

Characterization of a Naturally Occurring Mutation (Ile-12 to Thr) Close to Prosthetic Group FAD in Human Dihydrolipoamide Dehydrogenase

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD⁻ oxidoreductase, EC 1.8.1.4) is 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. It is a homodimeric flavoenzyme containing one FAD at each subunit.³ Patients with E3 deficiency have various clinical symptoms, ranging from severe neurological defects such as Leigh syndrome to less severe illness in childhood such as exertional fatigue. One substitution mutation of Ile-12 to Thr was found in two male second cousins with E3 deficiency.⁴

Site-directed mutagenesis method has been a useful tool for the structure-function study of human E3 and other proteins.⁵⁻¹⁰ By the site-specific mutations of Ile-12 to Thr, the effects of this naturally occurring mutation in human E3 structure and function were examined. The site-directed mutagenesis was performed using a mutagenesis kit according to the provided protocol. PCRs were carried out using the human E3 expression vector pPROEX-1:E3 as a template in a programmable PCR machine with the appropriate primers. Whole DNA sequence of human E3 coding region was sequenced to verify the integrity of DNA sequences other than the anticipated mutation.

The mutant (Ile-12 to Thr) was expressed in *E. coli* by IPTG (1 mM) induction. The purification of the mutant was performed using nickel affinity column according to the provided protocol. The mutant was eluted with the elution buffer containing 250 mM imidazole. SDS-PAGE showed that the mutant was highly purified. Its molecular weight was the same to that of wild-type human E3 on the SDS-PAGE gel. E3 assay was performed at 37 °C in a 50 mM potassium

phosphate buffer (pH 8.0) containing 1.5 mM EDTA with variable concentrations of the substrates, dihydrolipoamide and NAD⁻. The steady state kinetic parameters were determined using the SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA). Table 1 shows the kinetic parameters of both mutant and wild-type human E3s. The k_{cat} value was similar to that of normal human E3. The K_m value for dihydrolipoamide was slightly lower than that of normal human E3. However, the K_m value for NAD⁻ was about 2.4-fold larger than that of normal human E3, indicating that the mutation makes the enzyme less efficient to NAD⁻. In a

Table 1. Steady state kinetic parameters of both mutant (Ile-12 to Thr) and wild-type human E3s. E3 assay was performed at 37 °C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA with variable concentrations of the substrates.

Human E3s	k_{cat} (s ⁻¹)	K_m for DHL (mM)	K_m for NAD ⁻ (mM)
Wild-type	899	0.64	0.19
Mutant	915	0.48	0.45

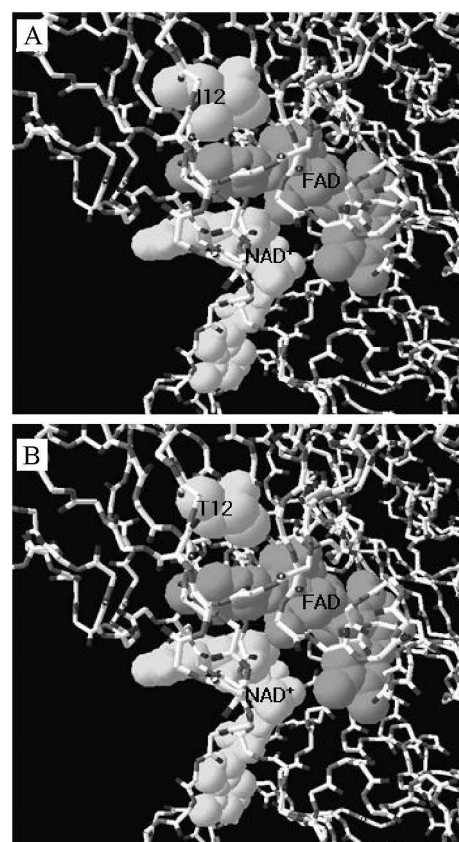


Figure 1. The location of Ile-12 in human E3 structure. The FAD, NAD⁻ and residue-12 are shown in space-filled structures and other residues are shown in backbone structures. A: Before the mutation, B: After the mutation (Ile-12 to Thr).

