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Characterization of Two Site-Specifically Modified Human Dihydrolipoamide Dehydrogenase Mutants (Pro-282 to Ala and Pro-298 to Ala)

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Dihydrolipoamide dehydrogenase, which plays important roles in the glycine cleavage system,[1] forms a subunit known as the E3 component, which is shared by several enzyme complexes (pyruvate, α-ketoglutarate, and branched-chain α-keto acid dehydrogenase complexes).[2] E3 is a homodimeric flavoenzyme containing a single FAD as a prosthetic group at each subunit. The subunit consists of 474 amino acids with a molecular mass of 50,216 daltons.[3] E3 belongs to the pyridine nucleotide-disulfide oxidoreductase family along with glutathione reductase, thioredoxin reductase, mercuric reductase and trypanothione reductase.[4] Figure S1 shows the sequence alignment of the Pro-282 and Pro-298 region of human E3 with the corresponding regions of E3s from other species. Pro-282 is absolutely conserved, whereas Pro-298 is not. Pro-282 forms a part of β-sheet F2 and is located close to FAD and NAD^+, as shown in Figure 1. Pro-298 is a component of a β-turn structure between α-helix 7 and β-sheet A5.

Site-directed mutagenesis is a useful tool for structure-function studies of human E3 and other proteins.[5-8] To examine the importance of the Pro-282 and Pro-298 residues on the human E3 structure and function, the Pro-282 and Pro-298 residues were mutated site-specifically to Ala by site-directed mutagenesis. Site-directed mutagenesis and purification of the mutants are described in the Supporting Information. An 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 substrates (dihydrolipoamide and NAD^+) to determine the kinetic parameters. The data was analyzed using the SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA), which generates double reciprocal plots, as shown in Figure S3. The plots showed parallel lines, indicating that both mutants also catalyze the reaction *via* a ping pong mechanism. The program also provides the kinetic parameters directly without the need for secondary plots. Table 1 lists the kinetic parameters of the mutant and normal human E3s. The k_{cat} value of the Ala-282 mutant was approximately 50% that of normal human E3, indicating that the mutation deteriorates the catalytic processes of the conversion of substrates to products. The K_m value of the Ala-282 mutant toward dihydrolipoamide was approximately 2.7-fold smaller than that of normal human E3, indicating that the mutation improves the efficiency of enzyme binding to dihydrolipoamide. On the other hand, the K_m value toward NAD^+ was approximately 2.1 times higher than that of normal human E3, indicating that the mutation results in less efficient enzyme binding to NAD^+. The catalytic efficiency (k_{cat}/K_m) of the Ala-282 mutant toward NAD^+ was 4.3-fold smaller than that of the normal enzyme, indicating that the mutant is a significantly less efficient enzyme toward NAD^+. The k_{cat} value of the Ala-298 mutant was approximately 1.2-fold smaller than that of normal human E3, indicating that the mutation makes the enzyme slightly less active. The K_m value toward dihydrolipoamide was similar to that of normal human E3, indicating that the mutation does not affect enzyme binding to dihydrolipoamide. The K_m value toward NAD^+ was approximately 3.2 times larger than that of normal human E3, indicating that the mutation induces enzyme binding to NAD^+ less efficient. The catalytic efficiency (k_{cat}/K_m) of the Ala-298 mutant toward NAD^+ was 3.9-fold smaller than that of the normal enzyme, indicating that the mutant is 3.9-fold less efficient toward NAD^+.

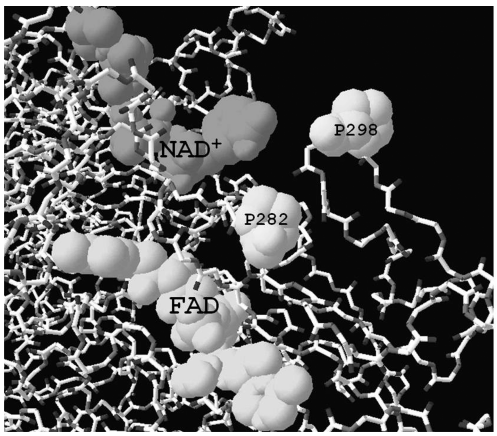

Figure 1. Location of Pro-282 and Pro-298 in human E3. A; FAD, NAD^+, Pro-282 and Pro-298 are shown in the space-filled structures and other residues are shown in the backbone structures. The structure was drawn using the Swiss-PdbViewer program (Swiss Institute of Bioinformatics).

