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Characterization of Two Naturally Occurring Mutations (Gly-101 Deletion and Glu-340 to Lys Substitution) in Human Dihydrolipoamide Dehydrogenase of a Patient with Metabolic Acidosis

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD^+ oxidoreductase; EC 1.8.1.4) is a common component in pyruvate, α-ketoglutarate, branched-chain α-keto acid dehydrogenase complexes[1]. E3 catalyzes the reoxidation of dihydrolipoyl groups in the acyltransferase components of the α-keto acid dehydrogenase complexes. E3 is a homodimeric flavoenzyme containing one FAD at each subunit, as shown in Figure S1. E3 belongs to the pyridine nucleotide-disulfide oxidoreductase family along with glutathione reductase, thioredoxin reductase, mercuric reductase and trypanothione reductase.[2] Patients with an E3 deficiency exhibit the increased urinary excretion of α-keto acids, and elevated blood lactate, pyruvate and branched chain amino acid level. These patients normally die at a young age because an E3 deficiency is a critical genetic defect affecting the central nervous system, such as the brain.

A boy with metabolic acidosis was diagnosed with low E3 activity.[3] At age 5 and half years, he died of an acute illness caused by the genetic defects in his E3 genes. Two independent mutations (Gly-101 deletion and Glu-340 to Lys substitution) were identified in his E3 alleles. Figure S2 shows the sequence alignments of the Gly-101 and Glu-340 regions of human E3 along with the corresponding regions of E3s from a range of sources. Gly-101 and Glu-340 are absolutely conserved, indicating their importance for the structure and function of human E3. Gly-101 is a member of long α-helix 3, and Glu-340 is a component of α-helix 8. They are located close to the dihydrolipoamide binding channel, as shown in Figure S1.

A site-directed mutagenesis method is a useful tool for a structure-function study of human E3 and other proteins.[4-7] This study examined the effects of the two naturally occurring mutations on the structure-function of human E3. Two site-specific mutations were produced by site-directed mutagenesis using two mutagenic primer pairs, as shown in Table S1. The PCR reactions were carried out using the human E3 expression vector, pPROEX-1:E3, as a template in a programmable PCR machine. The mutations were verified by DNA sequencing.

The mutants were expressed in *E. coli* by adding 1 mM IPTG and purified on a nickel affinity column. Several attempts at purifying the Gly-101 deletion mutant were unsuccessful. The SDS-PAGE gel showed small amounts of the mutant present in the elution solution after elution with 250 mM imidazole (Figure 1, lane 5). There was insufficient mutant present to be purified when its expression was induced in *E. coli*. This suggests that when its Gly-101 is deleted, human E3 becomes too unstable to be obtained easily from *E. coli*. The mutant was so unstable that it looked as though it degraded as soon as it was expressed in *E. coli*.

On the other hand, the Lys-340 mutant was expressed well in *E. coli.* To determine the steady state kinetic parameters, the E3 assay was performed at 37 °C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA. The data was analyzed using the SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA). The program generated double reciprocal plots, as shown in Figure S3, which revealed parallel lines, indicating that the mutant also catalyzes the reaction through a Ping Pong Bi Bi mechanism. The k_{cat} value was determined to be 637 ± 16 s^{-1}, which is

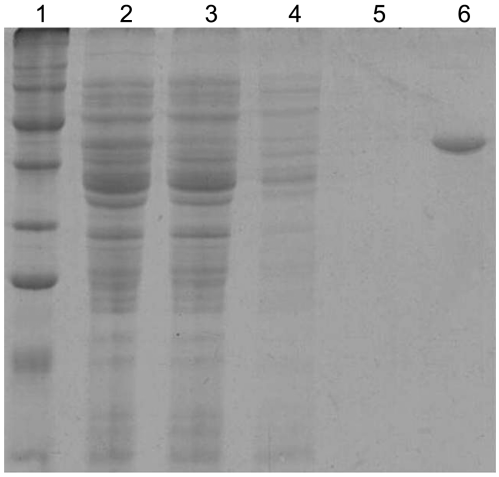

Figure 1. SDS-PAGE gel for purification of the Gly-101 deletion mutant. Lane 1, DokDo-MARK™ molecular weight markers (from bottom to top: 7, 15, 20, 25, 35, 50, 70, 100, 140, 240 kDa); lane 2, supernatant; lane 3, flow-through; lane 4, Binding buffer containing 50 mM imidazole; lane 5, Elution buffer containing 250 mM imidazole; lane 6, recombinant human E3 as a control.

29% lower than that of normal human E3 (899 ± 114 s^{-1}), indicating that the catalytic power of the mutant for the conversion of the substrates to products had deteriorated. The K_m value toward dihydrolipoamide was determined to be 0.46 ± 0.03 mM, which is approximately 28% lower than that of normal human E3 (0.64 ± 0.06 mM), suggesting that Glu-340 might be involved in enzyme binding to dihydrolipoamide. This is in accordance with the location of Glu-340, which is located close to the dihydrolipoamide binding channel. The K_m value toward NAD^+ was determined to be 0.20 ± 0 mM, which is similar to that of normal human E3 (0.19 ± 0.02 mM).