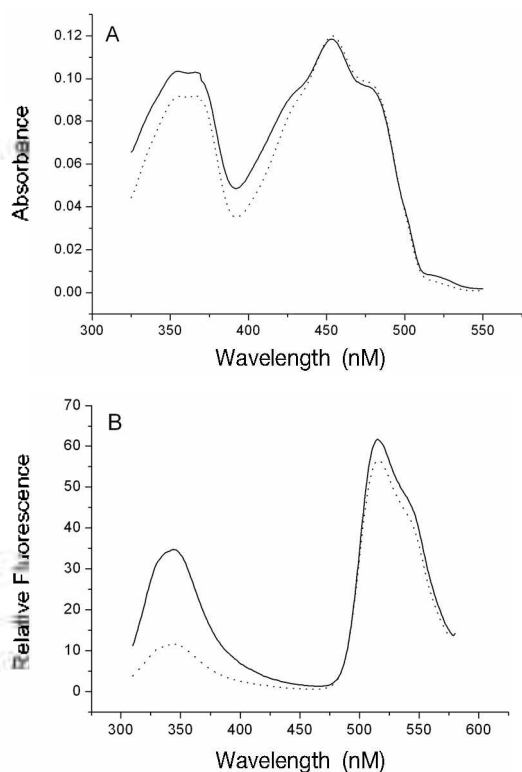


Figure 2. Spectroscopic properties of both mutant (Ile-12 to Thr) and wild-type human E3s. A: UV-visible spectra of the mutant (solid line) and wild-type (dotted line) human E3s. B: Fluorescence spectra of mutant (solid line) and wild-type (dotted line) human E3s.

human E3 structure. Ile-12 is located close to prosthetic group FAD as shown in Figure 1. It is part of a β -sheet secondary structure in the FAD domain. The amino acid volume of Ile is 166.7 \AA^3 while that of Thr is 116.1 \AA^3 . The mutation will give a vacancy of 50.6 \AA^3 at the residue-12. The OH group of Thr-12 side chain can form a new hydrogen bond with the CO group of Leu-145 backbone. The mutation of Ile-12, a large hydrophobic amino acid, to Thr, a smaller hydrophilic amino acid, could cause structural changes in the local structure of this region. These structural changes could be responsible for the alterations in kinetic parameters of the mutant.

To examine any structural changes occurring in the mutant, UV-visible absorption and fluorescence spectroscopies were performed. Because E3 contains FAD as a prosthetic group, human E3 shows a characteristic UV-visible absorption spectrum of flavoproteins as shown in Figure 2A. The overall shape of the mutant spectrum (solid line) was similar to that of the wild-type human E3 spectrum (dotted line). However, noticeable differences can be observed in the peak at around 350 nm. The peak of the mutant was slightly higher than that of the wild-type human E3. In case of the mutant (solid line), the ratio of the absorbance value of the second peak (at around 455 nm) to the absorbance value of the first peak (at around 350 nm) was about 1.15, which was slightly lower than the ratio (about 1.30) in case of the wild-type enzyme (dotted line). These differences in the UV-visible spectrum of the mutant indicated that structural changes could occur in the enzyme. More evidence of the structural changes in the mutant came from the fluorescence study. When enzymes

were excited at 296 nm, two fluorescence emissions were observed for both mutant and wild-type E3s, as shown in Figure 2B. The first emission from 305 nm to 400 nm was mainly Trp fluorescence. The second emission from 480 nm to over 550 nm was FAD fluorescence. In human E3, the Trp fluorescence was quenched due to fluorescence resonance energy transfer from Trp to FAD. When the fluorescence spectra of E3s were compared, a noticeable difference was found in the ratio between relative intensities of the first and second fluorescence emissions. The ratio (about 1.8) between relative intensities of the first and second fluorescence emissions of the mutant (solid line) was much smaller than that (about 4.9) of the wild-type enzyme (dotted line). This was mainly due to the increase in the first fluorescence of Trp residues. This also indicated that the energy transfer from Trp to FAD was disturbed in the Thr-12 mutant. The structural changes due to the mutation of Ile-12 to Thr could have affected the structure of human E3 so that the Trp fluorescence was increased and the fluorescence resonance energy transfer from Trp residues to FAD was interfered with.

In this study, the effects of a naturally occurring mutation (Ile-12 to Thr) in human E3 structure and function were examined using site-directed mutagenesis, E3 activity measurement and spectroscopic methods. The mutation of Ile-12 to Thr in human E3 resulted in the structural changes which altered the UV-visible spectrum, and increased the Trp fluorescence, and interfered with the efficient fluorescence resonance energy transfer from Trp residues to FAD. These structural changes could affect the kinetic parameters of the mutant, indicating that the mutant became less efficient to NAD^+ . These findings indicated that the conservation of Ile-12 residue in human E3 was important to its structure and function.

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