Deletion of the Last Five Amino Acid Residues in Human Dihydrolipoamide Dehydrogenase

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD^+ oxidoreductase; EC 1.8.1.4) is present as a common component in three α-keto acid dehydrogenase complexes (pyruvate, α-ketoglutarate and branched-chain α-keto acid dehydrogenase complexes)[1] and the glycine cleavage system.[2] It catalyzes the reoxidation of the dihydrolipoyl prosthetic group attached to the lysyl residue(s) of the acyltransferase components of the three α-keto acid dehydrogenase complexes and to the hydrogen-carrier protein of the glycine cleavage system.

E3 is a homodimeric enzyme containing one FAD as an essential prosthetic group at it each subunit. The subunit of human E3 consists of 474 amino acids with a molecular mass of 50,216 daltons calculated from the primary amino acid sequence.[3] It belongs to a pyridine nucleotide-disulfide oxidoreductase family along with glutathione reductase (GR), thioredoxin reductase, and trypanothione reductase.[4] Through the common components (FAD and the active disulfide center) in the active site, the enzymes catalyze electron transfers between pyridine nucleotides (NAD^+ or NADPH) and their specific substrates.

From the comparison studies of the structures of E3s and GRs from several sources, it becomes possible to predict the amino acid residues important in the human E3 function.[5,6] The crystal structure of *Pseudomonas putida* E3 Val shows that the last five amino acid residues form an arm folding back into the putative dihydrolipoamide binding site.[7] Several hydrogen bondings are observed between the last five residues and other residues of the enzyme. In order to examine the roles of the corresponding residues of human E3, the last five amino acid residues have been deleted by site-directed mutagenesis. The mutant shows a very low E3 activity and altered spectroscopic properties, indicating that this region is important to the human E3 structure and catalytic function.

Experimental Section

Materials. The electrophoresis reagents, imidazole, iminodiacetic acid sepharose 6B, lipoamide and NAD^+ were from Sigma Chemical Co. Dihydrolipoamide was synthesized according to Reed *et al.*[8] Isopropyl-β-D-thiogalactopyranoside (IPTG) was from POSCOCHEM R&D Center. *E. coli* XL1-Blue containing a human E3 expression vector pROEX-1:E3 was a generous gift from Dr. Mulchand S. Patel of State University of New York at Buffalo. Vent polymerase and T4 DNA ligase were from New England Biolab. Primers and dNTP were from Bioneer.

Site-directed mutagenesis and construction of the human E3 mutant expression vector pROEX-1 : E3 (K-470 - > Stop). Strategies for the site-directed mutagenesis and construction of the mutant expression vector are shown in Figure 1. Polymerase chain reaction (PCR) was performed with 5' primer (5'-TCCCAACGACCGAAAACC-TG-3') and 3' mutagenic primer (5'-GCGCGC**GGATCC**-TAGCCAAATGACGCAGCAAG-3': mismatched bases are underlined and *Bam*HI sequence is highlighted with bold letters). The reaction was carried out with Vent polymerase in a programmable PCR machine using the human E3 expression vector pROEX-1 : E3 as a template. After denaturation of the template DNA at 95 °C for 2 min, 33 rounds of temperature cycling were performed at 95 °C for 30 sec, 43 °C for 1 min, 72 °C for 90 sec and a final 7-min incubation at 72 °C was followed. The PCR generated a 1453 bp DNA fragment containing human E3 sequence of which codon (AAA) for Lys-470 was substituted to the amber stop codon (TAG). The fragment was digested with *Eco*RI and *Bam*HI to generate a 513 bp *Eco*RI/*Bam*HI fragment. The *Eco*RI/*Bam*HI

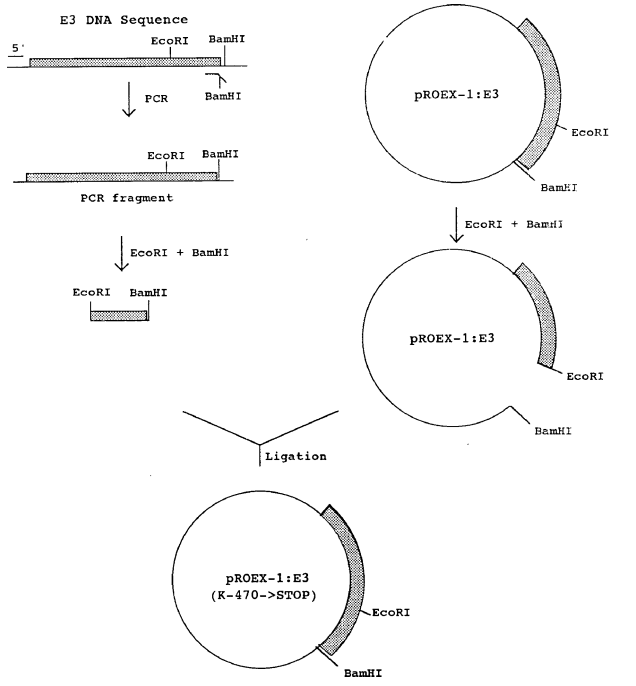

Figure 1. Strategies for PCR and the construction of the human E3 mutant expression vector pROEX-1 : E3 (K-470 - > Stop).: ■ E3 coding region.

