Impaired Assembly of E1 Decarboxylase of the Branched-chain α-Ketoacid Dehydrogenase Complex in Type IA Maple Syrup Urine Disease*

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The E1 decarboxylase component of the human branched-chain ketoacid dehydrogenase complex comprises two E1α (45.5 kDa) and two E1β (37.5 kDa) subunits forming an αβ₂₄ tetramer. In patients with type IA maple syrup urine disease, the E1α subunit is affected, resulting in the loss of E1 and branched-chain ketoacid dehydrogenase catalytic activities. To study the effect of human E1α missense mutations on E1 subunit assembly, we have developed a pulse-chase labeling protocol based on efficient expression and assembly of human (His₆)-E1α and untagged E1β subunits in Escherichia coli in the presence of overexpressed chaperonins GroEL and GroES. Assembly of the two ³⁵S-labeled E1 subunits was indicated by their co-extraction with Ni²⁺-nitrilotriacetic acid resin. The nine E1α maple syrup urine disease mutants studied showed aberrant kinetics of assembly with normal E1β in the 2-h chase compared with the wild type and can be classified into four categories of normal (N222S-α and R220W-α), moderately slow (G245R-α), slow (G204S-α, A240P-α, F364C-α, Y368C-α, and Y393N-α), and no (T265R-α) assembly. Prolonged induction in E. coli grown in the YTGK medium or lowering of induction temperature from 37 to 28 °C (in the case of T265R-α), however, resulted in the production of mutant E1 proteins. Separation of purified E1 proteins by sucrose density gradient centrifugation showed that the wild-type E1 existed entirely as αβ₂₄ tetramers. In contrast, a subset of E1α missense mutations caused the occurrence of exclusive αβ₂ dimers (Y393N-α and F364C-α) or of both αβ₂₄ and lower molecular weight species (Y368C-α and T265R-α). Thermal denaturation at 50 °C indicated that mutant E1 proteins aggregated more rapidly than wild type (rate constant, 0.19 min⁻¹), with the T265R-α mutant E1 most severely affected (rate constant, 4.45 min⁻¹). The results establish that the human E1α mutations in the putative thiamine pyrophosphate-binding pocket that are studied, with the exception of G204S-α, have no effect on E1 subunit assembly. The T265R-α mutation adversely impacts both E1α folding and subunit interactions. The mutations involving the C-terminal aromatic residues impede both the kinetics of subunit assembly and the formation of the native αβ₂ structure.

The mammalian mitochondrial branched-chain α-ketoacid dehydrogenase (BCKD) complex catalyzes the oxidative decarboxylation of the branched-chain α-ketoacids derived from the branched-chain amino acids, leucine, isoleucine, and valine (1). This multienzyme complex is organized around a dihydrolipoyl transacylase (E2) core, to which a branched-chained α-ketoacid decarboxylase (E1), a dihydrolipoamide dehydrogenase (E3), a specific kinase, and a specific phosphatase are attached through ionic interactions (2, 3). E1 is a thiamine pyrophosphate (TPP)-dependent enzyme comprising two E1α and two E1β subunits that assemble into an αβ₂₄ tetramer. E2 is a 24-meric protein consisting of identical lipoyl acid-bearing subunits arranged on octahedral 4,3,2-point group symmetry. Each E2 polypeptide contains three independently folded domains (i.e. lipoyl-bearing, E1/E3-binding, and inner core) that are highly conserved among E2 proteins of the α-ketoacid dehydrogenase complexes (4). These complexes include pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and BCKD complexes (3). E3 is a homodimeric flavoprotein that is common to members of the α-ketoacid dehydrogenase complexes (3). The kinase and the phosphatase are specific for the BCKD complex and regulate its activity through a reversible phosphorylation (inactivation) and dephosphorylation (activation) cycle (5).

In patients with maple syrup urine disease (MSUD) or branched-chain ketoaciduria, the activity of the BCKD complex is deficient. This leads to clinical manifestations including often fatal ketoacidosis, neurological derangements, and mental retardation (1). The molecular genetics of MSUD are heterogeneous as mutations in the E1α, E1β, E2, and E3 genes have been described (1, 6). Based on the locus affected, genetic subtypes of MSUD have been proposed, with type IA referring to mutations in the E1α gene, type IB to the E1β gene, type II to the E2 gene, and type III to the E3 gene (1). It has been suggested that certain type IA MSUD missense mutations, for example Y393N-α (7) and Y368C-α (8), may impede the assembly of mutant E1α with normal E1β subunit, resulting in the degradation of E1 subunits in patient’s cells.

