

Characterization of Site-Specific Mutations Affecting the Catalytic Efficiency of Human Dihydrolipoamide Dehydrogenase toward NAD⁺

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Dihydrolipoamide dehydrogenase (E3) (dihydrolipoamide: NAD⁺ oxidoreductase; EC 1.8.1.4) is a homodimeric enzyme containing one FAD as a prosthetic group at each subunit, as shown in Fig. 1. Each subunit of human E3 is comprised of 474 amino acids with a total molecular mass of 50,216 Daltons, as calculated from the primary amino acid sequence.¹ The human E3 gene is located on chromosome 7 and encodes a 509 amino acid protein.² E3 is a mitochondrial protein that is synthesized in precursor form in the cytoplasm. The precursor E3 for humans has a leader sequence composed of 35-amino acids at its amino-terminus, which is cleaved off during its import into the mitochondria matrix.³

E3 is a very important enzyme in the energy metabolism because it is a common component in three α -keto acid dehydrogenase complexes (pyruvate, α -ketoglutarate and branched-chain α -keto acid dehydrogenase complexes).⁴ E3 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. In particular, the pyruvate dehydrogenase complex links between the glycolysis and citric acid cycle by converting pyruvate to acetyl-CoA. Therefore, patients with an E3 deficiency normally die young because such a deficiency is a critical genetic defect that affects the central nervous system, and can manifest as Leigh syndrome with recurrent episodes of hypoglycemia and ataxia, permanent lactic acidemia, and mental retardation.⁵

E3 belongs to the pyridine nucleotide-disulfide oxidoreductase family along with glutathione reductase, thioredoxin reductase, mercuric reductase and trypanothione reductase.⁶ Through the FAD and active disulfide center, this family catalyzes electron transfer between the pyridine nucleotides (NAD⁺ or NADPH) and their specific substrates.

Proline (Pro), an imino acid, is unique among the 20

amino acids in proteins. The last carbon atom in its side chain links covalently the nitrogen atom of the peptide bond, exhibiting very strong conformational rigidity, which is normally observed in the β -turn structure. Fig. 2 presents the sequence alignments of the Pro-156 and Pro-303 regions of human E3 along with the corresponding regions of E3s from pigs, yeast, *Escherichia coli* and *Pseudomonas fluorescens*. Pro-156 is absolutely conserved, whereas Pro-303 is not. Pro-156 has a random coil structure between β -sheet F1 and α -helix 4, whereas Pro-303 forms a part of a random coil structure between α -helix 7 and β -sheet A5 (Fig. 3). They are located close to NAD⁺, indicating their involvement in enzyme binding to NAD⁺. This study examined the role and importance of Pro-156 and Pro-303 in the human structure and function by site-specific mutations to Ala. Ala was selected because it is a hydrophobic amino acid like Pro with a small volume decrease. The

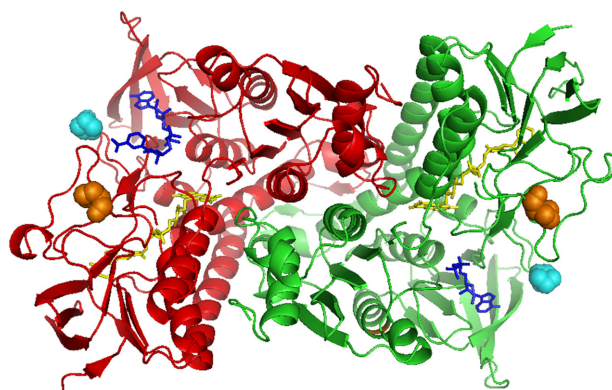


Figure 1. Location of Pro-156 and Pro-303 in human E3. Two subunits of the human E3 homodimeric structure are shown as cartoons, representing secondary structures, in a single color (red and green, respectively). FAD (yellow) and NAD⁺ (blue) are shown as sticks, whereas Pro-156 (cyan) and Pro-303 (orange) are shown as spheres. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

