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단신 (Notes)

Characterization of Site-specific Mutations in Human Dihydrolipoamide Dehydrogenase Significantly Destabilizing the Transition State of the Enzyme Catalysis

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD^+ oxidoreductase; EC 1.8.1.4) is a homodimeric flavoprotein containing a single FAD in each subunit.[1] E3 is an essential component in pyruvate, α-ketoglutarate and branched-chain α-keto acid dehydrogenase complexes.[2] E3 catalyzes the reoxidation of a dihydrolipoyl prosthetic group attached to the lysyl residue(s) of the acyltransferase components of the three α-keto acid dehydrogenase complexes. Because E3 is a common component in three α-keto acid dehydrogenase, a decrease in E3 activity affects the activities of all three complexes, which leads to increased urinary excretion of α-keto acids, elevated blood lactate, pyruvate, and branched chain amino acids. Patients with an E3 deficiency normally die young because an E3 deficiency is a critical genetic defect affecting the central nervous system. A deficiency in E3 results in Leigh syndrome with recurrent episodes of hypoglycemia and ataxia, permanent lactic acidaemia, and mental retardation.[3]

E3, glutathione reductase, thioredoxin reductase, mercuric reductase, and trypanothione reductase are members of the pyridine nucleotide-disulfide oxidoreductase family.[4] These enzymes consist of four structural domains (FAD, NAD, central, and interface domains). The catalytic mechanisms of these enzymes are similar. All are homodimeric flavoenzymes containing an active disulfide center and a FAD in their each subunit. They catalyze electron transfer between pyridine nucleotides (NAD^+ or NADPH) and their specific substrates via the FAD and active disulfide center.

Fig. 1 presents the sequence alignment of the Cys-50 region of human E3 with the corresponding regions of the E3s from a range of sources, such as pigs, yeast, *Escherichia coli*, and *Pseudomonas fluorescens*. Cys-50 is absolutely conserved in all E3s, highlighting its importance to the structure and function of the E3s, including human E3. Cys-50 is a component of the very long a-helix structure 2, which is composed of 25 amino acids. Cys-50 forms an active disulfide center with Cys-45, as shown in *Fig.* 2. A Cys-45 to Ala mutation results in a large decrease in human E3 activity and changes in the spectroscopic properties of human E3.[5] On the other hand, the effects of Cys-50 mutations on the catalytic function and structure of human E3 have not been studied clearly. The following two questions were investigated by the site-specific mutations of Cys-50 to Ala and Thr in human E3. First, to what extent is the catalytic function of human E3 affected by these mutations? Second, do these mutations cause changes in the spectroscopic properties of human E3?.

EXPERIMENTAL SECTION

Materials

Imidazole, iminodiacetic acid sepharose 6B, lipoamide, electrophoresis reagents, and NAD^+ were obtained from

E3	Amino acid sequence
	50
sp|P09622|DLDH_HUMAN	LGGTCLNVGCIPSKALLNNS
sp|P09623|DLDH_PIG	LGGTCLNVGCIPSKALLNNS
sp|P09624|DLDH_YEAST	LGGTCLNVGCIPSKALLNNS
sp|P0A9P0|DLDH_ECOLI	LGGVCLNVGCIPSKALLHVA
sp|P14218|DLDH_PSEFL	LGGTCLNVGCIPSKALLDSS
	————————
	α2

***Figure* 1.** Sequence alignment of the Cys-50 regions of human E3 with the corresponding regions of the E3s from various sources (from top to bottom; human, pigs, yeast, *Escherichia coli* and *Pseudomonas fluorescens*). The UniProtKB ID and amino acid sequence from residues-41 to -60 are shown. Cys-50 and the corresponding residues are underlined. Alignment analysis was performed using the MAFFT program on the ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

