and intercepts.

Thermal Inactivation of Wild-type and MSUD Mutant E1 Proteins—The purified wild-type and mutant E1 proteins (32 μ g/ml) were incubated for various times in an MJR PTC-100 thermal cycler equilibrated at 42 °C. At different time points, aliquots were removed and added to a spectrophotometric assay mixture (21). The reduction of NAD⁺ absorbance at 340 nm at 30 °C was used to determine residual E1 activity by reconstitution with E2 and E3. Rate constants (min⁻¹) were derived from the slopes of the pseudo-first order activity decay, as determined by curve fitting using the program Cricket Graph III for the Macintosh computer.

Unfolding of Wild-type and Mutant E1 Proteins with GdnHCl—An 8 M stock of GdnHCl was prepared in 50 mM potassium phosphate, pH 7.5, 100 mM KCl, 0.1 mM EDTA, and 10 mM dithiothreitol. Wild-type and mutant E1 proteins (72 μ g/ml) were incubated at 25 °C for 2 h in the same buffer containing increasing concentrations of GdnHCl. Emission spectra of tryptophan fluorescence over a range of 300–400-nm wavelengths were obtained with a PerkinElmer luminescence spectrometer at an excitation wavelength of 282 nm as described previously (22). Decreases in intensity of the tryptophan fluorescence were used to calculate the ratio of unfolded to folded protein as a function of increasing GdnHCl concentrations, based on the equation $f_u = (x \times x_0)/(x_u \times x_0)$, where f_u represents the fraction of unfolded protein; x is the relative fluorescence emission; x_0 is the relative fluorescence emission in the absence of GdnHCl, and x_u is the relative fluorescence emission of the completely unfolded E1 protein at 4 M GdnHCl. The free energy of denaturation (ΔG_{GdnHCl}) was calculated from the fraction of folded E1 protein over the denaturation transition region (23). The value $\Delta G_{\text{GdnHCl}, 0}$ was obtained by extrapolating to the zero concentration of the denaturant.

TABLE I
Clinical phenotypes and type IB mutations in MSUD patients

Patient	Age	Sex	Clinical phenotype	Percentage of normal activity ^a	Ethnic origin	Mutation	Genotype
	years						
A. S.	5	Male	Classic	0.5	Non-Jewish Druze (Israel)	S289L- β (TCG \rightarrow TTG)	Homozygote
C. G.	11	Male	Intermittent	0	Same as above	S289L- β	Compound-heterozygote
M. N.	7	Female	Classic	0	Same as above	S289L- β	Homozygote
F. N.	5	Female	Classic	0	Same as above	S289L- β	Homozygote
N. P.		Female	Classic	0	Ashkenazi-Jewish (Israel)	R133P- β (CGG \rightarrow CCG)	Compound-heterozygote
H. D.		Female	Classic	6	Jewish (U.S.)	R133P- β	Homozygote

^a The decarboxylation rate of α -keto[1-¹⁴C]isovalerate with lymphoblasts from normal subjects is in the range of 0.114–0.144 nmol CO₂/min/mg of protein.

RESULTS

Identification of Mutations in Different Subunits of the BCKD Complex—We studied four unrelated MSUD patients (A. S., C. G., M. N., and F. N.) from the non-Jewish Druze kindred in Israel, an Ashkenazi-Jewish patient (N. P.) also from Israel, and a Jewish patient (H. D.) in the United States. Lymphoblasts from these patients were cultured and assayed for the rate of decarboxylation using KIV as substrate. The four cell lines from the Druze kindred and the Israeli Jewish patient (N. P.) exhibit absent or nearly absent decarboxylation activity compared with normal cells (Table I). The results correlate with a classic MSUD phenotype except C. G., who has an intermittent MSUD phenotype. The United States Jewish patient (H. D.) shows significant residual activity (6% of normal) that does not correlate with the classic MSUD phenotype in this patient.

To locate the subunit of the BCKD complex affected in these patients, cell lysates were subjected to SDS-PAGE, followed by Western blotting using polyclonal antibodies to E1 (specific for both α and β subunits) or E2 as a probe (24, 13). The level of E1 β subunit ranges from nearly absent to absent in the above six MSUD cell lines, whereas the E1 α subunit is present at reduced amounts compared with normal (data not shown). By contrast, the amount of the E2 subunit was normal in these mutant cell lines. The results indicate that the E1 β subunit may be affected in these MSUD patients. To identify putative mutations in this subunit, two rounds of polymerase chain reaction were performed to amplify 5'- and 3'-terminal regions of the E1 β cDNA synthesized from patients' total RNAs. Nucleotide sequencing of patients' E1 β cDNAs disclosed a type IB S289L- β substitution (TCG \rightarrow TTG) in three homozygous patients (A. S., M. N., and F. N.) and a compound-heterozygous patient (C. G.), all from the Druze kindred (Table I). A second type IB mutation that results in an R133P- β (CGG \rightarrow CCG) substitution is present in one allele of the non-Druze Israeli patient (N. P.) and both alleles of the United States Jewish patient (H. D.).