fragment was ligated with pROEX-1 : E3 of which the corresponding normal *Eco*RI/*Bam*HI fragment had been removed by *Eco*RI and *Bam*HI digestions. The ligation resulted in the construction of the human E3 mutant expression vector pROEX-1 : E3 (K-470 - > Stop).

Expression and purification of the human E3 mutant. Three mL of an overnight culture of *E. coli* XL1-Blue containing the mutant expression vector were used to inoculate 600 ml of LB medium containing ampicilin (100 μg/ml). Cells were grown at 37 °C to an absorbance of 0.7 at 595 nm and IPTG was added to a final concentration of 1 mM. The growing temperature was shifted to 30 °C and cells were allowed to grow overnight. The overnight culture was harvested by centrifugation at 4000 g for 5 min. Cell pellets were washed with 50 mM potassium phosphate buffer (pH 8.0) containing 100 mM NaCl (binding buffer) and then recollected by centrifugation at 4000 g for 5 min. They were resuspended in 10 ml of binding buffer. They were lysed by a sonication treatment and centrifuged at 10,000 g for 20 min. The supernatant was loaded on a nickel iminodiacetic acid sepharose 6B column. The column had been washed with 2 column volumes of distilled water and then equilibrated with 5 column volumes of binding buffer. After loading of the supernatant, the column was washed with 10 column volumes of binding buffer and then with the same volume of binding buffer containing 150 mM imidazole. The E3 mutant was eluted with binding buffer containing 500 mM imidazole.

E3 assay and spectroscopic study. E3 activity was assayed at 27 °C in 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA. The activity was recorded spectrophotometrically by observing the reduction of NAD^+ at 340 nm with a Shimazu UV 160A recording spectrophotometer. The UV-visible absorption spectrum was recorded using the same spectrophotometer. The fluorescence spectra were recorded using a Fluoromax spectrofluorometer (Industies, Inc., Edison, NJ, USA). E3 was excited at 296 nm and the emission was recorded from 302 nm to 580 nm. The data were transferred to an ASCII file and the spectra were drawn using the MicroCal Origin program (Photon Technology International, South Brunswick, NJ, USA).

Results and Discussion

Table 1 shows carboxy-terminus regions of several E3s and human GR. Among those E3s, the crystal structure of *P. putida* E3 Val has been revealed.[7] The structure shows that the last five amino acids form an arm folding back into the putative dihydrolipoamide binding site. Several hydrogen bondings are observed between the amino acids of the carboxy-terminus region and those of other regions of the enzyme. Some hydrogen bondings involve interactions with amino acids from the other subunit. His-457 forms weak hydrogen bonding with the hydroxyl group of Tyr-18 of the other subunit. It also interacts with Glu-446, a component in the last helical structure. The peptide backbone between Ala-455 and Leu-456 interacts with Gln-26 of the other subunits and with the Glu-446. GR, having a good structural homology to E3, lacks the corresponding carboxy-terminus region (Table 1).

Table 1. Comparison of amino acid sequences at carboxy-terminus region of E3s from several sources and human glutathione reductase (GR). The deleted residues in human liver E3 and the corresponding residues in other E3s are highlighted by bold letters

Source	Amino acid sequence
Pseudomonas putida	444-E A V Q E A A L G **H A L H I**-458
Sachromyces cerevisiae	465-E A N M A A Y D - **K A I H C**-478
Pig heart	461-E A N L A A S I G **K A I N F**-474
Human liver	461-E A N L A A S F G **K S I N F**-474
Human GR	476-T L R-478