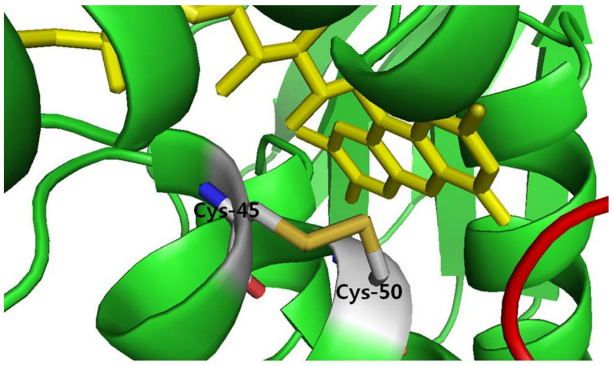

***Figure* 2.** Location of Cys-50 in human E3. The two subunits are shown as cartoons, representing the secondary structures in a single color (red and green, respectively). FAD (yellow), Cys-45 and Cys-50 are shown as sticks. Cys-50 and Cys-45 are connected by an active disulfide bond (the blue, red, yellow and white color represents nitrogen, oxygen, sulfur and carbon, respectively). The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

Sigma-Aldrich (St. Louis, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Promega (Madison, USA). Dihydrolipoamide was synthesized by the reduction of lipoamide using sodium borohydride. The primers and dNTP were purchased from Bioneer (Daejeon, Korea). The Muta-Direct™ Site-Directed Mutagenesis Kit was supplied by iNtRON Biotechnology (Seongnam, Korea). Ni-NTA His-Bind Resin was acquired from QIAGEN (Hilden, Germany). The *E. coli* XL1-Blue containing a human E3 expression vector, pPROEX-1:E3, was a generous gift from Dr. Mulchand S. Patel of the University at Buffalo, the State University of New York.

Site-Directed Mutagenesis

Site-directed mutagenesis was performed using a mutagenesis kit (iNtRON Biotechnology, Sungnam, ROK) with two mutagenic primer pairs, as listed in *Table* 1. The mutations were confirmed by DNA sequencing.

Expression and Purification of the Human E3 Mutant

Three mL of an overnight culture of *E. coli* XL1-Blue containing the human E3 mutant expression vector was used to inoculate 1 L of LB medium containing ampicillin (100 μg/mL). The cells were grown at 37 °C to an absorbance of 0.7 at 595 nm, and IPTG was then added to a final concentration of 1 mM. The growth temperature was shifted to 30 °C and the cells were allowed to grow overnight. The overnight culture was harvested by centrifugation at 4000×g for 5 min. The cell pellets were then washed with a 50 mM potassium phosphate buffer (pH 8.0) containing 100 mM NaCl and 20 mM imidazole (Binding buffer), and then recollected by centrifugation at 4000×g for 5 min. The pellets were resuspended in 10 ml of Binding buffer. The cells were lysed by sonication and centrifuged at 10,000×g for 20 min.

The supernatant was loaded onto a Ni-NTA His-Bind Resin column that had been washed with 2 column volumes of distilled water and then equilibrated with 5 column volumes of Binding buffer. After loading the supernatant, the column was washed with 10 column volumes of Binding buffer and then with the same volume of Binding buffer containing 50 mM imidazole. The E3 mutant was eluted with Binding buffer containing 250 mM imidazole. The E3 mutant was then eluted with Binding buffer containing 250 mM imidazole and then dialyzed three times against 50 mM potassium phosphate buffer (pH 8.0) containing 0.25 mM EDTA to remove the imidazole.

SDS-Polyacrylamide Electrophoresis

SDS-PAGE analysis of the proteins was performed in 12% SDS-PAGE gel. The gel was stained with Coomassie blue after electrophoresis.[6]

E3 Assay

The kinetic parameters were determined by an E3 assay 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^+. The activity was recorded spectrophotometrically by observing the reduction of NAD^+ at 340 nm using a SPECORD200 spectrophotometer (Analytik Jena AG, Jena, USA). One unit of activity is defined as 1 μmol of NAD^+ reduced per

***Table* 1.** Primers for the site-directed mutagenesis. The mismatched bases are underlined

Mutations/Primers	Primer Sequences
Cys-50 to Ala mutation	
sense	5'-CATGCTTG AATGTTGGTGCTATTCCTTCTAAGGC-3'
antisense	5'-GCCTT AGAAGGAATAGCACCAACATTCAAGCATG-3'
Cys-50 to Thr mutation	
sense	5'-CATGCTTGAATGTTGGTACTATTCCTTCTAAGGC-3'
antisense	5'-GCCTTAGAAGGAATAGTACCAACATTCAAGCATG-3'

min. The data was analyzed using the SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA).