Expression of Mutant E1 Carrying the S289L- β or R133P- β MSUD Mutation—The His₆-tagged S289L- β mutant E1 expressed at 28 °C was extracted from the *E. coli* lysate with Ni²⁺-NTA, followed by FPLC gel filtration on a Superdex-200 column. Fig. 1A shows that the wild-type human E1 heterotetramer migrates as a single species and peaks at fraction 32. In contrast, the mutant E1 carrying the S289L- β mutation eluted at fraction 34 as a heterodimer. The same mutant E1 also migrated as a heterodimeric species, when separated on a 10–30% sucrose density gradient by ultracentrifugation (data not shown). The mutant E1 that contains the R133P- β substitution was also expressed at 28 °C using the same expression system. The FPLC gel filtration profile shows that this mutant E1 peaks at fraction 32 as a heterotetramer, similar to wild-type E1.

Temperature-dependent Folding Defects in the R133P- β Subunit—The potential effect of the kink introduced by a proline in

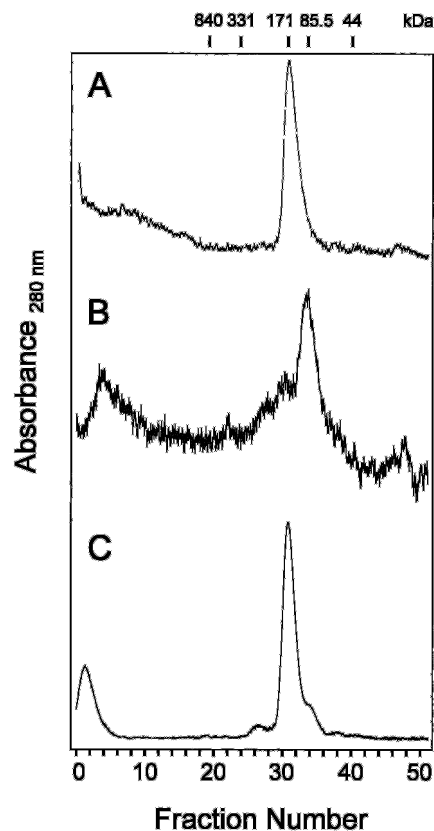


FIG. 1. Elution profiles of wild-type and MSUD mutant E1 from an FPLC gel filtration column. His₆-tagged wild-type or MSUD mutant E1 proteins were expressed in *E. coli* CG-712 (ES¹⁸) co-transformed with pGroESL plasmid overexpressing chaperonins GroEL and GroES. Cell lysates were treated with Ni²⁺-NTA resin, and the bound E1 proteins were eluted with a 25–250 mM imidazole gradient. The extracted E1 proteins were further separated on a Superdex 200 column in an FPLC system. Gel filtration profiles show that the wild-type E1 migrates as an $\alpha_2\beta_2$ heterotetramer and peaks at fraction 32 (A). The mutant E1 containing the S289L- β mutation is eluted as a $\alpha\beta$ heterodimeric species and peaks at fraction 34 (B). The mutant E1 carrying the R133P- β substitution behaves as a heterotetramer, similar to the wild-type E1 (C). The molecular mass markers used were as follows: ovalbumin, 44 kDa; Y393N- α E1, 85.5 kDa; His₆-tagged wild-type E1, 171 kDa; MBP-E1, 331 kDa; and GroEL, 840 kDa.

the R133P- β mutation on the folding and assembly of E1 was investigated. Cells co-transformed with pHis-TEV-E1 and pGroESL plasmids were induced with isopropyl-1-thio- β -D-galactopyranoside overnight at different temperatures for the expression of wild-type and mutant E1. Fig. 2A shows that the expression of wild-type E1 activity in the Ni²⁺-NTA extract remains relatively constant in the temperature range of 23–37 °C. However, the expression of residual R133P- β mutant E1 activity is temperature-dependent, with equally high activity obtained at 23 and 28 °C and very low activity at 37 °C. SDS-