We have recently established that chaperonins GroEL and

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The abbreviations used are: BCKD, branched-chain α-ketoacid dehydrogenase; E1, branched-chain α-ketoacid decarboxylase; E2, dihydrolipoyl transacylase; E3, dihydrolipoamide dehydrogenase; HPLC, high performance liquid chromatography; Hsp, heat shock protein; IPTG, isopropyl β-D-thiogalactopyranoside; K⁺, potassium phosphate; MBP, maltose-binding protein; MSUD, maple syrup urine disease; NTA, nitrilotriacetic acid; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TEV, tobacco etch virus; TPP, thiamine pyrophosphate.
GroES is essential for efficient folding and assembly of the E1 tetramer in Escherichia coli (9) and the E2 24-mer in vitro (10). To gain insight into the biochemical basis of the apparently impaired assembly of E1 in type I A MSUD, we have co-expressed both mature mutant E1α and normal E1β in E. coli co-transformed with a second plasmid overproducing chaperonins GroEL and GroES. Pulse-chase labeling of both E1 sub-units was carried out to measure the kinetics of assembly of the mutant E1α with normal E1β in the bacterial cell. The results showed a marked reduction in the rate of E1 assembly in certain E1α mutants compared with normal. It was also found that a subset of E1α mutations affect the assembly state of mutant E1 after the prolonged induction in E. coli. Thermostability and protease digestion studies further indicated these slowly assembled mutant E1 proteins had less stable conformations than the wild type. These results define the residues that are critical for subunit interactions and stability of E1 and have implications for understanding chaperonin-mediated biogenesis of hetero-oligomeric structures.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Cultures—** Fibroblasts of classic MSUD patients were kindly provided by the following physicians: B.A. by Dr. David Valle, John F. Kennedy Institute, Baltimore, MD; C.Q. by Dr. Robin Casey, University of Saskatchewan, Canada; and K.U. by Dr. Selma Snyderman, New York University Medical Center, New York, NY. Fibroblasts of classic MSUD patient L.C. were obtained from Cell Repository of McGill Medical Center, Montreal, Canada. Amniocytes of the fetus-at-risk for classic MSUD (F.J.) were kindly provided by Dr. Casey, University of Saskatchewan, Canada; and K.U. by Dr. Selma Valle, John F. Kennedy Institute, Baltimore, MD; C.Q. by Dr. Robin Casey, University of Saskatchewan, Canada; and K.U. by Dr. Selma Snyderman, New York University Medical Center, New York, NY.

**Construction of pHisT-E1 Prokaryotic Expression Vector—** The 5′-portion of the mature human E1α cDNA sequence was amplified from the pMAL-c2-hE1α expression vector (12) using an internal 22-mer anti-sense primer with sequence 5′-GTACACAGATGACCCCTGTTGCT-3′, and a 49-mer sense primer with sequence 5′-GGCTCTGAGAGCTGAGAATTCTTTATTTCCTCACACGCTCACCAGGCTTT-3′ to yield a 601-bp product. The sense primer adds exogenous sequence (shown in uppercase) to the 5′-terminus of the mature E1α open reading frame (shown in lowercase). This exogenous sequence includes an XhoI restriction site (shown in uppercase) to the 5′-terminus of the mature E1α open reading frame (shown in lowercase).

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**Analysis of Assembly State by Sucrose Density Gradient Centrifugation—** Wild-type and mutant E1 proteins were fractionated on a 10–25% sucrose density gradient (10 ml). Gradients were poured in 50 ml C2 broth minimal medium (13) and the wild-type or mutant E1 expression vectors were co-transformed into E. coli strains CG712 (ES ts) and the plasmid pGroESL, which overexpresses GroESL in E. coli. Pure GroESL was induced with 1 mM IPTG at 37 °C for 15–20 h.

