http://dx.doi.org/10.5012/bkcs.2012.33.5.1445

Characterization of a Naturally Occurring Mutation (Ile-358 to Thr) in Human Dihydrolipoamide Dehydrogenase of a Patient with Leigh Syndrome

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Received January 6, 2012, Accepted January 30, 2012

Key Words : Dihydrolipoamide dehydrogenase, Pyridine nucleotide-disulfide oxidoreductase, Site-directed mutagenesis, Dihydrolipoamide deficiency, α-Keto acid dehydrogenase complexes

Dihydrolipoamide dehydrogenase (E3) is a common flavoprotein component in three α-keto acid dehydrogenase complexes, such as pyruvate, α-ketoglutarate and branched-chain α-keto acid dehydrogenase complexes.[1] E3 is a homodimeric enzyme containing one FAD at each subunit that catalyzes the reoxidation of the dihydrolipoyl group of the acyltransferase components of the α-keto acid dehydrogenase complexes. One mutation observed in a boy with low E3 activity was the substitution of Ile-358 with Thr.[2] The boy had Leigh syndrome with recurrent episodes of hypoglycaemia and ataxia, permanent lactic acidaemia and mental retardation. The aim of this study was to understand the effects of an Ile-358 to Thr substitution mutation in human E3, which leads to Leigh syndrome. Figure 1 shows the sequence alignment of the Ile-358 region of human E3 with the corresponding regions of the E3s from a range of sources. Ile-358 is highly conserved, suggesting that it might be important to the structure and function of human E3.

A site-directed mutagenesis method is a useful tool for structure-function studies of human E3 and other proteins.[3-7] The effects of this naturally occurring mutation in the human E3 structure and function were examined using a site-specific Ile-358 to Thr mutation. Site-directed mutagenesis was performed using a mutagenesis kit (iNtRON Biotechnology) with two mutagenic primers (Primer A (5'-GTGTGCCATCAGTGACTTACACACACCCTGAAG-3': the mismatched bases are underlined) and Primer B (5'-CTTCAGGGTGTGTGTAAGTCACTGATGGCACAC-3')). After overnight induction with 1 mM IPTG, the cells were harvested and lysed by sonication. The supernatant was loaded onto to a nickel affinity column. The mutant was then eluted with the binding buffer containing 250 mM imidazole. SDS-PAGE gel showed that the mutant was highly purified (Figure. S1).

The E3 assay was performed at 37 °C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA with various concentrations of the substrates, dihydrolipoamide and NAD^+ to determine the kinetic parameters. Double reciprocal plots were drawn using the SigmaPlot Enzyme Kinetics Module program (Systat Software Inc., San Jose, USA), as shown in Figure S2. The graph exhibited parallel lines, indicating that an Ile-358 to Thr mutation did not affect the kinetic mechanism of human E3, which was a Ping Pong Bi Bi mechanism. The k_{cat} was determined to be 927 ± 83 s^{-1}, which is similar to that (899 ± 114 s^{-1}) of normal human E3. The K_m value toward dihydrolipoamide was determined to be 0.46 ± 0.03 mM, which was slightly smaller than that (0.64 ± 0.06 mM) of normal human E3. On the other hand, the K_m value toward NAD^+ was determined to be 0.48 ± 0.06 mM, which was approximately 2.5 times higher than that (0.19 ± 0.02 mM) of normal human E3, indicating that the mutation caused less efficient enzyme binding to NAD^+. The NAD^+ concentration in cells was determined to be approximately 0.37 mM.[8] This NAD^+ concentration is sufficient for the efficient catalytic function of normal human E3, whose K_m value toward NAD^+ is 0.19 mM. On the other hand, the catalytic function of the Thr-358 mutant becomes severely deteriorated because the K_m value toward NAD^+ (0.48 mM) is larger than the NAD^+ concentration in the cells (0.37 mM). This deteriorated catalytic function of the mutant can be responsible for the low E3 activity observed in the reported patient.[2]

Ile-358 is located in a hydrophobic environment close to both FAD and NADH (Figure S3). The main-chain oxygen of its neighboring Val-357 forms a hydrogen bond with the nitrogen of the exocyclic amide of the bound NADH. Ile-358 forms van der Waals interactions with Val-188, which also form van der Waals contacts with the nicotinamide group of NADH. The mutation of Ile-358, a large hydro-