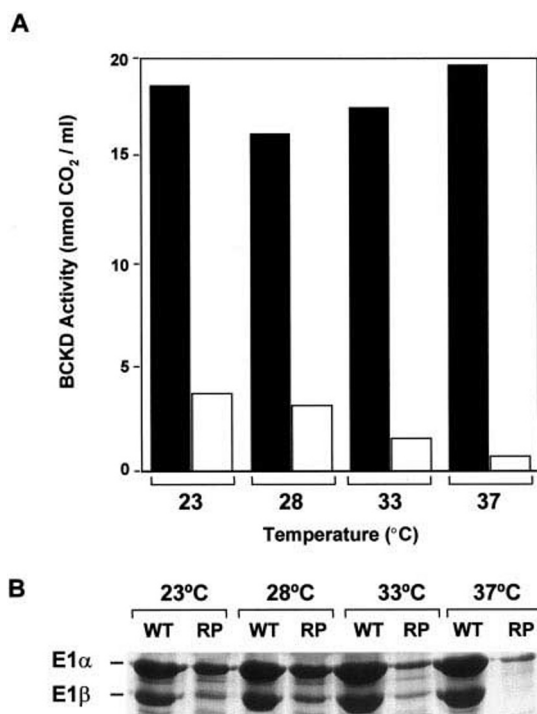


FIG. 2. Expression at different temperatures of wild-type E1 and mutant E1 carrying the R133P- β mutation. *E. coli* CG-712 cells co-transformed with pGroESL (overexpressing GroEL and GroES) and the pHisT-E1 plasmids expressing wild-type or the R133P- β mutant E1 were grown at 37 °C until $A_{590} = 0.6$ was reached. Cells were treated with 1 mM isopropyl-1-thio- β -D-galactopyranoside to induce the expression of wild-type and mutant E1, followed by an overnight incubation at indicated temperatures. Cell lysates prepared in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 mM benzamide) were treated with Ni^{2+} -NTA. The extracted wild-type and mutant E1 proteins were assayed for BCKD activity by reconstitution with E2 and E3 (A; wild type (solid bar) and R133P- β mutant (open bar)) or subjected to SDS-PAGE and Coomassie Blue staining (B; wild type (WT) and R133P- β mutant (RP)).

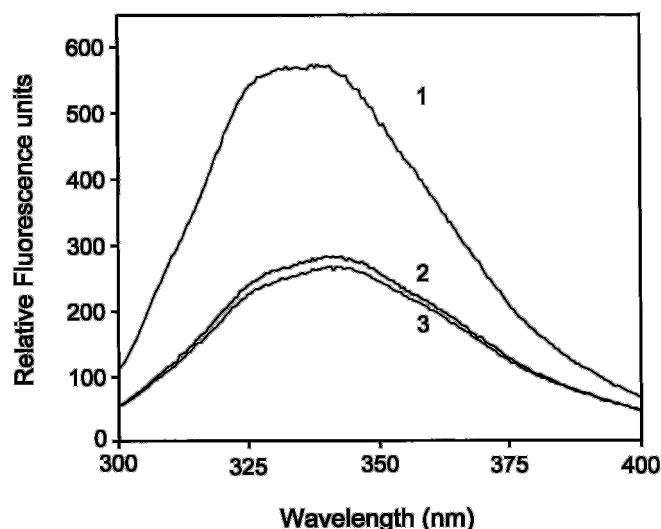


FIG. 3. Emission spectra of tryptophan fluorescence for wild-type and mutant E1 proteins. Wild-type and mutant E1 proteins carrying MSUD mutations were dissolved in 50 mM potassium phosphate, pH 7.5, 100 mM KCl, 0.1 mM EDTA, and 10 mM dithiothreitol to identical protein concentrations (75 μ g/ml). The protein solutions were excited at 282 nm in a PerkinElmer Life Sciences luminescence spectrometer. The tryptophan emission spectra were scanned over the range of 300–400 nm. Curve 1, wild-type E1; curve 2, S289L- β mutant E1; curve 3, Y393N- α mutant E1. Wild-type and Y393N- α mutant E1s are known $\alpha_2\beta_2$ heterotetrameric and $\alpha\beta$ heterodimeric species, respectively.

TABLE II
Kinetic constants (k_{cat} and K_m) for wild-type and MSUD mutant E1

Kinetic constants for substrate α KIV were determined by measuring reconstituted BCKD activity spectrophotometrically in the presence of excess E2 and E3. Kinetic constants for cofactor TDP were determined using radiochemical assays for reconstituted BCKD activity. The kinetic constants are averages of three separate determinations.