**Pulse-Chase Labeling to Determine Kinetics of E1α and E1β Subunit Assembly—** CG712 cells were transformed with the pGroESL plasmid and pHisT-hE1 expression vectors carrying the normal mature E1β cDNA and either normal or mutant His-tagged mature E1α cDNA. Cells were grown at 42 °C to an A600 of 0.8 in C2 broth minimal media. The C2 broth minimal medium was modified from the low sulfate and low amino acid content C2 broth medium described by Guzman-Verduzco and Kupersztch (18) and contained the following per liter: 2 g of NH₄Cl, 6 g of NaHPO₄ · 2H₂O, 3 g of KH₂PO₄, 3 g of NaCl, 6 g of yeast extract, 40 μmol of TPP, 50 mg of carbenicillin, and 50 mg of chloramphenicol. Cells were pelleted and resuspended in one-fifth original volume of the same media without antibiotics and allowed to recover with shaking for 5 min at 37 °C. Cells were subsequently induced with 2 mM IPTG for 5 min, pulsed with 50 μCi/ml [35S]Met (ICN Radiochemicals, Costa Mesa, CA) for 1 min, and chased with 3 volumes of the same media (without antibiotics) supplemented with 8 mg/ml each of non-radioactive l-cysteine and l-methionine. At specified time points following the chase, cell samples (1.5 ml) were taken and frozen in liquid N₂. Thawed samples were lysed by sonication, and supernatants after microcentrifugation were treated batchwise with an excess (15 μl) of Ni²⁺-NTA resin. The resin was washed three times (total volume: 2.4 ml) with 15 mM imidazole in 100 mM KCl, pH 7.5, containing 2 mM MgCl₂, 0.1 mM EDTA, 0.2 mM TTP, and 2 mM β-mercaptoethanol. Bound (His)₆-tagged E1α and assembled eukaryotic polypeptides were eluted with 30 mM Tris-HCl, pH 7.5, containing 15 mM Imidazole. Eluted labeled polypeptides were analyzed by SDS-PAGE, and autoradiograms were obtained by storage phosphorimaging.

**Determination of Thermal Denaturation of Normal and Mutant E1 Proteins—** Thermal aggregation was monitored by measuring absorbance at 360 nm versus time in a Gilford response spectrophotometer equipped with a Peltier heating device as described previously (20). Wild-type or mutant E1 proteins (1.2 μM, final concentration) were added to a buffer preheated to 50 °C, which contained 50 mM KCl, 250 mM KCl, 0.5 mM β-mercaptoethanol, 0.2 mM EDTA, and 10% glycerol at pH 7.5. A final volume of 1 ml (200 μl of the temperature of samples in glass cuvettes (2 mm in width and 10 mm in light path length) was measured using a small-bead thermocouple. The effects of cofactors on thermal denaturation and aggregation was studied by adding 2 mM TTP and 1 mM MgCl₂ to the incubation mixture. Thermal denaturation curves were analyzed as follows. The aggregation for each sample was allowed to proceed until no additional
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RESULTS

Identification of E1α Mutations in Type IA MSUD Patients—
Type IA (E1α-deficient) MSUD was initially suggested by a reduced level or absence of the E1-α subunit in cells from the patients. Missense mutations were identified by DNA sequencing with subclones of the RT-PCR product or by direct sequencing of that product. Y393N-α (codon TAC→AAC), F364C-α (TTC→TGC), Y368C-α (TAT→TGT), and G245R-α (GGG→AGG) mutations were reported previously (8, 21, 22). The N222S-α (AAT→AGT) is present in a homozygous classic MSUD patient F.J. G204S-α (GGG→AGG), R222W-α (CGG→TGG), and A240P-α (GCA→CCA) are present in one allele of classic patients L.C., B.A., and K.U., respectively. The other allele in these three patients is the prevalent Y393N-α mutation previously reported to occur in homozygous Menonite MSUD patients (23, 24). The T265R-α mutation (ACA→AGA) is in one allele of a classic patient C.Q. The second allele in this patient is a single C nucleotide insertion in exon 2 (the 144insC allele) that we reported previously (8). The four novel missense mutations (G204S-α, R220W-α, N222S-α, and A240P-α) as a cause of MSUD were studied by transfection of the full-length human E1α cDNA carrying one of these mutations into type IA MSUD lymphoblasts (22). Transfected cells were unable to decarboxylate the α-keto-1,4-Cisjuglurate (data not shown). As a positive control, decarboxylation activity in type IA MSUD cells was complemented by transfection with the normal pEBOhβE1-α plasmid (7).

Expression and Purification of Normal (His)_6-Human E1—In our earlier study of chaperonin-augmented expression of mammalian E1, a maltose-binding protein (MBP) ligand was fused to the N terminus of the E1α subunit. The presence of the MBP sequence increases the solubility of MBP-E1 and facilitates its purification by amylose resin affinity chromatography (12). In the present study, the MBP ligand is replaced with a (His)_6-tag, which is linked to the N terminus of the mature E1α subunit through a TEV-protease recognition sequence (LENLYFQ). Co-expression of (His)_6-E1α and untagged E1β (the pHis T-Ε1 plasmid) was carried out in an E. coli CG712 host, which contained a second plasmid GroESL that overproduced chaperonins GroEL and GroES. The cells grown in the YTGM medium were heat-shocked at 42 °C for 4 h, followed by induction with IPTG for 5 min at 37 °C. The cells were washed with the phosphate buffer at pH 7.5 containing 50 mM KCl and 15 mM imidazole and dialyzed against the same buffer (see “Experimental Procedures”). (His)_6-E1α was eluted with an imidazole gradient of 15–200 mM. Column fractions (20 ml each) were analyzed by SDS-PAGE. E1 activity in each fraction was assayed by a spectrophotometric method, in which the reduction of 2,6-dichlorophenol indophenol was monitored at 600 nm. The upper panel shows the Coomassie Blue staining of the SDS-PAGE profile. The lower panel depicts the activity and A_600 elution profiles.