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E3s                          Amino acid sequence
                                      358
sp| P09622 |DLDH_HUMAN       AVHIDYNCVPSVIYTHPEVAWVGK
sp| P09623 |DLDH_PIG         AVHIDYNCVPSVIYTHPEVAWVGK
sp| P09624 |DLDH_YEAST       HGHVNYNNIPSVMYSHPEVAWVGK
sp| P18925 |DLDH_AZOVI       KAQMNYDLIPAVIYTHPEIAGVGK
sp| P14218 |DLDH_PSEFL       KAQMNYDLIPSVIYTHPEIAWVGK
                               : :*: :*:*:*:***:*:***
                                  -->    ----->
                                  βG1    βG2
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Figure 1. Sequence alignment of the Ile-358 region of human E3 with the corresponding regions of E3s from various sources (from top to bottom; human, pigs, yeast, *Azotobacter vinellandii* and *Pseudomonas fluorescens*). Ile-358 and corresponding residues are underlined. The conserved residues are indicated by asterisk. Alignment analysis was performed using the MAFFT program on the ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

phobic amino acid, to Thr, a smaller hydrophilic amino acid, can affect these interactions by two factors. The first factor is the creation of a vacancy. The amino acid volume of Ile and Thr is 166.7 Å^3 and 116.1 Å^3, respectively. The mutation will results in a vacancy of 50.6 Å^3 at residue-358. This vacancy should be filled by a rearrangement of this region. The second factor is the introduction of a hydrophilic hydroxyl group (OH-) in the hydrophobic environment of this region. These factors can cause structural rearrangements in the local structure of this region. These structural changes can be responsible for the observed changes in the kinetic parameters of the mutant, particularly a large increase in the K_m value toward NAD^+.

UV-visible absorption and fluorescence spectroscopy were performed to examine the structural changes occurring in the 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 can be observed in the overall ranges of the spectra. The ratio (1.12) of the absorbance of the first and second peaks of the mutant (solid line) at approximately 350 nm and 455 nm, respectively, was lower than that (1.30) of the normal enzyme (dotted line). These differences in the UV-visible spectrum of the mutant indicate that structural changes might have occurred in the enzyme. Further evidence of the structural changes in the mutant came from the fluorescence study. When the enzymes were excited at 296 nm, two fluorescence emissions were observed for both the mutant and normal E3s, as shown in Figure 2(b). The first emission from 305 nm to 400 nm was due mainly to Trp. The second emission from 480 nm to more than 550 nm was due to FAD. When the fluorescence spectra of E3s were compared, a noticeable difference in the ratio was observed between the relative intensities of the first and second fluorescence emissions. The ratio (3.5) 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 fluorescence resonance energy transfer (FRET) from Trp to FAD was disturbed in the mutant, indicating that structural changes might have occurred in the mutant. The structural changes due to an Ile-358 to Thr mutation might have affected the structure of human E3 and interfered with the FRET from the Trp residues to FAD.

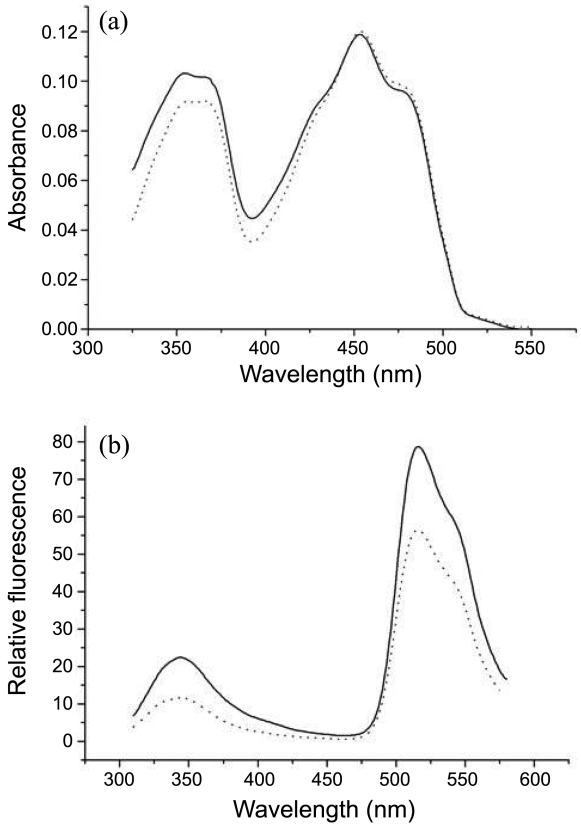

Figure 2. Spectroscopic properties of both mutant and normal human E3s. (a) UV-visible spectra of the mutant (solid line) and normal (dotted line) human E3s. The spectra were recorded using a SPECORD200 spectrophotometer and the data from 325 nm to 550 nm was transferred to an ASCII file. (b) Fluorescence spectra of the 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 a naturally occurring mutation (Ile-358 to Thr) on the structure and function of human E3 were examined by site-directed mutagenesis, E3 activity measurements and spectroscopic methods. The low E3 activity of the patient was attributed to the 2.5 times higher K_m value toward NAD^+ due to an Ile-358 to Thr mutation. This indicates that Ile-358 is important for enzyme binding to NAD^+. The mutation alters the UV-visible spectrum of the mutant and interferes with the FRET from Trp residues to FAD, indicating that structural changes occur in the mutant. This suggests that Ile-358 is important for the structure and function of human E3.

Acknowledgments. This research was supported in part by the Daegu University Research Grant, 2011.

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