Enzyme	KIV			TDP		
	K_m	k_{cat}	k_{cat}/K_m ($\times 10^3$)	K_m	k_{cat}	k_{cat}/K_m ($\times 10^3$)
	μ M	s^{-1}	$s^{-1} \mu$ M $^{-1}$	μ M	s^{-1}	$s^{-1} \mu$ M $^{-1}$
Wild type	62	8.25	140	0.66	8.16	12,400
R133P- β	34	1.16	34.7	0.8	1.78	2,225
S289L- β		0			0	

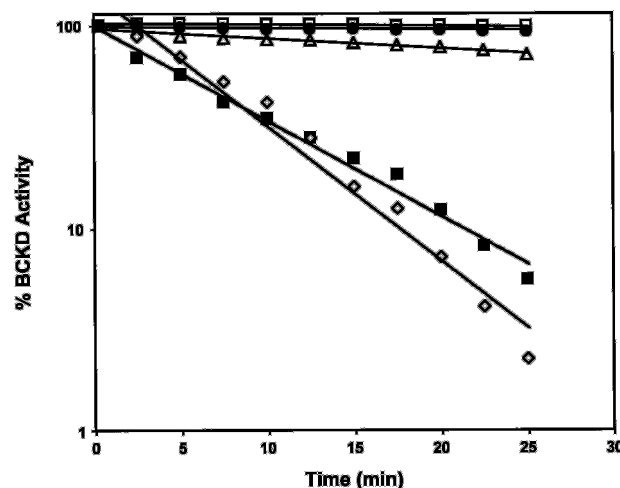


FIG. 4. Thermal inactivation of wild-type and MSUD mutant E1 proteins at 42 °C. Wild-type and MSUD mutant E1 proteins at 32 μ g/ml in 50 mM potassium phosphate, pH 7.5, 100 mM KCl, 0.1 mM EDTA, and 10 mM dithiothreitol were incubated at 42 °C for up to 30 min. Remaining BCKD activity was assayed spectrophotometrically by reconstitution with E2 and E3. The reduction of NAD^+ was monitored by the increase in absorbance at 340 nm. Pseudo-first order decay constants (k_{obs}) represent the slopes after curve fitting using the program Cricket Graph III for the Macintosh. The k_{obs} values are as follows: wild type (\square), 0.025 min^{-1} ; N222S- α (\bullet), 0.028 min^{-1} ; R133P- β (\diamond), 0.58 min^{-1} ; A209D- α (\triangle), 0.051 min^{-1} ; and R252H- α (\blacksquare), 0.38 min^{-1} .

PAGE analysis of the extracts shows that the levels of the wild-type E1 α and E1 β subunits at \sim 1:1 stoichiometry are similar at different temperatures (Fig. 2B). The slightly lower levels of E1 α and E1 β subunits at 23 °C than at higher temperatures are due to a slower growth of *E. coli* at 23 °C. Since only the E1 α subunit contains the His₆ tag at the N terminus, the untagged E1 β subunit isolated in the Ni^{2+} -NTA extract is assembled with the E1 α subunit. In contrast, the level of the assembled mutant E1 β subunit in R133P- β E1 is sharply reduced, compared with the normal E1 α subunit in the mutant. Levels of both the wild-type E1 α and the mutant E1 β subunits are decreased as the expression temperature is elevated. In particular, the assembled mutant E1 β is present at significant levels at 23 and 28 °C but is virtually absent at 37 °C.

Tryptophan Fluorescence Measurements of Wild-type and Mutant E1—The E1 α and E1 β subunits each contains four tryptophan residues, which provide a useful fluorophore for structural studies. Fig. 3 shows that wild-type E1 (curve 1) when excited at 282 nm emits a relatively broad fluorescence spectrum, with two discernible peaks at the 335 nm and 341 nm. The S289L- β mutant E1 (curve 2) shows about one-half of the intensity for tryptophan fluorescence, relative to the same concentration of wild-type E1, with a single peak at 341 nm. The results suggest that about one-half of the tryptophan res-