E3s	Amino Acid Sequences
	156
sp P09622 DLDH_HUMAN	IATGSEVTPFPGITIDEDTIV
sp P09623 DLDH_PIG	IATGSEVTPFPGITIDEDTVV
sp P09624 DLDH_YEAST	VATGSEVTPFPGIEIDEEKIV
sp POA9P0 DLDH_ECOLI	NAAGSRP IQLPFI PHEDPRIW
sp P14218 DLDH_PSEFL	IASGSRPVEI PPAPLSDDI IV
	-->
	<u>βF1</u>
	303
sp P09622 DLDH_HUMAN	IELDPRGRIPVNTRFQTKI
sp P09623 DLDH_PIG	IELDPRGRIPVNTRFQTKI
sp P09624 DLDH_YEAST	LEVDKRGRLYIDDQFNSKF
sp POA9P0 DLDH_ECOLI	VEVDDRGFIRVDKQLRTNV
sp P14218 DLDH_PSEFL	VTLDERGFIVVDDHCKTSV

Figure 2. Sequence alignments of the Pro-156 and Pro-303 regions of human E3 with the corresponding regions of E3s from various sources (from top to bottom; human, pigs, yeast, *Escherichia coli* and *Pseudomonas fluorescens*). The UniProtKB ID is shown and the Pro residues are underlined. Alignment analysis was performed using the MAFFT program at the ExPASy Proteomics Server (Swiss Institute of Bioinformatics).

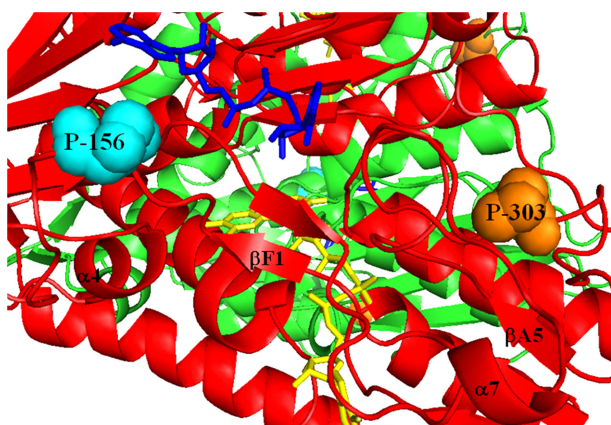


Figure 3. Location of Pro-156 and Pro-303 in human E3. Two subunits of the human E3 homodimeric structure are shown as cartoons, representing secondary structures, in a single color (red and green, respectively). FAD (yellow) and NAD⁺ (blue) are shown as sticks whereas Pro-156 (cyan) and Pro-303 (orange) are shown as spheres. Pro-156 is a random coil structure between β -sheet F1 and α -helix 4, whereas Pro-303 forms a part of a random coil structure between α -helix 7 and β -sheet A5. The structure was drawn using the PyMOL program (DeLano Scientific LLC). The PDB ID for the human E3 structure is 1ZMC.

mutation of Pro to Ala will result in a small conformational change, compared to the mutations to other amino acids.

Experimental Section

Materials

The electrophoresis reagents, imidazole, iminodiacetic acid sepharose 6B, lipoamide and NAD⁺ were obtained from

Sigma-Aldrich (St. Louis, USA). Dihydropolipoamide was synthesized by the reduction of lipoamide using sodium borohydride. Isopropyl- β -D-thiogalactopyranoside (IPTG) was purchased from Promega (Madison, USA). 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. The primers and dNTP were purchased from Bioneer (Daejeon, Korea). The Muta-DirectTM Site-Directed Mutagenesis Kit was supplied by iNtRON Biotechnology (Seongnam, Korea). Ni-NTA His-Bind Resin was obtained from QIAGEN (Hilden, Germany).

Site-Directed Mutagenesis

Site-directed mutagenesis was carried out using a mutagenesis kit (iNtRON Biotechnology, Sungnam, ROK) with two mutagenic primer pairs, as listed in Table 1. The entire DNA sequence of the human E3 coding region was sequenced to confirm the integrity of the DNA sequences other than the anticipated mutations.