Spectroscopic Study

The UV-visible 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 (Photon Technology International, South Brunswick, USA). The fluorescence spectra were recorded using a FP-6300 spectrofluorometer (Jasco Inc., Easton, USA). The samples were excited at 296 nm and the emissions were recorded from 305 nm to 580 nm. The data was transferred to an ASCII file and the spectra were drawn using the MicroCal Origin program.

RESULTS AND DISCUSSION

Site-directed mutagenesis has been used for structure-function studies of human E3 and other proteins.[7–9] The site-directed mutagenesis with the mutagenic primers listed in *Table* 1 resulted in the construction of the mutant E3 expression vectors. The entire DNA sequence of the human E3 coding region was sequenced to confirm the integrity of the DNA sequences. No other mutations were found except for the site-directed mutations at residue-50. The mutants were expressed in *Escherichia coli* and purified on a Ni-NTA His-Bind Resin column. The purification steps were followed by SDS-PAGE (*Fig.* 3). The gel (12%) confirmed the purity of the mutants. An E3 assay was performed, as described in the *Experimental Section*, and the data was analyzed using the SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA). *Table* 2 lists the kinetic parameters and the free energy difference (D*G*) for the mutant and wild-type human E3s. The k_{cat} values of both mutants were reduced by approximately 2000-fold, suggesting that Cys-50 is very important to the efficient catalytic step of the conversion of the substrates to products. The K_m values toward the dihydrolipoamide of the mutants decreased by 3- to 4-fold, indicating that the mutations improve the efficiency of enzyme binding to dihydrolipoamide. Cys-50 is located the end of the dihydrolipoamide binding channel, as shown in *Fig.* 4. The location of Cys-50 may be responsible for these changes in the K_m values toward the dihydrolipoamide of the mutants. The K_m value toward NAD^+ of the C50A mutant increased 2.5-fold, indicating that the mutation makes enzyme binding to NAD^+ less efficient, whereas that of the C50T mutant was similar, suggesting that the mutation does not have a significant effect on enzyme binding to NAD^+. The catalytic efficiency (k_{cat}/K_m) of the C50A mutant toward NAD^+ decreased 5317-fold, indicating that the mutation makes the enzyme substantially inefficient to NAD^+. This corresponds to the destabilization of the transition state of human E3 catalysis by approximately 5.3 kcal mol^{-1}. The catalytic efficiency of C50T mutant toward NAD^+ was reduced 2057-fold, indicating that the mutant became substantially inefficient toward NAD^+. This corresponds to destabilization of the transition state of human E3 catalysis by approximately 4.7 kcal mol^{-1}. The results suggest that the conservation of Cys-50 in human

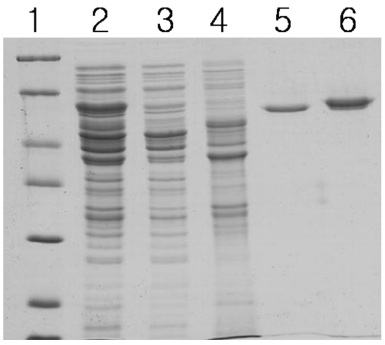

***Figure* 3.** SDS-PAGE gel (12%) for the purification of C50T mutant E3. Lane 1, molecular weight markers (from top to bottom, β-galactosidase 116.3 kDa, bovine serum albumin 66.2 kDa, ovalalbumin 45.0 kDa, lactate dehydrogenase 35.0 kDa, REase Bsp981 25 kDa, β-lactoglobulin 18.4 kDa, lysozyme 14.4 kDa); lane 2, supernatant; lane 3, flow-through; lane 4, Binding buffer containing 50 mM imidazole; lane 5, Binding buffer containing 250 mM imidazole; lane 6, previously purified recombinant human E3 as a control.