![Fig. 1. Purification of recombinant human (His)_6-E1 by Ni^{2+}-NTA affinity chromatography.](Image)

The efficient expression of (His)_6-E1 in the presence of excess chaperonins GroEL and GroES was achieved. CG712 cells co-expressing (His)_6-E1α, untagged E1β, GroEL, and GroES were grown in the C2 broth minimal medium and heat-shocked at 42 °C for 4 h, followed by induction with IPTG for 5 min at 37 °C. The cells were pulsed with [35S]cysteine/[35S]methionine for 1 min and then chased with unlabeled amino acids from 2 to 120 min. Lysates prepared from cells harvested at different times were purified by Ni^{2+}-NTA affinity chromatography. The eluted radiolabeled polypeptides were separated by SDS-PAGE, and autoradiograms were obtained by storage phosphorimaging. Since the E1β subunit was untagged, the co-purification of this subunit with the (His)_6-E1α subunit by Ni^{2+}-NTA indicated assembly of the two polypeptides synthesized during the 1-min pulse. Fig. 2 shows that the assembly of normal E1β with normal (His)_6-E1α occurs as early as 10 min in the chase and reaches a plateau at 30 min. Similar results were obtained with N222S-α (second panel from the top) and R220W-α (data not shown). These two mutations are in the category of a normal assembly with the E1β subunit. A second group of mutations previously reported by the G245R-α showed that significant assembly with E1β did not occur until 30 min into the chase and plateaued at 60 min. Mutations that produce this sluggish assembly kinetics belong to the category of moderately slow assembly. A third group of E1α mutations comprising G204S-α, A240P-α, F364C-α, Y368C-α, and Y393N-α did not generate detectable assembly with normal E1β during the 2-h chase. This is indicated by the absence of the E1β subunit in the autoradiogram. This group of mutations is classified as slow assembly.
assembly, as they produce the assembled mutant E1 only after a prolonged growth of the transformed cells at 37 °C for 16 h (see below). The fourth category of no assembly is represented by the T265R-α subunit, which is not soluble when cells are grown at 37 °C, as indicated by the rapid disappearance of the mutant E1α subunit in the chase. This resulted in a complete absence of assembly with the normal E1β subunit, even after a prolonged growth at 37 °C. However, a significant amount of the assembled T265R-α E1 was produced, when the induction temperature was lowered from 37 to 28 °C (see below).

Measurements of Total and Soluble E1α and E1β Subunits—The levels of total recombinant E1α and E1β polypeptides in E. coli cells were measured by Western blotting. Cells co-transformed with pHisT-E1 and pGroESL plasmids and grown in the C2 broth minimal medium were heat-shocked as described above and induced with IPTG for 12 h. Cells were harvested and total lysates prepared by sonication followed by solubilization in an SDS-PAGE sample buffer. After SDS-PAGE, the samples were subjected to Western blotting using the antibody to E1α or E1β as a probe. Fig. 3A shows that the levels of normal and mutant E1α are comparable. More significantly, the levels of normal E1β in cells expressing normal or mutant E1α are relatively constant. The data rule out the possibility that the reduced level or absence of normal E1β assembled with the mutant E1α subunits (Fig. 2) is caused by aberrant E1β expression. Fig. 3B shows Western blotting of total soluble E1α (normal or mutant) and E1β (normal) subunits in E. coli cells when co-expressed at 37 °C. Five (G204S-α, R220W-α, N222S-α, A240P-α, and G245R-α) of the nine E1α mutants studied are associated with wild-type levels of soluble E1α and E1β subunits. In contrast, the level of T265R-α is markedly reduced with a concomitant near-absence of the normal E1β subunit. The results support the conclusion drawn from pulse-chase labeling (Fig. 2), which indicates that the mutant T265R-α is largely insoluble at 37 °C and fails to assemble with the normal E1β subunit. The unassembled E1β subunit, while expressed at a normal rate (Fig. 3A), became aggregated and was removed from the supernatant after centrifugation. As for the F364C-α, Y368C-α, and Y393N-α mutants (Fig. 3B), the levels of both soluble mutant E1α subunits and the soluble E1β subunit are decreased, compared with the wild-type E1α.