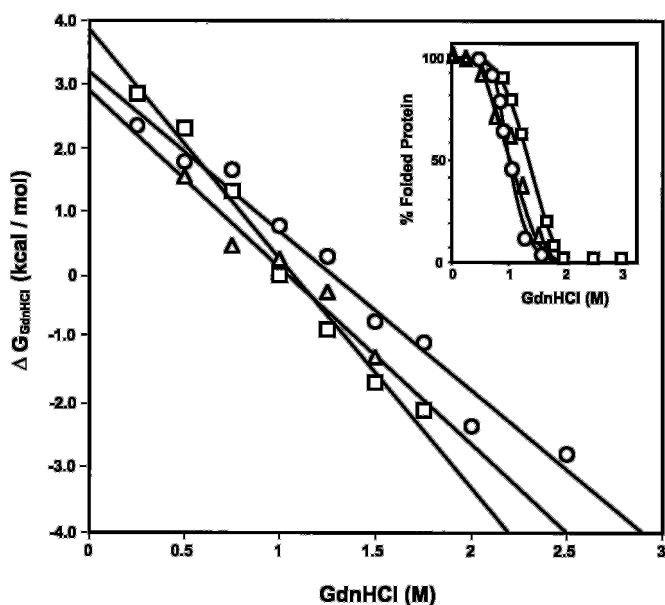


FIG. 5. Denaturation of wild-type and mutant E1 proteins carrying type IB MSUD mutations at 72 $\mu\text{g}/\text{ml}$ were incubated at 25 $^{\circ}\text{C}$ for 2 h in increasing concentrations of GdnHCl. Tryptophan emission fluorescence (excitation and emission at 282 and 431 nm, respectively) of denatured proteins at a given GdnHCl concentration was used to calculate the percentage of folded protein (*inset*) as described under "Experimental Procedures." The Gibbs' free energy change of denaturation (ΔG_{GdnHCl}) was calculated from the fraction of the folded E1 protein at each GdnHCl concentration. \square , wild-type; \circ , S289L- β ; \triangle , R133P- β .

TABLE III

Free energy changes for wild-type and MSUD mutant E1 in GdnHCl

The free energy change of denaturation in GdnHCl (ΔG_{GdnHCl}) is calculated according to the equation $\Delta G = -RT \ln(1/f_N - 1)$, where $1/f_N$ represents the fraction of folded protein. The $\Delta G_{\text{GdnHCl},0}$ values are obtained by extrapolating the slopes in Fig. 5 to zero concentration of the denaturant. The difference in free energy changes ($\Delta\Delta G_{\text{GdnHCl}}$) between the mutant and the wild type is calculated as $\Delta G_{\text{GdnHCl},0}$ (mutant) - $\Delta G_{\text{GdnHCl},0}$ (wild type). The C_m values are the half-maximal denaturant concentration for unfolding. The m values are the slope of $\ln(1/f_N - 1)$ plotted against the denaturant concentration. The free energy changes are averages of two separate determinations.

	$\Delta G_{\text{GdnHCl},0}$	$\Delta\Delta G_{\text{GdnHCl},0}$	C_m	m
	kcal/mol	kcal/mol	M	kcal/mol/M
Wild type	3.8		1.4	3.5
S289L- β	3.2	-0.6	1.0	2.4
R133P- β	2.8	-1.0	1.1	2.7

idues in the wild-type E1 become exposed and are quenched by the solvent in the S289L- β heterodimer. Similar reduced tryptophan fluorescence was observed with the established heterodimeric Y393N- α E1 (*curve 3*). The data confirm that the S289L- β mutant E1 exists as a heterodimer in solution.

Kinetic Studies of Type IB MSUD Mutants—The E1 active site that contains the cofactor TDP-binding pocket is at the interface between two heterodimers that are assembled through α/β' and α'/β subunit interactions in native E1 (8). As expected, heterodimers of the S289L- β mutant E1 are enzymatically inactive. The heterotetrameric R133P- β mutant exhibits significant residual BCKD activity when reconstituted with E2 and E3 components. The k_{cat} values for substrate KIV and TDP for R133P- β E1 are 14 and 21% of the wild-type E1, respectively (Table II). The K_m for KIV and TDP are comparable between R133P- β and wild-type E1. Therefore, the catalytic efficiency (k_{cat}/K_m) of R133P- β E1 is significantly reduced at 26% for KIV and 18% for TDP of the wild-type. The data

indicate that the R133P- β alteration also affects catalytic function in the assembled mutant heterotetramer.