***Table* 2.** Steady state kinetic parameters and the free energy difference (ΔG) for mutant and wild-type 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. Values are mean of three independent determinations

Human E3s	k_{cat} (s^{-1})	K_m toward dl[a] (mM)	K_m toward NAD^+ (mM)	k_{cat}/K_m toward NAD^+ (s^{-1}/M)	ΔG^b (kcal mol^{-1})
Wild-type	899	0.64	0.19	4.73×10^6	NA[c]
C50A mutant	0.42	0.22	0.47	0.89×10^3	5.3
C50T mutant	0.46	0.16	0.20	2.30×10^3	4.7

[a]Dihydrolipoamide.
[b]Calculated from the equation: $\Delta G = -RT \ln((k_{cat}/K_m)_{mutant}/((k_{cat}/K_m)_{wild\text{-}type}$ using k_{cat}/K_m values for NAD^+ as described in references 10 and 11.
[c]Not applicable.

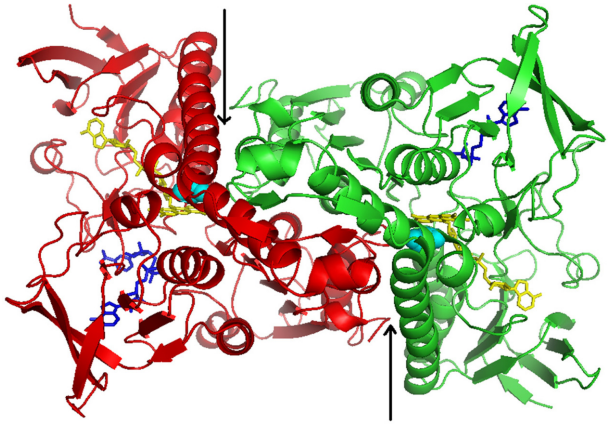

***Figure* 4.** Homodimeric structure of human E3. Two subunits are shown as cartoons, representing secondary structures in a single color (red and green, respectively). FAD (yellow), NAD^+ (blue) and Cys-50 (cyan) are shown as sticks and spheres, respectively. The arrows indicate the dihydrolipoamide binding channels. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

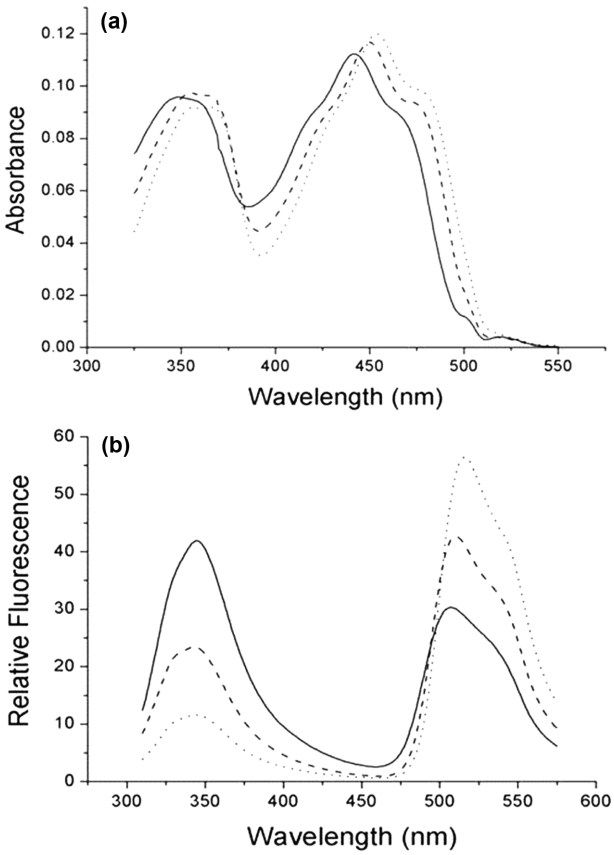

***Figure* 5.** Spectroscopic properties of human E3s. (a) UV-visible spectra of the C50A mutant (dashed line), the C50T mutant (solid line) and wild-type (dotted line) human E3s. The spectra were recorded using a SPECORD200 spectrophotometer, and the data from 325 nm to 550 nm was transferred to an ASCII file. The spectra were then drawn using the MicroCal Origin program. (b) Fluorescence spectra of the C50A mutant (dashed line), the C50T mutant (solid line) and wild-type (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.