UV-visible absorption and fluorescence spectroscopy were performed to examine the structural changes occurring in the mutants. Human E3 has a characteristic UV-visible absorption spectrum of flavoproteins due to the prosthetic

Table 1. Steady state kinetic parameters of mutant and normal human E3s. The E3 assay was performed at 37 °C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA

Human E3s	k_{cat} (s^{-1})	K_m toward DHL (mM)	K_m toward NAD^+ (mM)	k_{cat}/K_m toward DHL (s^{-1}/mM)	k_{cat}/K_m toward NAD^+ (s^{-1}/mM)
Normal	899	0.64	0.19	1405	4732
Ala-282 mutant	438	0.24	0.40	1825	1095
Ala-298 mutant	736	0.64	0.60	1150	1227

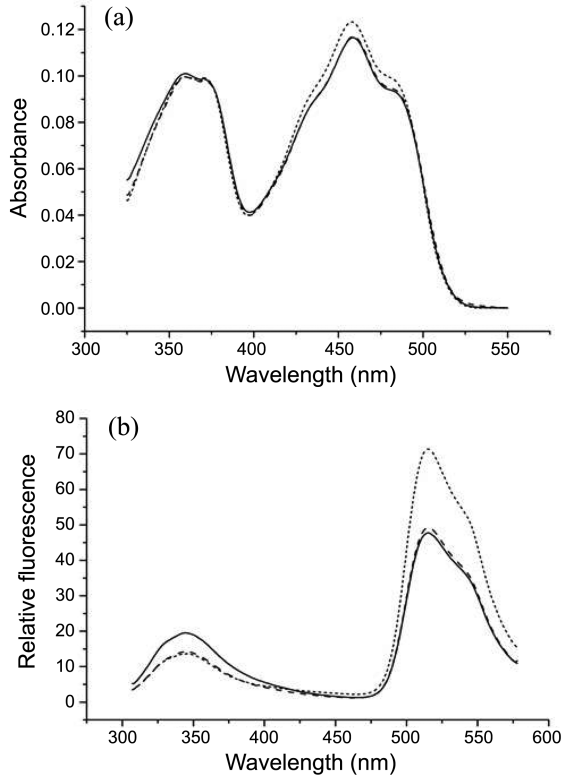

Figure 2. Spectroscopic properties of human E3s. A; UV-visible spectra of the Ala-282 mutant (solid line), Ala-298 mutant (dashed line) and normal (dotted line) human E3s. The spectra were recorded using a SPECORD200 spectrophotometer and the data from 325 nm to 550 nm was transferred to an ASCII file. The spectra were then drawn using the MicroCal Origin program. B; Fluorescence spectra of the Ala-282 mutant (solid line), Ala-298 mutant (dashed line) and normal (dotted line) human E3s. 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.

group, FAD, as shown in Figure 2(a). The overall shapes of the Ala-282 (solid line) and Ala-298 (dashed line) mutant spectra were similar to those of the normal human E3 spectrum (dotted line). On the other hand, the second peaks of the mutants at 452 nm were slightly lower in intensity than that of normal human E3, indicating structural changes in the mutants. Further evidence of the structural changes in the mutants came from a fluorescence study. When the enzymes were excited at 296 nm, two fluorescence emissions were observed for 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 > 550 nm was assigned to FAD. In human E3, Trp fluorescence was quenched due to fluorescence resonance energy transfer (FRET) from Trp to FAD. When the fluorescence spectra were compared, there was a large difference in the ratio 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 Ala-282 mutant (dashed line) was smaller than that (~5.2) of the normal enzyme (dotted line). This suggests that FRET from Trp to FAD was disturbed in the mutant. The structural changes due to a Pro-282 to Ala mutation might have affected the structure of human E3, interfering with energy transfer from the Trp residues to FAD. The ratio (~2.4) between the relative intensities of the first and second fluorescence emissions of the Ala-298 mutant (solid line) was also smaller than that (~5.2) of the normal enzyme (dotted line). This suggests that FRET from Trp to FAD was also disturbed in the Ala-298 mutant. The small structural changes due to a Pro-298 to Ala mutation might have affected the structure of human E3, interfering with FRET from the Trp residues to FAD.

This study examined the effects of the Pro-282 to Ala and Pro-298 to Ala mutations on the human E3 structure and function using site-directed mutagenesis, E3 activity measurements and spectroscopic methods. The substitution of Pro-282 with Ala in human E3 reduced the catalytic efficiency of the enzyme toward NAD^+ and caused structural changes that interfered with efficient FRET from the Trp residues to FAD. A Pro-298 to Ala mutation in human E3 also deteriorated the catalytic efficiency of the enzyme toward NAD^+ and caused structural changes that interfered with efficient FRET from the Trp residues to FAD. This suggests that both Pro-282 and Pro-298 residues are important for the efficient catalytic process of the enzyme toward NAD^+ and for efficient FRET from the Trp residues to FAD.

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