UV-visible absorption and fluorescence spectroscopy were performed to examine the structural changes occurring in the Lys-340 mutant. Human E3 has a characteristic UV-visible absorption spectrum of flavoproteins due to the prosthetic group, FAD, as shown in Figure 2(a). The overall shape of the mutant spectrum (solid line) was similar to that of the normal human E3 spectrum (dotted line). On the other hand, slight differences were observed in overall ranges of the spectra. The ratio (1.23) between the absorbance of the first peak at ~350 nm and the second peak at ~455 nm (solid line) of the mutant was slightly lower than that (1.30) of the normal enzyme (dotted line). These differences in the UV-visible spectrum of the mutant indicated that structural changes can occur in the enzyme. A fluorescence study provided further evidence of structural changes. After exciting human E3 at 296 nm, two fluorescence emissions (Trp emission from 305 nm to 400 nm and FAD emission from 480 nm to over 550 nm) were observed, as shown in Figure 2(b). Trp fluorescence was quenched due to fluorescence resonance energy transfer (FRET) from Trp to FAD. A comparison of the fluorescence spectra of the human E3s revealed a significant difference in the ratio between the relative intensities of the first and second fluorescence emissions. The ratio (2.9) between the relative intensities of the first and second fluorescence emissions of the mutant (solid line) was lower than that (4.9) of the normal enzyme (dotted line). This suggests that the FRET from Trp to FAD was disturbed in the mutant. This again indicates that structural changes can occur in the mutant. The structural changes due to a Glu-340 to Lys mutation might have affected the structure of human E3, which interfered with FRET. The amino acid volume of Glu is 138.4 $Å^3$, whereas that of Lys is 168.6 $Å^3$. The mutation will add 30.2 $Å^3$ in volume to the residue-340. The mutation also changes the negatively charged side chain of Glu to a positively charged side chain of Lys. These alterations can cause structural changes in human E3, which might also be responsible for the alterations in the kinetic parameters of the mutant.

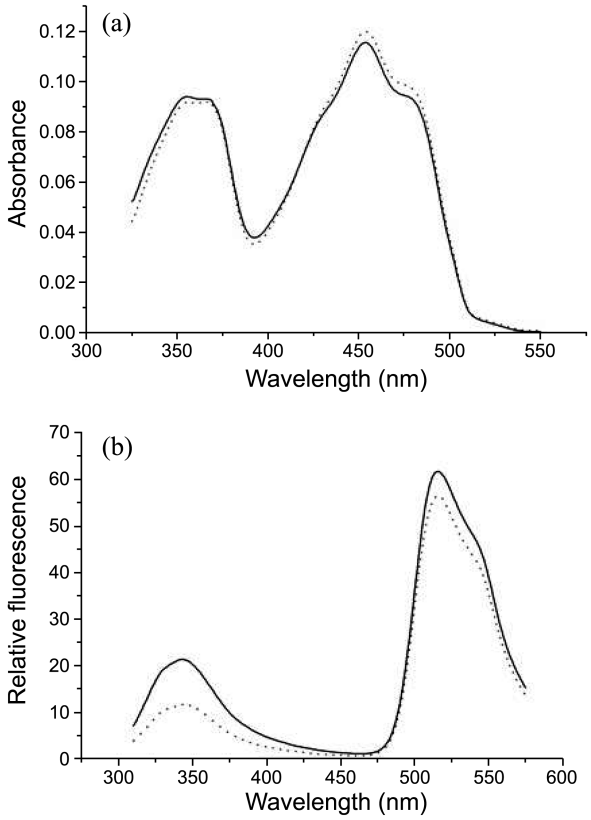

Figure 2. Spectroscopic properties of human E3s. (a) UV-visible spectra of the Lys-340 mutant (solid line) and normal (dotted line) human E3s. The spectra were recorded using a SPECORD200 spectrophotometer and the data from 325 nm to 550 nm was transferred to an ASCII file. The spectra were then drawn using the MicroCal Origin program. (b) Fluorescence spectra of the Lys-340 mutant (solid line) and normal (dotted line) human E3s. The enzymes were excited at 296 nm and the emissions were observed from 305 nm to 575 nm. The data was transferred to an ASCII file and the spectra were then drawn using the MicroCal Origin program.

The effects of two naturally occurring mutations (Gly-101 deletion and Glu-340 to Lys substitution) on the human E3 structure and function were examined by site-directed mutagenesis, SDS-polyacrylamide gel electrophoresis, E3 activity measurements and spectroscopic methods. A deletion of the Gly-101 residue in human E3 was detrimental enough to destroy the stable expression of the enzyme in *E. coli*, indicating that a Gly-101 deletion in human E3 results in an unstable enzyme. The Glu-340 to Lys mutation deteriorated the catalytic power of the enzyme, caused structural changes that altered the UV-visible spectrum, and interfered with the efficient FRET from the Trp residues to FAD. These results indicate that the conservation of both Gly-101 and Glu-340 residues is important for the structure and function of human E3.

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