Expression and Purification of the Human E3 Mutant

Three ml of an overnight culture of *Escherichia coli* XL1-Blue containing the human E3 mutant expression vector was used to inoculate 1 L of LB medium containing ampicillin (100 g/ml). The cells were grown at 37 °C to an absorbance of 0.7 at 595 nm and IPTG was 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 \times g for 5 min. The cell pellets were 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 \times 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 \times g for 20 min.

The supernatant was loaded onto a Ni-NTA His-Bind Resin column, which 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 and then dialyzed three times against 50 mM potassium phosphate buffer (pH 8.0) containing 0.25 mM EDTA to remove 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.⁷

E3 Assay

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. E3 activity was measured 16 times (4×4) in one assay experiment set with 4 different concentrations of the substrates, dihydropyridine (0.100, 0.154, 0.286, and 2.00 mM) and NAD⁺ (0.100, 0.154, 0.286, and 2.00 mM), respectively, to determine the kinetic parameters. 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 mmol of NAD⁺ reduced per min. The data was analyzed using the SigmaPlot Enzyme Kinetics Module (Systat Software Inc., San Jose, USA).

Fluorescence Spectroscopic Study

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 (Photon Technology International, South Brunswick, USA).

RESULTS AND DISCUSSION

Site-directed mutagenesis is a useful tool for the structure-function studies of human E3 and other proteins.^{8–10} To examine the importance of Pro-156 and Pro-303 on the human E3 structure and function, Pro-156 and Pro-303 were mutated site-specifically to Ala by site-directed mutagenesis. The site-directed mutagenesis with the mutagenic primers listed in Table 1 resulted in the construction of the mutant E3 expression vectors. The mutants were expressed in *Esch-*

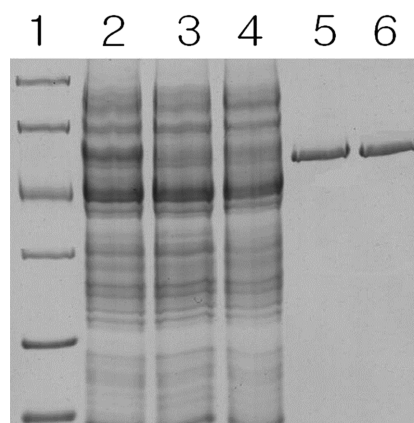


Figure 4. SDS-polyacrylamide gel (12%) for the purification of the P156A mutant E3. Lane 1, molecular weight markers (from top to bottom, β -galactosidase 116.3 kDa, bovine serum albumin 66.2 kDa, ovalalbumin 45.0 kDa, lactate dehydrogenase 35.0 kDa, REase Bsp981 25 kDa, b-lactoglobulin 18.4 kDa, lysozyme 14.4 kDa); lane 2, supernatant; lane 3, flow-through; lane 4, Binding buffer containing 150 mM imidazole; lane 5, Binding buffer containing 500 mM imidazole; lane 6, previously purified recombinant human E3 as a control.

erichia coli and purified by a Ni-NTA His-Bind Resin column. The purification steps were followed by SDS-PAGE (Fig. 4). The gel (12%) revealed the mutants to be highly purified.

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). The double reciprocal plots showed parallel lines, suggesting that both mutants catalyze the reaction via a ping pong mechanism. The program also provides the kinetic parameters without the need for secondary plots. Table 2 lists the kinetic parameters of the mutant and normal human E3s. The k_{cat} value of the P156A mutant was 9% lower than that of normal human E3, indicating that the mutation makes the enzyme less active. The K_m value toward dihydropyridine was similar to that of normal human E3. The K_m value of NAD⁺ was 26% higher than that of normal human E3, suggesting that the mutation reduces the efficiency of enzyme binding to NAD⁺ significantly.