Expression and Assembly State of Wild-type and Mutant E1
**Proteins**—The co-purification of (His)$_6$-E1$_a$ with untagged E1$_b$ cannot discern the assembly state of assembled E1 subunits. To address this question, *E. coli* cells in the C2 broth medium which expressed wild-type E1 subunits were grown for 20 h. E1 subunits in cells harvested at different induction times were purified by Ni$^{2+}$-NTA resin and subsequently subjected to size fractionation by HPLC on a TSK-G3000SW XL column. Fractions collected at different retention times were analyzed by SDS-PAGE and Coomassie Blue staining. E1 activity in eluted fractions was assayed using a reconstituted system with addition of recombinant E2 and recombinant E3. Radiolabeled α-keto-[1-$^{14}$C]isovalerate was used as a substrate, and enzyme activity of the BCKD complex was measured. A, elution profile of assembled E1 species. The molecular mass markers (indicated on top) are GroEL (840 kDa), thyroglobulin (669 kDa), catalase (232 kDa), aldolase (158 kDa), and GroES (70 kDa).

**Fig. 4** Assembled wild-type E1 species during 20 h induction in the C2 broth minimal medium. CG712 *E. coli* cells co-transformed with the pHis T-E1 and the pGroESL plasmids were induced for the expression of wild-type (His)$_6$-E1$_a$ and E1$_b$ subunits with IPTG in the C2 broth minimal medium. Cells were harvested at different times during the 20 h induction. Assembled E1 subunits were extracted from the cell lysate with Ni$^{2+}$-NTA resin. Purified E1 species in 50 mM potassium phosphate, pH 7.5, 0.2 mM EDTA, and 250 mM KCl were subjected to size fractionation by HPLC on a TSK-G3000SWXL column. Fractions collected at different retention times were analyzed by SDS-PAGE and Coomassie Blue staining. E1 activity in eluted fractions was assayed using a reconstituted system with addition of recombinant E2 and recombinant E3. Radiolabeled α-keto-[1-$^{14}$C]isovalerate was used as a substrate, and enzyme activity of the BCKD complex was measured. A, elution profile of assembled E1 species. The molecular mass markers (indicated on top) are GroEL (840 kDa), thyroglobulin (669 kDa), catalase (232 kDa), aldolase (158 kDa), and GroES (70 kDa). B, BCKD activity in fractions collected at different retention times. ◆, reconstituted E1 activity after 2 h induction; ▲ after 3 h induction; ◆, after 20 h induction.

Despite the slow assembly of mutant E1$_a$ subunits with normal E1$_b$ as determined by pulse-chase labeling, prolonged induction with IPTG in *E. coli* grown in the YTGK medium resulted in the production of mutant E1 proteins. Bacterial lysates prepared from *E. coli* cells after the 16-h induction at 37 or 28 °C were purified by Ni$^{2+}$-NTA column, followed by gel filtration on Sephacryl S-100 column. The purified wild-type and mutant E1 proteins were subjected to sucrose density gradient centrifugation to analyze their subunit assembly state. Fig. 5 depicts the sedimentation profiles of E1 proteins as determined by SDS-PAGE analysis of gradient fractions after the centrifugation. The wild-type E1 protein induced at 37 °C migrated as a tetrameric species of 165 kDa with the αβ$_2$ structure (fractions 7–9). The trace amounts of E1$_a$ and E1$_b$ subunits at the bottom of the gradient were the result of slight aggregation that occurred during the centrifugation. In contrast, the Y393N-$\alpha$ mutation E1 expressed at 37 °C migrated as an αβ$_2$ dimeric species with a molecular mass of 83 kDa (fractions 3–6). The mutant E1 with the F364C-$\alpha$ mutation also occurred entirely as αβ dimers in sucrose density gradient centrifugation (data not shown). Interestingly, the mutant
T265R-α, when expressed at 28 °C, was able to remain soluble and assemble with the normal E1β subunit. The sedimentation profile indicated that T265R-α mutant E1 migrated predominantly as tetramers, although lesser amounts of lower molecular weight species were present and sedimented in early gradient fractions. Similarly, the mutant E1 bearing the Y368C-α mutation sedimented as approximately equal amounts of both tetramers and lower molecular weight species when expressed at 37 °C. The mutant E1 proteins carrying the remaining E1α mutations (R220W-α, N222S-α, G245R-α, and A240P-α) were present only as tetramers (Table I). The assembly state of the above wild-type and mutant E1 proteins was confirmed by the elution profiles from TSK-G3000SWxL sizing column on HPLC and Sephacryl S-100 column (data not shown).