E3 is very important to the efficient catalytic process of the enzyme.

The changes in the spectroscopic properties of the mutants were examined by UV-visible absorption and fluorescence spectroscopy. Human E3 contains a single FAD as a prosthetic group in each subunit. Therefore, it shows the characteristic UV-visible absorption spectrum for flavoproteins, as shown in *Fig.* 5(a) (dotted line). The overall shapes of the C50A mutant (dashed line) and C50T mutant (solid line) spectra were similar to that of the wild-type human E3 spectrum (dotted line) but with two noticeable differences. First, the ratios (2.6 for C50A mutant and 2.1 for C50T mutant) between the absorbance of the valley (near 390 nm) and peak (near 450 nm) of the mutants were significantly smaller than those of the wild-type enzyme (3.4). Second, the spectra of the mutants were blue-shifted by 4 nm for the C50A mutant and 10 nm for the C50T mutant, indicating that the environment of FAD becomes more hydrophilic due to these mutations. This agrees well with the nature of these mutations, which are expected to provide a more hydrophilic environment near FAD of human E3.

Two fluorescence emissions were observed when human E3 was excited at 296 nm, as shown in *Fig.* 5(b). The first emission from 305 nm to 400 nm was mainly Trp fluorescence. The second emission from 480 nm to > 550 nm was assigned to FAD fluorescence. The Trp fluorescence was quenched due to fluorescence resonance energy transfer (FRET) from Trp to FAD. A comparison of the fluorescence spectra of E3s revealed a significant difference in the ratio between the relative intensities of the first and second fluorescence emissions. The ratios (1.8 for C50A mutant and 0.7 for C50T mutant) between the relative intensities of the first and second fluorescence emissions of the mutants were much smaller than those of the wild-type enzyme (4.9). This suggests that FRET from Trp to FAD was disturbed significantly in the mutants, indicating that the mutations affected the environment of FAD in human E3 so energy transfer from Trp residues to FAD was severely affected. Cys-50 is located very close to FAD within the van der Waals distance, as shown in *Fig.* 2. The Cys-50 to Ala or Thr mutations will result in a more hydrophilic environment near FAD. The mutations also destroy the active disulfide

center between Cys-45 and Cys-50, which restricts the freedom of Cys-50. This destruction will impart freedom to the substituted amino acids at residue-50, which can also be responsible for the altered spectroscopic properties.

In this study, the effects of Cys-50 to Ala and Thr mutations in human E3 on the catalysis and structure of the enzyme were examined by site-directed mutagenesis, E3 activity measurements and spectroscopic methods. The Cys-50 to Ala and Thr mutations in human E3 result in substantially reduced k_{cat} values, indicating that the conservation of Cys-50 is very important for the efficient catalytic function of the enzyme. The mutations make enzyme binding to dihydrolipoamide more efficient. This suggests that Cys-50 is involved in enzyme binding to dihydrolipoamide. This is consistent with the location of Cys-50, which is located at the end of the dihydrolipoamide binding channel. The catalytic efficiencies toward NAD^+ of the mutants were also deteriorated severely, indicating that the mutations significantly destabilize the transition state of human E3 catalysis by 5.3 kcal mol^{-1} (C50A mutant) and 4.7 kcal mol^{-1} (C50T mutant). The spectroscopic studies of the mutants suggest that the environment near FAD becomes more hydrophilic as a result of these mutations so the UV-visible spectra of the mutants are blue-shifted and the efficient FRETs between Trp and FAD are deteriorated.

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