Stability Measurements of Wild-type and Mutant E1—Thermal stability was studied by incubating wild-type and MSUD mutant E1 at 42 $^{\circ}\text{C}$ for different lengths of time. The remaining BCKD enzyme activity was measured by reconstitution with E2 and E3. Inactivation curves of both wild-type and mutant E1 follow pseudo-first-order kinetics as a function of time (Fig. 4). The wild-type E1 and the type IA MSUD mutant N222S- α , which affects the E1 active site (8), are similarly stable with inactivation rate constants, k_{obs} , of 0.025 and 0.028 min^{-1} , respectively. The A209D- α type IA mutation, which impedes the α/α' subunit interaction in E1, produces a mutant E1 that is less stable than the wild-type with a k_{obs} of 0.051 min^{-1} . The R133P- β type IB mutation renders the mutant E1 markedly unstable, with a k_{obs} value of 0.58 min^{-1} . For comparison, the R252H- α type IA MSUD mutation, which also disrupts the α/α' subunit interaction (8), results in a mutant E1 that is as unstable as the R133P- β E1 with a k_{obs} value of 0.38 min^{-1} .

The Gibbs' free energy of denaturation for wild-type E1 and type IB MSUD mutants was determined by chemical denaturation of the proteins in the chaotropic reagent GdnHCl. The ratio of unfolded to folded proteins in increasing concentrations of the denaturant was determined by decreases in tryptophan fluorescence (Fig. 5, *inset*). The ratio was used to calculate the free energy change of denaturation (ΔG_{GdnHCl}) at a given GdnHCl concentration (Fig. 5). The free energy change of denaturation in the absence of the denaturant ($\Delta G_{\text{GdnHCl},0}$) for the wild-type heterotetrameric E1, when extrapolated to zero GdnHCl concentration, is 3.8 kcal/mol (Table III). The heterodimeric S289L- β mutant E1 is slightly less stable, with a $\Delta G_{\text{GdnHCl},0}$ of 3.2 kcal/mol. The heterotetrameric R133P- β is the least stable, with a $\Delta G_{\text{GdnHCl},0}$ of 2.8 kcal/mol and $\Delta\Delta G_{\text{GdnHCl}}$ of -1.0 kcal/mol, relative to the wild-type E1 (Table III). The results support the conclusion from the thermal inactivation studies that the R133P- β mutation adversely affects stability of the mutant E1.

DISCUSSION

The aim of the present study was to determine the molecular and biochemical basis of MSUD in the Israeli population. The occurrence of the homozygous type IB S289L- β mutations in three of the four unrelated Druze patients studied strongly suggests that this allele is prevalent in the non-Jewish kindred, presumably through the practice of consanguinity. The second type IB mutation R133P- β is present in the compound-heterozygous Israeli Jewish patient and in the homozygous United States patient of European-Jewish descent. The data suggest that the R133P- β allele segregates in the Israeli Jewish population outside the Druze kindred. The identification of these two type IB MSUD alleles will facilitate DNA-based diagnosis for this metabolic disorder in the Israeli population in general and the Druze kindred in particular.

The recent determination of the three-dimensional structure of human E1 has provided a structural basis for the two type IB MSUD mutations reported here. As shown in Fig. 6A, the Ser-289 residue is located in the beginning of helix 11. The two helices 11, each from β and β' subunits, are contacting one another along a pseudo-2-fold axis of symmetry. Ser-289 in the β subunit is hydrogen-bonded the side chain of Glu-290 in the homologous β' subunit (Fig. 6B). In addition, Arg-309 in the β -subunit forms a salt bridge with Glu-290 in the β' subunit. The same type of interaction occurs involving Ser-289 in the β' subunit. The substitution of Ser-289 with a larger hydrophobic Leu residue is likely to disrupt the above polar and ionic interactions at the β/β' subunit interface, thereby preventing the assembly of $\alpha\beta$ and $\alpha'\beta'$ heterodimers into a native $\alpha_2\beta_2$ heterotetramer. The