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

Mutations/Primers	Primer Sequences
Pro-156->Ala	
Sense	5'-GAAGTTACTCCTTTT <u>Q</u> CTGGAATCACGATAGATG-3'
Antisense	5'-CATCTATCGTGATTCCAG <u>C</u> AAAAGGAGTAACTTC-3'
Pro-303->Ala	
Sense	5'-CCAGAGGTAGAATT <u>Q</u> CAGTCAATACCAGATTTC-3'
Antisense	5'-GAAATCTGGTATTGACTG <u>C</u> AATTCTACCTCTGG-3'

Table 2. 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. Values are means of three independent determinations

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
P156A mutant	817	0.63	0.24	1295	3400
P303A mutant	629	0.55	0.42	1144	1498

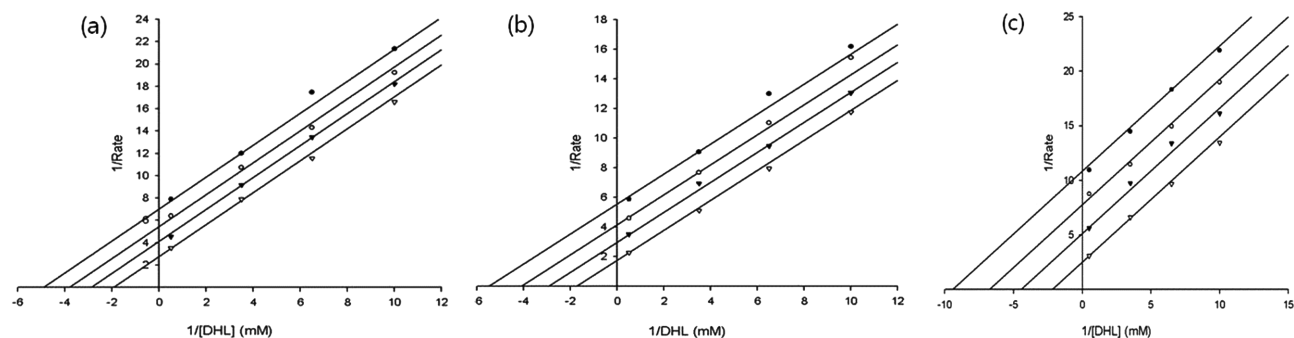


Figure 5. Double reciprocal plots for the normal (a), P156A (b) and P303A (c) mutant human E3s. E3 activities were determined at 37 °C in a 50 mM potassium phosphate buffer (pH 8.0) containing 1.5 mM EDTA with variable concentrations of the substrates, dihydrolipoamide (DHL) and NAD^+ . The plots were drawn using the SigmaPlot Enzyme Kinetics Module program. The NAD^+ concentrations from the top to bottom are 0.100, 0.154, 0.286, and 2.00 mM. The DHL concentrations from right to left are 0.100, 0.154, 0.286, and 2.00 mM.

The catalytic efficiency (k_{cat}/K_m) of the P156A mutant toward dihydrolipoamide was 8% lower than that of the normal enzyme, indicating that the mutant is less efficient toward dihydrolipoamide. The catalytic efficiency (k_{cat}/K_m) of the mutant toward NAD^+ was considerably lower (28%) than that of the normal enzyme, suggesting that the mutant is a significantly less efficient enzyme toward NAD^+ . The k_{cat} value of the P303A mutant was 30% lower than that of normal human E3, indicating that the mutation makes the enzyme significantly less active. The K_m value toward dihydrolipoamide was 14% lower than that of normal human E3, indicating that the binding of the mutation enzyme to dihydrolipoamide is slightly more efficient. The K_m value toward NAD^+ was 121% larger than that of normal human E3, indicating that the mutation results in significantly less efficient enzyme binding to NAD^+ . The catalytic efficiency (k_{cat}/K_m) of the P303A mutant toward dihydrolipoamide was 19% lower than that of the normal enzyme, indicating that the mutant is less efficient toward dihydrolipoamide. The catalytic efficiency (k_{cat}/K_m) of the P303A mutant toward NAD^+ was 68% lower than that of the normal enzyme, suggesting that the mutant is significantly less efficient toward NAD^+ . The mean NAD^+ concentration in cells is 0.37 mM.^{11,12} Therefore, this significantly lower catalytic efficiency toward NAD^+ is more detrimental inside the cells because of the low cellular NAD^+ concentration.