To examine the importance of the corresponding region in human E3 structure and function, the last five amino acids were deleted by site-directed mutagenesis. The site-directed mutagenesis and construction of the mutant expression vector, pROEX-1 : E3 (K-470 → Stop), were described in Figure 1. The site-directed mutagenesis was performed using PCR as described in *Experimental Section*. As shown in Figure 2 lane 2, the PCR generated an 1453 bp E3 DNA fragment in which codon (AAA) for Lys-470 was substituted to the amber stop codon (TAG). The fragment was digested with *Eco*RI and *Bam*HI and the resulting 513 bp *Eco*RI/*Bam*HI fragment with the mutation was isolated by agarose gel electrophoresis. The human E3 expression vector pROEX-1 : E3 was digested with *Eco*RI and *Bam*HI to remove the corresponding normal *Eco*RI/*Bam*HI fragment. The resulting human E3 expression vector lacking the normal *Eco*RI/*Bam*HI fragment was isolated by agarose gel electrophoresis. The isolated vector was ligated with the previously isolated *Eco*RI/*Bam*HI fragment, resulting in the

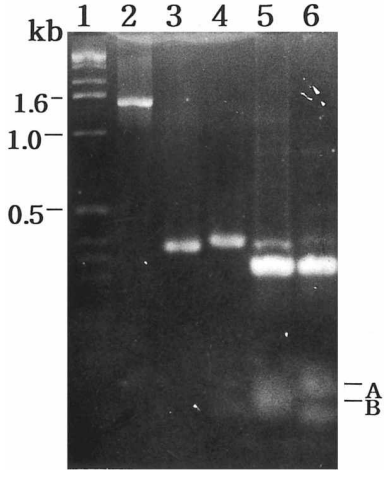

Figure 2. Electrophoretic analysis of PCR products. DNA fragments were analyzed on a 2.0% agarose gel in 40 mM Tris-acetate and 1 mM EDTA and visualized by ethidium bromide staining. Lane 1, molecular weight marker (1 kb ladder); lane 2, 1453 bp fragment; lane 3, 370 bp fragment; lane 4, 385 bp fragment; lane 5, *Hind*III digestion products of the 370 bp fragment; lane 6, *Hind*III digestion products of the 385 bp fragment.

construction of the mutant expression vector pROEX-1 : E3 (K-470 → Stop).

The mutation resulted in a deletion of five amino acids at carboxy-terminus of human E3, which equaled to a deletion of 15 bp in nucleotide level. This difference in nucleotide length was used for the screening and verification of pROEX-1 : E3 (K-470 → Stop). *E. coli* XL1-Blue was transformed with the ligation mixture. Plasmids were isolated from the transformed cells and used as template DNAs for the following PCR for screen. PCR was performed with primer 5' (5'-ACCCTGAAGTTGCTGCAGTTGGCAAATCAG-3') and primer 3' (5'-GCCAAAACAAGCCAAGCTTGG-3'). This PCR was to amplify DNA sequence for the carboxy-terminus region of human E3 containing the last five amino acids. PCR with pROEX-1 : E3 (K-470 → Stop) should produce a DNA fragment of 370 bp (Figure 2, lane 3) while the corresponding PCR with pROEX-1 : E3 should generate a DNA fragment of 385 bp (lane 4). Even though the 370 bp fragment seemed to move faster than the 385 bp fragment did, it was difficult to determine clearly the difference on 2% agarose gel. Therefore the PCR products were digested with *Hind*III and analyzed on the agarose gel. The 370 bp fragment generated an expected 65 bp fragment (lane 5, here indicated by letter B) due to the deletion of the 15 bp while the 385 bp fragment produced an expected 80 bp fragment (lane 6, here indicated by letter A) instead of the 65 bp fragment. The band, shown below the 80 bp fragment in lane 6, was a PCR side product irrelevant to this study. It could be also observed in lane 4 on the gel by bare eye. The mutation was also confirmed by a dideoxy sequence analysis.

The expression of the mutant in *E. coli* was induced by adding IPTG. Since the expression vector pROEX-1 was designed to insert six histidines to the amino-terminus of any expressed proteins, the mutant could be purified using immobilized metal affinity chromatography.[9] After equilibration with binding buffer, supernatant solution was applied to the column. *E. coli* proteins were washed out with binding buffer and then with binding buffer containing 150 mM imidazole. The mutant was eluted with binding buffer containing 500 mM imidazole. Purification was followed by SDS-polyacrylamide gel electrophoresis (data not shown). The gel showed that the E3 mutant was highly purified.

E3 assay was performed at 27 °C because E3 activity of the mutant seemed to be unstable above 27 °C. The E3 activity was determined as 0.8 unit/mg at substrate concentrations of 2 mM dihydrolipoamide and 3 mM NAD^+. This value was about 600-fold lower than that of normal human E3 activity determined at the same condition, indicating that the last five amino acids were important to the efficient and stable catalytic function of human E3. Due to the very low E3 activity and the instability of the mutant, it was difficult to determine steady-state kinetic parameters.