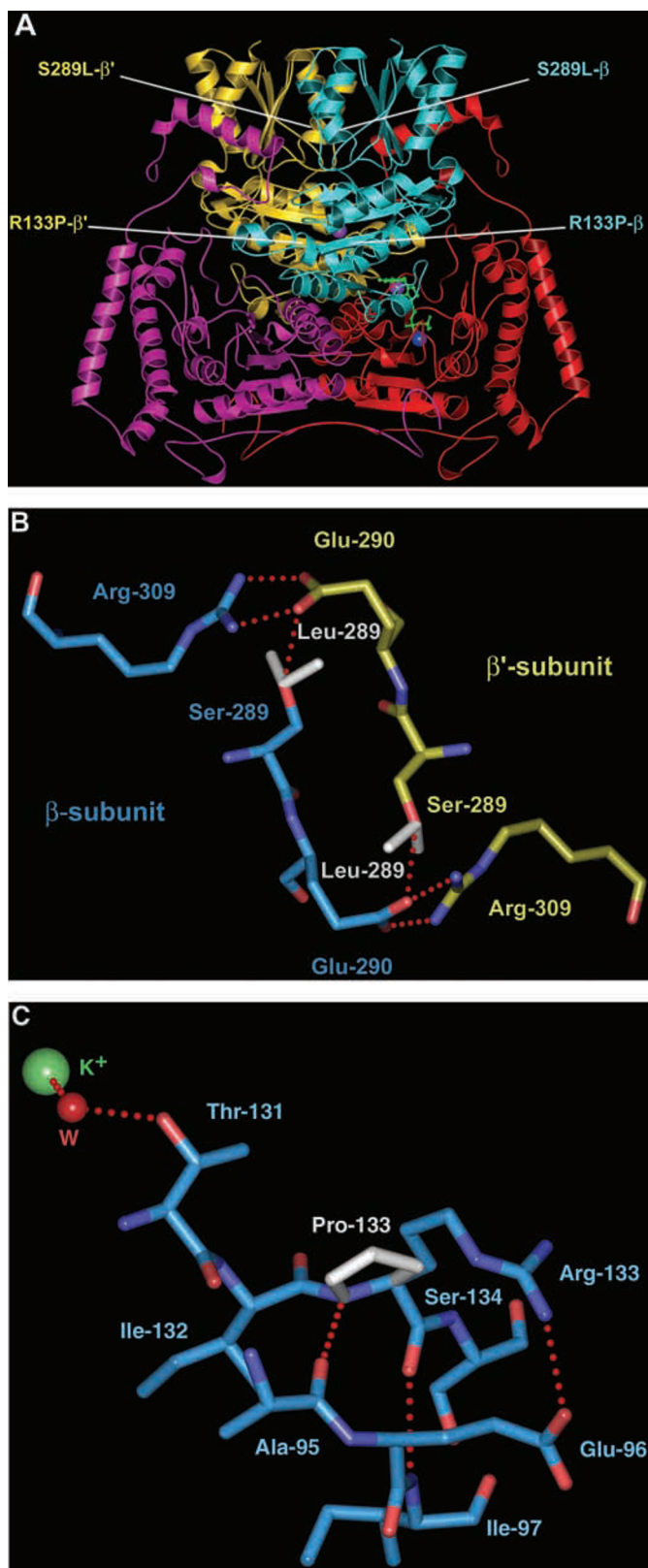


FIG. 6. The three-dimensional structure of the E1 heterotetramer and the structural basis of type IB MSUD mutations. *A*, the three-dimensional organization of α (magenta), α' (red), β (blue), and β' (yellow) subunits in the $\alpha_2\beta_2$ heterotetramer. The S289L- β mutation is located in helix 11 at the interface between β and β' subunits. The R133P- β mutation is situated in strand E in the β or β' subunit. *B*, the putative effect of S289L- β mutation on the β/β' subunit interactions. The Ser-289 residue in the β subunit (blue) is hydrogen-bonded (red dots) to Glu-290 in the β' subunit (yellow). Moreover, Arg-309 in the β subunit forms ionic interactions (red dots) with Glu-290 in the β' subunit. Parallel polar and ionic interactions involving the

trapped heterodimers are presumably in a low energy minimum, and are reasonably stable as indicated by the Gibbs' free energy change of denaturation ($\Delta G_{\text{GdnHCl}, 0}$) of 3.2 kcal/mol, compared with 3.8 kcal/mol for the wild-type E1 heterotetramer. Previously, we reported that the hydrogen bonding of Tyr-393- α to Asp-328- β' is essential for α/β' subunit interaction, which is disrupted by the Y393N- α type IA MSUD mutation in the United States Mennonite population (8). As a result, the mutant E1 is also locked in the inactive heterodimeric conformation. The exclusive presence of inactive E1 heterodimers is consistent with the severe classic phenotype in the Druze and Mennonite MSUD patients, homozygous-affected by the type IB S289L- β and type IA Y393N- α mutations, respectively. Thus, studies of the naturally occurring MSUD mutations have established that both Ser-289- β and Tyr-393- α residues are critical for α/β' and β/β' subunit interactions, respectively, and these interactions are essential for the heterotetrameric assembly of native E1. The results illustrate the power of molecular genetics in identifying amino acid residues that are critical for subunit interactions and protein oligomerization.