Fluorescence spectroscopy was carried out to detect the

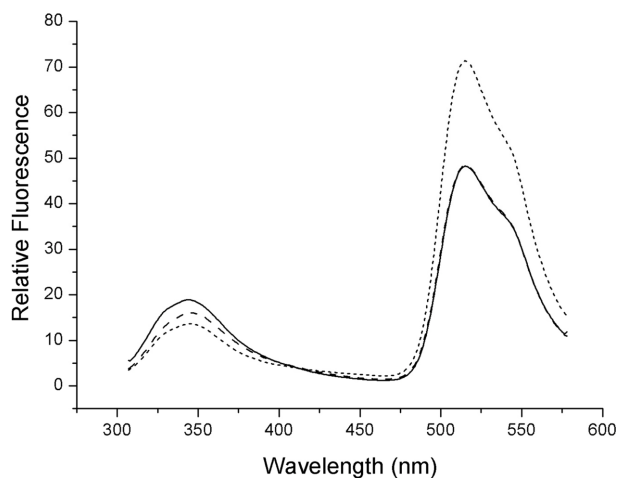


Figure 6. Fluorescence spectra of the P156A mutant (solid line), P303A mutant (dashed line) and normal (dotted line) human E3s. The enzymes were excited at 296 nm and emission was observed from 305 nm to 575 nm. The data was transferred to an ASCII file and the spectra were then drawn using the Micro-Cal Origin program.

structural changes in the mutants. The enzymes were excited at 296 nm resulting in two fluorescence emissions, as shown in Fig. 6. The emissions from 305 nm to 400 nm and from 480 nm to > 550 nm were assigned to Trp and FAD, respectively. Trp fluorescence was quenched due to fluorescence resonance energy transfer (FRET) from Trp to FAD. When the fluorescence spectra were compared, there was a con-

siderable difference in ratio between the relative intensities of the first and second fluorescence emissions. The ratio (3.0) between the relative intensities of the first and second fluorescence emissions of the P156A mutant (dashed line) were smaller than that (5.2) of the normal enzyme (dotted line). This indicates that FRET from Trp to FAD is disturbed in the mutant. The structural changes due to a Pro-156 to Ala mutation might have affected the structure of human E3, interfering with energy transfer from the Trp residues to FAD. The ratio (2.5) between the relative intensities of the first and second fluorescence emissions of the P303A mutant (solid line) were also lower than that (5.2) of the normal enzyme (dotted line). This indicates that the FRET from Trp to FAD was also disturbed in the P303A mutant. The small structural changes due to a Pro-303 to Ala mutation affects the structure of human E3, interfering with FRET from the Trp residues to FAD. The amino acid volume of Pro was 112.7 Å³, whereas that of Ala was 88.6 Å³.¹³ A Pro to Ala mutation will result in a 24.1 Å³ vacancy at the mutated residue, which will remove the conformational rigidity of Pro at the mutation site. This vacancy and conformation freedom might induce structural changes at the mutation sites, which might alter the kinetic parameters of the mutants. The precise structural changes occurring due to the mutation can only be revealed by an X-ray crystallographic study.

The effects of Pro-156 to Ala and Pro-303 to Ala mutations on the human E3 structure and function were examined by site-directed mutagenesis, E3 activity measurements and spectroscopic methods. The substitution of Pro-156 with Ala in human E3 resulted in a deterioration of the catalytic efficiency of the enzyme toward NAD⁺ as well as structural changes that interfered with efficient FRET from the Trp residues to FAD. A Pro-303 to Ala mutation in human E3 also resulted in a significant decrease in the catalytic efficiency of the enzyme toward NAD⁺ and interfered with efficient FRET from the Trp residues to FAD. This indicates that both Pro-156 and Pro-303 are important to the catalytic efficiency of the enzyme toward NAD⁺ and to efficient FRET

from the Trp residues to FAD.

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