**Activity Levels and Stability of Wild-type and Mutant E1 Proteins**—Wild-type and mutant E1 proteins produced in *E. coli* grown on the YTgk medium were purified by Ni²⁺-NTA affinity column and gel filtration on Sephacryl S-100 column. The E1 activity of normal and mutant proteins was assayed by the radiochemical method with 2,6-dichlorophenol indophenol as an electron acceptor. As shown in Table I, only the mutant E1 carrying N222S-α or G245R-α has residual E1 activity (1.37 and 2.66% of normal activity, respectively). The mutant E1 proteins containing each of the remaining seven E1α mutations do not have detectable E1 catalytic activity.

The thermal stability of the above purified normal and mutant E1 proteins was studied by heat denaturation at 50 °C. Light scattering at 360 nm as a result of protein aggregation was monitored. The fraction of soluble proteins was calculated from the progress curve and expressed as log % values versus the incubation time. Fig. 6 shows that the wild-type E1 at 1.21 μm in the presence of 2 mM TPP is most stable with a denaturation rate constant of 0.08 min⁻¹. Similar denaturation rate constants were obtained at 0.97, 0.48, 0.24, and 0.12 μm concentrations of E1 (data not shown). The data support the thesis that the aggregation of E1 is caused by heat-induced conformational changes rather than high concentrations of E1. In the absence of TPP, the denaturation rate constant of wild-type E1 increased to 0.19 min⁻¹. TPP had no effect on the thermal stability of mutant E1 proteins. Among the nine mutant E1 proteins studied, the one containing N222S-α was more stable than wild-type E1 (in the absence of TPP) with a denaturation rate constant of 0.14 min⁻¹. The mutant E1 protein carrying the remaining E1α mutations are less stable than the wild-type E1 (±TPP). The mutant E1 that harbors the T265R-α mutation is the least stable with a denaturation rate constant of 4.45 min⁻¹.

The susceptibility of wild-type E1 tetramers and Y393N-α E1 dimers to proteolysis was also studied. The E1 proteins were incubated with different concentrations of trypsin (protein/trypsin = 250 to 25,000:1, w/w) at 0 °C for 20 min, followed by termination of the digestion with 10 mM PMSF. Fig. 7 shows that Y393N-α dimers are markedly more susceptible to the tryptic digestion than wild-type tetramers as analyzed by SDS-PAGE.

**DISCUSSION**

The major focus of this investigation is to characterize the effect of human E1α mutations in type IA MSUD on the assembly and stability of mutant E1 proteins. For these studies,
we have developed efficient bacterial expression systems for folding and assembly of E1 \(\alpha\) and E1 \(\beta\) subunits. We showed previously that co-expression of mature MBP-E1 \(\alpha\) and E1 \(\beta\) sequences of the human BCKD complex in the same E. coli cells resulted in a 500-fold increase in the yield of active MBP-E1 tetramers (9). In the present study, a \((\text{His})_6\) affinity tag is fused to the mature E1 \(\alpha\) N terminus through a TEV protease recognition site. Co-transformation with the pGroESL plasmid was also found necessary and sufficient for productive folding and assembly of \((\text{His})_6\)-E1. The results argue against the suggestion that the dependence of human E1 on chaperonins for a high yield is due to the presence of the MBP sequence in the chimeric E1 \(\alpha\) polypeptide (23). Our recent in vitro refolding results indicate that the reconstitution of untagged E1, MBP-E1, and \((\text{His})_6\)-E1 show the same chaperonin-dependent kinetics.\(^2\) The findings further established that productive folding and assembly of mature human E1 have an absolute requirement for enrichment for chaperonins GroEL and GroES and are not affected by the presence of affinity tags. Thus, the pulse-chase labeling protocol developed in this study provides the first approximation of the rate of E1 subunit assembly under optimal conditions through the augmentation of bacterial chaperonins that are homologue of mitochondrial chaperonins Hsp60 and Hsp10, respectively.