Residue Arg-133- β , on the other hand, is located in the middle of strand E in the β subunit (Fig. 6A). This residue is in close proximity (10 Å in distance) to the novel K^+ ion present in the β subunit (Fig. 6C). The side chain of Arg-133- β is ion-paired to the side chain of Glu-96- β in strand D of the same β subunit. Intrasubunit hydrogen bonds occur between the main-chain imino group of Arg-133- β and the main-chain carbonyl group of Ala-95- β as well as between the main-chain carbonyl group of Arg-133- β and the imino group of Ile-97- β . The introduction of a Pro residue at position 133 of the β subunit in the type IB R133P- β mutation produces a kink in the main chain of the β subunit. The altered conformation potentially abolishes the cross talks of Arg-133- β with Ala-95- β , Glu-96- β , and Ile-97- β , which are critical for strand-strand interactions within each individual β subunit. Moreover, Thr-131- β coordinates through a water molecule to the K^+ ion in the β subunit. The displacement of strand E carrying the Thr-131- β residue, as a result of the impaired strand-strand interactions may also prevent efficient binding of the K^+ ion essential for E1 activity. The structural defects caused by the R133P- β MSUD mutation explain the thermal instability and the significant $\Delta\Delta G_{\text{GdnHCl}}$ value of -1.0 kcal/mol, relative to the wild-type E1. This mutation does not appear to hinder the heterotetrameric assembly, since residue Arg-133- β is internal and distant from the subunit interfaces. However, the altered structure caused by the R133P- β substitution also has an adverse effect on catalysis as indicated by the markedly reduced k_{cat} of the mutant enzyme. At present, we cannot delineate the inconsistency between significant residual activity in cultured lymphoblasts and the severe classic phenotype of the homozygous patient

Ser-289 residue also occur in the β' subunit. The S289L substitution (with the Leu residue in white) in either β or β' subunit abrogates the above β/β' subunit interactions, preventing the assembly of $\alpha\beta$ and $\alpha'\beta'$ heterodimers into a native heterotetramer. *C*, the proposed effect of R133P- β mutation on E1 structure and function. The main chain of Arg-133 in strand E of the β subunit is 10 Å away from the novel K^+ ion (green sphere) bound by this subunit. The side chain of Arg-133 is ion-paired (red dots) to the side chain of Glu-96 in strand D of the same β subunit. An intrasubunit hydrogen bond (red dots) also occurs between the main-chain imino group of Arg-133 and the main-chain carbonyl of Ala-95 as well as between the main-chain carbonyl group of Arg-133 and the imino group of Ile-97. The kink introduced by a Pro residue (in white) at position 133 in the R133P- β mutation apparently abolishes the interactions of Arg-133 with Ala-95, Glu-96, and Ile-97 that are necessary for stand-to-strand cross-talks within the same β subunit. Thr-131 coordinates to the K^+ ion through a water molecule (red sphere). The displacement of strand E is likely to also affect K^+ ion binding, which is essential for E1 activity.

carrying the R133P- β mutation (Table I). One can speculate that instability associated with the mutant E1 may result in a rapid turnover in tissues, which accounts for the inability to degrade BCKAs in the patient.

We have shown previously that folding and assembly of wild-type E1 heterotetramers is dependent on the presence of chaperonins GroEL and GroES either in *E. coli* (17) or *in vitro* (25). The bacterial chaperonins promote dissociation/reassociation cycles of the heterodimeric intermediate to facilitate its assembly into the native heterotetramer (26, 27). Despite the presence of overexpressed chaperonins, the recombinant mutant E1 containing the S289L- β mutation is trapped in permanent heterodimeric intermediate conformation. The results support the concept that molecular chaperones do not contain steric information capable of correcting the aberrant conformation dictated by the mutation. On the other hand, the expression of the mutant E1 containing the R133P- β substitution in *E. coli* is facilitated by lowering the expression temperature. At a higher expression temperature (*e.g.* 37 °C), the overexpressed mutant E1 β subunit is misfolded and degraded or targeted to the inclusion bodies. The unassembled wild-type E1 α subunit is unstable at 37 °C and is therefore present in a markedly reduced amount in the supernatant compared with the wild-type heterotetramer (Fig. 2B). At low expression temperature (*e.g.* 23 or 28 °C), a fraction of folded R133P- β mutant E1 β subunit is able to assemble with the wild-type E1 α subunit to produce the partially active heterotetramer (Fig. 2A). A large excess of unassembled E1 α subunit is stable at 23 and 28 °C and remains in the supernatant, although at lower than wild-type levels. The expression data, taken together, strongly suggest that the R133P- β mutation results in a folding defect, which is partially ameliorated by the slow folding kinetics, when overexpressed at ambient temperatures and assisted by chaperonins GroEL and GroES.

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