To examine any conformational changes in the mutant due to the deletion, UV-visible absorption and fluorescence spectra were observed as described in *Experimental Section*. Due to the prosthetic group FAD, human E3 shows a characteristic UV-visible absorption spectrum of flavoproteins as

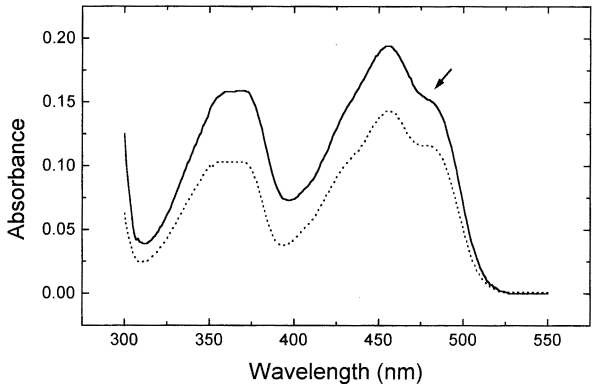

Figure 3. UV-visible spectra of the mutant (17.2 mM, solid line) and normal (12.5 mM, dotted line) recombinant human E3s. The spectra were recorded using Shimazu UV 160A spectrophotometer and the data from 350 nm to 550 nm were transferred to an ASCII file and the spectra were drawn using the MicroCal Origin program. The arrow indicates the unique shoulder region.

shown in Figure 3 (dotted line). The overall shape of the mutant spectrum (solid line) was similar to that of the normal recombinant human E3 spectrum (dotted line). However, an alteration was found in the region of the unique shoulder between 465 nm and 485 nm as indicated by an arrow in Figure 3. This unique shoulder is a characteristic feature in the spectra of many flavoproteins including human E3. The strong association of FAD with the protein is suggested to be responsible for this feature.[4] The alteration of this unique shoulder implied that conformational changes could occur in the mutant. The changes could affect the association of FAD with the enzyme so that the alteration in the unique shoulder region of the UV-visible spectrum of the mutant occurred.

Another evidence for the conformational changes in the mutant came from the fluorescence study. With proteins, fluorescence study can provide very useful information such as conformational changes, ligands binding and molecular interactions.[10] E3 is a good subject for the fluorescence study because it contains a useful intrinsic fluor FAD in addition to aromatic amino acids. E3s were excited at 296 nm and the fluorescence emissions were observed from 302 nm to 580 nm. As shown in Figure 4, two fluorescence emissions were observed for both E3s. The first emission from 300 nm to 400 nm is due to aromatic amino acids, mainly tryptophans. The second emission from 480 nm to over 550 nm is due to FAD. When the fluorescence spectra of both E3s were compared, a noticeable difference was found in the intensities of the first fluorescence emissions. Even though considering the difference in the sample concentrations (about 2-fold), intensity of the first emission of the mutant (solid line) was much higher than that of the first emission of the normal enzyme (dotted line). This difference in the fluorescence spectra implied again that the conformational changes could occur in the mutant. Similar high increase in the first fluorescence emission had been reported in the spectroscopic studies of the human E3 mutant in which Glu-457 residue had been substituted to Gln.[11]

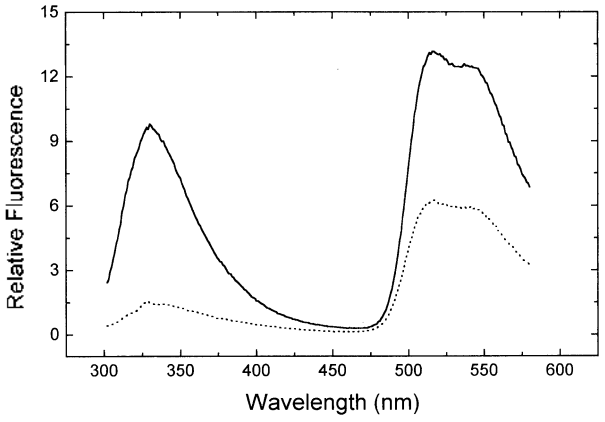

Figure 4. Fluorescence spectra of the mutant (17.2 mM, solid line) and normal (8.7 mM, dotted line) recombinant human E3s. Enzymes were excited at 296 nm and the emissions were observed from 302 nm to 580 nm. The data were transferred to an ASCII file and the spectra were drawn using the MicroCal Origin program.

From these results, the following conclusions can be made. 1. The last five amino acids are important to the stable structure of human E3, by presumably making several interactions with the neighboring amino acids as observed in the three dimensional structure of *P. putida* E3 Val. 2. These interactions may be important to the proper association of FAD with the enzyme. 3. The proper structure formed by the last five amino acids is critical to the efficient and stable E3 catalytic function of human E3.

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