The wild-type and mutant human E1\(\alpha\) and the wild-type E1\(\beta\) subunits are expressed at relatively equal efficiencies and are stable, as indicated by Western blotting of the total crude lysates prepared 12 h after induction (Fig. 3A). This allows one to follow the fates of E1\(\alpha\) and E1\(\beta\) subunits synthesized during the 1-min window of pulse-labeling. The presence of the \((\text{His})_6\)-tag in the wild-type and mutant E1\(\alpha\) subunits facilitates the isolation of the \(^{35}\text{S}\)-labeled subunit. One can measure the kinetics of the E1\(\beta\) assembly with the wild-type and mutant E1\(\alpha\) subunits by the co-purification of the untagged E1\(\beta\) with \((\text{His})_6\)-E1\(\alpha\) with the Ni\(^{2+}\)-NTA resin as a function of time. The equally strong signals of E1\(\alpha\) and E1\(\beta\) subunits during the 2-h chase (Fig. 2A) indicate that both subunits are efficiently synthesized. The total numbers of cysteine and methionine residues in the E1\(\alpha\) and E1\(\beta\) subunits are similar, which are 16 and 15, respectively. The autoradiogram of the co-purified E1 subunits as separated by SDS-PAGE cannot discern the assembly state of the associated subunits. However, size fractionation of the pulse-chase labeled products by HPLC show that the wild-type E1\(\alpha\) and E1\(\beta\) assemble during the 2-h chase predominantly as inactive \(\alpha\)\(\beta\) dimers, which are later converted to active \(\alpha\)\(\beta\) tetramers (Fig. 4, A and B). It is noteworthy that a significant amount of the wild-type \(\alpha\)\(\beta\) dimeric intermediate was observed only when E. coli cells were grown on the C2 broth minimal medium. When the bacterial cells were cultured on the YTGK medium, wild-type E1 was expressed predominantly as inactive \(\alpha\)\(\beta\) dimers, which are later converted to active \(\alpha\)\(\beta\) tetramers (Fig. 5) with little or no accumulation of \(\alpha\)\(\beta\) dimers during the 16-h induction period (data not shown). The factors responsible for the apparent effects of culture media on the accumulation of the wild-type dimeric intermediate during E1 assembly are currently unknown. The major differences between the two bacterial culture media lie in the fact that the C2 minimal medium is low in the content of \(\text{SO}_4^{2-}\) and amino acids when compared with the YTGK medium (18). Possible effects of these ingredients on the dimerization of wild-type \(\alpha\)\(\beta\) dimers are under investigation.

It is of interest that a weak GroEL signal co-purifies with wild-type (\(\text{His})_6\)-E1\(\alpha\) and E1\(\beta\) at 120 min into the chase (Fig. 2A). This apparent ternary complex is also observed in the early fractions of the Imidazole gradient during purification of wild-type (\(\text{His})_6\)-E1 (Fig. 1). The GroEL-E1\(\alpha\)-E1\(\beta\) ternary complex is a productive intermediate at a later step of the chaperonin-mediated assembly of E1 \(\alpha\)\(\beta\) tetramers.\(^2\) Only a

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\(^2\) J. L. Chuang, R. M. Wynn, and D. T. Chuang, manuscript in preparation.
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FIG. 8. Sequence alignment of the GDG (X)_22–28 NN motif in different TPP-dependent enzymes. Sequences of the GDG(X)_22–28 NN motif for binding the pyrophosphate moiety of cofactor TPP in the nine selected TPP-dependent enzymes were searched and aligned using an advanced version of BLASTP 2.0.3 employing the SWISS-PROT protein data base (38). Residue numbers for the N and C termini of the motif in each sequence are indicated. Residues that are boxed are absolutely conserved in all TPP-dependent enzymes. The abbreviations used are: BDH, branched-chain α-ketoadipic dehydrogenase; KDH, α-ketoglutarate dehydrogenase; TNK, transketolase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; POX, pyruvate oxidase.

GroEL-E1α binary complex is observed in F364C-α because the assembly of E1β with mutant E1α did not occur within the 2-h chase. The weak and sub-stoichiometric signal of GroEL relative to E1 subunits is a result of isotopic dilution by the over-abundance of unlabeled GroEL in E. coli.

The assembled mutant αβ dimers were produced in E. coli grown on the YTGK medium after the 16-h induction with “slow assembly” E1α mutants including Y393N-α and F364C-α. The results indicate that this group of mutations not only reduces the rate of the assembly of the mutant E1α with normal E1β but also prevents conversion of the dimeric assembly intermediate into the stable αβ2 structure of wild-type E1. The production of these mutant αβ dimers apparently is not affected by growth media, as the expression of mutant E1 carrying these mutations in E. coli grown in the C2 broth minimal medium also resulted in the expression of exclusive dimers. In vitro reconstitution of the 6 M urea-denatured mutant Y393N-α E1 in the presence of chaperonins GroEL and GroES also resulted exclusively in mutant αβ dimers.2 The unstable Y393N-α dimeric intermediate as demonstrated by its propensity for thermal aggregation and proteolytic digestion compared with the wild-type tetramer explains the markedly reduced levels E1α and E1β subunits in cells from Menonnette MSUD patients homozygous for the Y393N mutation (24, 25). The present study establishes that C-terminal aromatic residues (F364-α, Y368-α, and Y393-α) in the E1α subunit are crucial for proper E1 assembly. The important roles of the C terminus in subunit assembly and protein interactions have been demonstrated for example, the C-terminal 25 amino acid residues of the herpes simplex virus type 1 UL26.5 protein are required for the assembly of the icosaedral capsid shell (26). In the case of the E2 core of the related pyruvate dehydrogenase complex from Azotobacter vinelandii, the C-terminal residues 632–637 comprise a 3_10-like helix (H6) which acts as a “hydrophobic knob” that fits into a “hole” in the 2-fold related subunit to produce the 24-mer cubic assembly (27). Introduction of a polyhistidine tag into the C terminus of BCKD-E2, which is highly homologous to the bacterial pyruvate dehydrogenase-E2 results in the formation of stable trimers instead of the native 24-mer structure (data not shown).

The T265R-α subunit, when expressed at 37 °C, was largely insoluble even in the presence of excess chaperonins GroEL and GroES. This was indicated by the gross disappearance of the mutant E1α signal in the soluble fraction during the 2-h chase (Fig. 2A); however, the level of the T265R-α subunits in total crude lysates was comparable to that of wild type (Fig. 3A). These results strongly suggest that the T265R-α residue is important for proper folding of the E1α subunit. It is also of interest that lowering of the induction temperature from 37 to 28 °C resulted in the production of a significant amount of assembled mutant E1 protein carrying the T265R-α mutation. The yield of the mutant E1 was 5 mg/liter culture at 28 °C compared with 20–40 mg/liter culture for the wild-type E1 expressed at 37 °C (data not shown). The finding is consistent with the thesis that lowering the expression temperature slows the folding kinetics of the nascent peptide, thereby reducing the probability of the off-pathway folding reactions, as demonstrated by the expression of rabbit muscle phosphorylase (28) and the human E1β (13). However, the assembled T265R-α mutant E1 has a grossly altered conformation, which renders it very unstable as indicated by its most rapid thermal aggregation at 50 °C among the nine E1α mutants studied. This unstable conformation is also manifested by the apparent dissociation of the mutant tetramers to lower molecular weight species as detected by sucrose density gradient centrifugation. The current data indicate that the T265R-α residue also plays a key role in subunit interactions and are consistent with the location of this residue at the putative subunit-interaction site conserved between BKCD and pyruvate dehydrogenase E1 proteins (29).

The crystal structure of the E1 αβ2 has not been solved, but structures are known for the related TPP-dependent proteins transketolase (30) and pyruvate decarboxylase (31) from Saccharomyces cerevisiae and pyruvate oxidase from Lactobacillus plantarum (32). The transketolase is a homodimer, whereas the human E1 is a tetramer made up of two non-identical subunits. Sequence alignment between the two enzymes shows that the highly conserved TPP-binding pocket in E1 is composed of residues from both E1α and E1β subunits (33, 34). Aromatic residues from E1β form a hydrophobic pocket to accommodate the pyrimidium and thiazolium rings of cofactor TPP. On the other hand, the highly conserved TPP-binding motif GDG(X)_22–28NN, which was first described by Hawkins et al. (35) and is essential for binding the pyrophosphate moiety, is located in the E1α subunit (Fig. 8). It should be mentioned that a D440E mutation introduced via mutagenesis into this motif in pyruvate decarboxylase from Zymomonas mobilis yielded a homodimeric enzyme with reduced affinity for TPP, in contrast to the wild-type enzyme which exists as a homotetramer (36). It was proposed that deficient TPP binding may have caused a failure in the conversion of the mutant dimeric forms into native tetramers. However, the occurrence of αβ dimers in the mutant E1 carrying Y393N-α and F364C-α substitutions is likely through a different mechanism, since these
residues are not involved in TPP binding and are located in the C-terminal region. G204S-α, R220W-α, and N222S-α that are affected in type IA MSUD are residues within the TPP-binding motif (Fig. 8). Specifically, N222S-α aligns with Asn-187 in the yeast transketolase and provides a ligand to this pentameric coordination involving the Mg²⁺ cation. The N222S-α mutation conceivably disrupts the pentameric coordination, resulting in the inability of E1 to bind the pyrophosphate moiety of TPP and the loss of E1 catalytic function. However, the N222S-α mutation is without effect on the assembly of the mutant E1α with normal E1β, as determined by pulse-chase labeling (Fig. 2A). The R220W-α mutation, which is also located in the pyrophosphate moiety binding site, also has no adverse effect on E1 subunit assembly. In contrast, G204S-α mutation, which is presumably located at the interface between the two non-identical subunits of E1, based on the yeast transketolase structure (37), impedes the assembly of the mutant E1α subunit with the normal E1β unit.

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